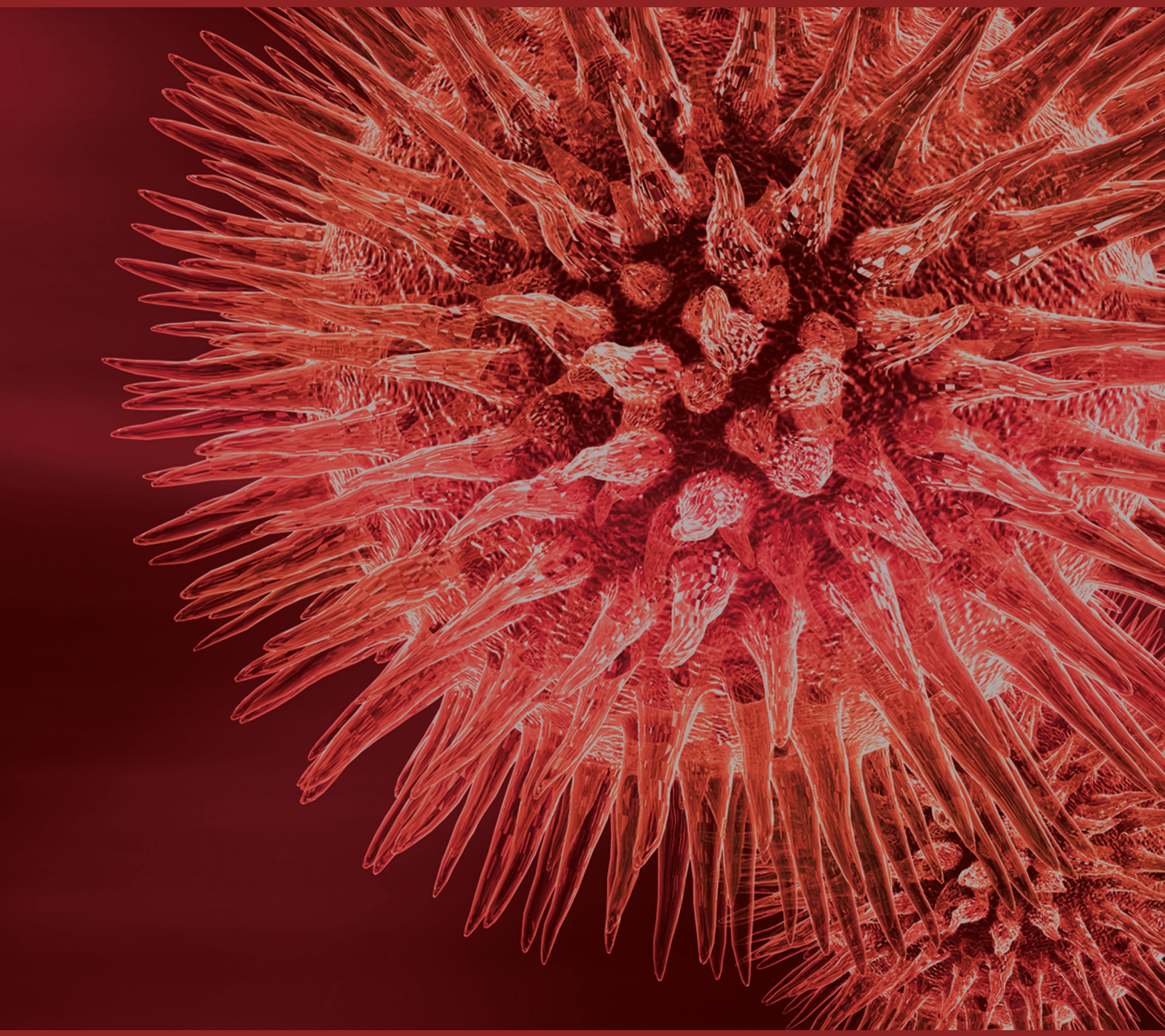
BioMed Research International

# Pharmacological Application of Growth Factors: Basic and Clinical

Guest Editors: Jian Xiao, Yadong Wang, Saverio Bellusci, and Xiaokun Li



# Pharmacological Application of Growth Factors: Basic and Clinical

BioMed Research International

# Pharmacological Application of Growth Factors: Basic and Clinical

Guest Editors: Jian Xiao, Yadong Wang, Saverio Bellusci, and Xiaokun Li

# Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 141794, 2 pages
http://dx.doi.org/10.1155/2015/141794

*Editorial*

# Pharmacological Application of Growth Factors: Basic and Clinical

**Jian Xiao,[1] Yadong Wang,[2] Saverio Bellusci,[3] and Xiaokun Li[1]**

[1]*School of Pharmacy, Wenzhou Medical University, Chashan University-Town, Wenzhou 325035, China*
[2]*Department of Bioengineering, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[3]*Excellence Cluster Cardio Pulmonary System, University Justus Liebig Giessen, Aulweg 130, 35392 Giessen, Germany*

Correspondence should be addressed to Xiaokun Li; xiaokunli@163.net

Received 29 September 2014; Accepted 29 September 2014



Growth factors are signaling molecules that are typically secreted at the site of repair by many different cell types including platelets, stem cells, and fibroblasts. Since Montalcini and Hamburger first described the nerve growth factor (NGF) in 1951, this epoch-making discovery has initiated the detection of a multitude of other growth factors that alter cell growth, cell differentiation, and proliferation, which is essential for wound healing and tissue repair and regeneration. Several growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), and nerve growth factor (NGF) play a key role in brain or spinal cord injury and acute organ injuries. Vascular endothelial growth factor (VEGF) increases vascular permeability, induces angiogenesis, vasculogenesis, which is applied in treatment of ischemic heart disease.

Treatment with growth factors is beginning to gain worldwide prevalence, mainly in plastic and reconstructive surgery. However, the molecular mechanisms of growth factors treatment are still undefined. Thus, further investigations on mechanisms of growth factors in basic and clinical research are urgent. Therefore, we have invited the researchers to contribute few research/review papers to provide evidence that supports the application of growth factor in prevention or treatment of diseases.

In this special issue, we have invited some papers hoping to shed light on some aspects of this very interesting field. We have collected 8 papers by scientists from 4 countries. In the submitted research papers, H. Wang et al. summarize the current understanding of the NGF signaling in retina and the therapeutic implications in the treatment of glaucoma. NGF offers the promise of actually restoring visual function through acting on the TrkA receptor; however, the future of NGF-dependent treatments in the armamentarium of glaucoma therapy as most of the present studies were in animal models, hence, randomized, controlled glaucoma clinical trials need to be performed to evaluate the therapeutic effect of NGF in the treatment of glaucoma. While M. Ammendola et al. review antitumor and antiangiogenic potential of three agents which are able to inhibit the functions of mast cells (MCs) tryptase: gabexate mesylate, nafamostat mesylate, and tranilast, the authors suggest that future awaited clinical studies aim to evaluate the truly efficacy of the tryptase inhibitors as a novel tumor antiangiogenic therapy. J. Cai et al. concluded the neuroprotective efficacy of neurotrophins (NTs) (NGF, BDNF, FGF-2, IGF, NT3, and NT4/5) in animal models, highlighted outstanding technical challenges, and discussed more recent attempts to harness the neuroprotective capacity of endogenous NTs using small molecule inducers and cell transplantation. On the other hand, J.-C. Chen and colleagues demonstrated that NGF exist multiple bioactivity except for the neuronprotective activity. They found NGF accelerates the healing of skin excisional wounds in rats and the fibroblast migration induced by NGF may contribute to this healing process; moreover, the activation of PI3K/Akt, Rac1, JNK, and ERK may be involved in the regulation of NGF-induced fibroblast migration. In two very interesting research papers, Z.-G. Feng et al. have shown that tobacco plants express Keratinocyte Growth Factor (KGF1) via Agrobacterium-mediated transformation using a Potato virus X- (PVX-) based vector (pgR107). The plant-derived

KGF1 promotes the proliferation of NIH/3T3 cells and significantly stimulates wound healing in the diabetic wounded rat model. This finding indicated that KGF1 from tobacco maintains its biological activity, implying prospective industrial production in a plant bioreactor. While X.S Wang suggested endoplasmic reticulum (ER) stress is the key mechanism for regulating FGF21 in several metabolic diseases. This study showed FGF21 is the target gene for activating transcription factor 4 (ATF4) and CCAAT enhancer binding protein homologous protein (CHOP). ER stress increased the half-life of mRNA of FGF21, which may partly explain the mechanism of increasing FGF21 levels in metabolism disease. In the following papers, H. Nawa et al. discussed neuregulin-1 (NGR1) and EGF to rodent pups, juveniles, and adults and characterized neurobiological and behavioral consequences. The cytokine-driven dopaminergic dysfunction might illustrate some of the psychopathological features of schizophrenia, although it is possible that the responsible factors might be other cytokines other than EGF, NRG1, or virokine. L.-J. Xiang ea al. investigated the hair growth promoting activities of three approved growth factor drugs, FGF-10, FGF-1, and FGF-2. They observed that FGFs promoted hair growth by inducing the anagen phase in telogenic C57BL/6 mice. FGFs-treated group showed earlier induction of $\beta$-catenin and Sonic hedgehog (Shh) in hair follicles, suggesting that FGFs promotes hair growth by inducing the anagen phase in resting hair follicles and might be a potential hair growth-promoting agent. Finally, J. Song et al. summarized the recent findings on the association between risk factors for vascular dementia and adiponectin including aging, diabetes, hypertension, atherosclerosis, and stroke. The authors suggested that further studies are necessary to examine the role of adiponectin in vascular dementia, and the regulation of adiponectin levels and receptors of adiponectin would be important for the prevention and treatment of vascular dementia.

## Acknowledgments

Finally, we would like to thank all contributors to this special issue for their participation. We hope that this special issue will be helpful for the growth factor basic research and the development of new therapeutic drugs for clinical treatment.

*Jian Xiao*
*Yadong Wang*
*Saverio Bellusci*
*Xiaokun Li*

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 730139, 9 pages
http://dx.doi.org/10.1155/2015/730139

*Research Article*

# Fibroblast Growth Factors Stimulate Hair Growth through β-Catenin and Shh Expression in C57BL/6 Mice

**Wei-hong Lin,[1] Li-Jun Xiang,[2] Hong-Xue Shi,[2] Jian Zhang,[2] Li-ping Jiang,[3] Ping-tao Cai,[2] Zhen-Lang Lin,[4] Bei-Bei Lin,[2] Yan Huang,[2] Hai-Lin Zhang,[4] Xiao-Bing Fu,[5] Ding-Jiong Guo,[6] Xiao-Kun Li,[2] Xiao-Jie Wang,[2] and Jian Xiao[2]**

[1]*Department of Nurse, The first Affiliated Hospital, Wenzhou Medical University, Wenzhou 325035, China*
[2]*Molecular Pharmacology Research Center, School of Pharmacy, Wenzhou Medical University, Wenzhou 325035, China*
[3]*School of Nurse, Wenzhou Medical University, Wenzhou 325035, China*
[4]*Department of Pediatrics, The Second Affiliated Hospital, Wenzhou Medical University, Wenzhou 325000, China*
[5]*Wound Healing and Cell Biology Laboratory, Institute of Basic Medical Science, Chinese PLA General Hospital, Beijing 100853, China*
[6]*Department of General Surgery, Cixi People's Hospital, Wenzhou Medical University, Ningbo, China*

Correspondence should be addressed to Xiao-Jie Wang; wangxiaojie1972@126.com and Jian Xiao; xfxj2000@126.com

Received 7 March 2014; Accepted 31 August 2014

Academic Editor: Saverio Bellusci



Growth factors are involved in the regulation of hair morphogenesis and cycle hair growth. The present study sought to investigate the hair growth promoting activities of three approved growth factor drugs, fibroblast growth factor 10 (FGF-10), acidic fibroblast growth factor (FGF-1), and basic fibroblast growth factor (FGF-2), and the mechanism of action. We observed that FGFs promoted hair growth by inducing the anagen phase in telogenic C57BL/6 mice. Specifically, the histomorphometric analysis data indicates that topical application of FGFs induced an earlier anagen phase and prolonged the mature anagen phase, in contrast to the control group. Moreover, the immunohistochemical analysis reveals earlier induction of β-catenin and Sonic hedgehog (Shh) in hair follicles of the FGFs-treated group. These results suggest that FGFs promote hair growth by inducing the anagen phase in resting hair follicles and might be a potential hair growth-promoting agent.

## 1. Introduction

Hair is considered accessory structure of the integument along with sebaceous glands, sweat glands, and nails. Hair follicle morphogenesis requires the intricately controlled regulation of apoptosis, proliferation, and differentiation. Hair follicles are miniorgans that, during postnatal life, cycle through periods of anagen (growth phase), catagen (regression phase), and telogen (resting phase) [1, 2]. Hair loss is generally not a life-threatening event, but the number of patients suffering from it has increased dramatically. Meanwhile, hair loss takes impact on social interactions and patients' psychological well beings. To date, there are only two anti-hair loss drugs, finasteride and minoxidil, which have been used in clinical, but the effect of these drugs is limited, transient, and somewhat unpredictable [3]. Therefore, it is urgent to develop novel pharmacological treatments.

Various cytokines and growth factors are involved in the regulation of hair morphogenesis and cycle hair growth. The fibroblast growth factor (FGF) family is composed of 22 members with a wide range of biological functions involved in angiogenesis, embryonic development, cell growth, and tissue repair [4, 5]. The early literature reported that acidic fibroblast growth factor (aFGF or FGF-1) and basic fibroblast growth factor (bFGF or FGF-2) may affect the growth of hair follicles, but people have different conclusion. D.C DL demonstrated that exogenous FGF-1 and FGF-2 interfere with follicle morphogenesis and ultimately suppress the hair cycle [6, 7]. On the other hand, Katsuoka et al. reported that FGF-2 promotes papilla cell proliferation and the increase in the

size of hair follicle in mice [8, 9]. The controlled release study also showed that gelatin hydrogel enables FGF-2 to positively act on the hair growth cycle of mice [10, 11]. Recent evidence explored another FGF, named keratinocyte growth factor 2 (KGF-2 or FGF-10), which also significantly stimulated human hair-follicle cell proliferation in organ culture [12]. Moreover, FGF-1 has been identified as a crucial endogenous mediator of normal hair follicle growth, development, and differentiation [13], but KGF is not required for wound healing [14]. Therefore, it is necessary for enhanced *in vivo* efficacy to contrive the administration form of FGF.

It should be noted that FGF-1, FGF-2, and FGF-10 have been approved by SFDA for wound healing, and China is the only country in the world for clinical application of these drugs. Thus, the commercial growth factors were used in this study, the hair growth promoting activity was scientifically proven, and the mechanism of action was investigated.

## 2. Materials and Methods

*2.1. Materials.* The DAB chromogen kit (ZSGB-BIO, Beijing, China) was purchased. Anti-$\beta$-catenin (rabbit polyclonal antibody, Abcam, UK), anti-FGF9 (rabbit polyclonal antibody, Abcam, UK), and anti-Shh (rabbit polyclonal antibody, Santa Cruz Biotech, Santa Cruz, CA, USA) antibodies were purchased. Hematoxylin (Beyotime Institute of Biotechnology, China) and eosin (Beyotime Institute of Biotechnology, China) were purchased. FGF-1 (Shanghai Wanxing Co.), FGF-2 (Zhuhai Essexbio Co.), and FGF-10 (Anhui Xinhuakun Co.) were applied to the experimental group. The concentration of FGFs was diluted at 500 $\mu$g/mL.

*2.2. Experimental Animals.* Healthy C57BL6/N mice (6-week-old, 15 mice per group) were obtained from Laboratory Animals Center of Wenzhou Medical University. All animals were from the Laboratory Animals Center of Wenzhou Medical University and were treated strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were carried out with the approval of the Animal Experimentation Ethics Committee of Wenzhou Medical University. Temperature ($23 \pm 2^{\circ}$C), humidity (35–60%), and photoperiod (12 h light and 12 h darkness cycle) were kept constant.

*2.3. Experimental Studies with FGFs.* 60 animals in 4 randomized groups ($n = 15$) were used for the study of hair promoting activity. All animals were shaved using depilatory cream (Veet, USA) at 6 weeks of age, at which all hair follicles were synchronized in the telogen stage. FGF-10, FGF-1, FGF-2 (all 500 $\mu$g/mL dissolved in 10 $\mu$L normal saline, 5 $\mu$g/12 cm$^2$ per mouse), or vehicle (100 $\mu$L normal saline) was applied topically on dorsal skin of C57BL/6N mice with subcutaneous injection for 14 d. At every 1, 7, 14, and 28 d, three mice of each group were sacrificed to obtain skin specimen. Visible hair growth was recorded at every 1, 7, 14, and 28 d.

*2.4. Histological Studies.* Dorsal skin was excised after topical application with FGFs at the indicated time points. Dorsal skin was maintained in 4% paraformaldehyde at 4°C and embedded in paraffin blocks to obtain longitudinal and transverse section. 5 mm sections were stained with hematoxylin and eosin (H&E). The other skin was stored at −80°C for protein extraction. Digital photomicrographs were taken from representative areas at a fixed magnification of 100x.

*2.5. Hair Follicle Count.* The H&E stained slides were photographed using a digital photomicrograph and all of the images were cropped in a fixed area of 300 pixels width. We counted hair follicles manually in a fixed area (0.09 mm$^2$). Digital photomicrographs were taken from representative areas at a fixed magnification of 100x.

*2.6. Hair Length Determination.* Hairs were plucked randomly from shaved dorsal area at 1, 7, 14, 21, and 28 days. After plucking 20 hairs per mouse, we measured the average hair length manually.

*2.7. Immunohistochemistry.* Dorsal skins were stained with anti-$\beta$-catenin and Sonic hedgehog (Shh) antibodies. Sections were dewaxed and hydrated. To quench endogenous peroxidase activity, deparaffinized sections were pretreated with 3% peroxidase for 10 min. After washing with PBS, the sections were incubated with serum to block nonspecific binding of biotinylated secondary antibody for 30 min and then incubated with anti-$\beta$-catenin (1 : 200) and Shh (1 : 500) antibodies overnight at 4°C. Slides were incubated with biotinylated secondary antibody for 30 min. After incubating with HRP-streptavidin complex to detect secondary antibody for 30 min, slides were developed until light brown staining was visible with DAB chromogen kit. The immunopositivity in fields was counted for per sections using Image-Pro Plus software (Nikon, Tokyo, Japan).

*2.8. Statistical Analysis.* Results are expressed as mean ± SD. Statistical significance was determined with Student's $t$-test when there were two experimental groups. For more than two groups, statistical evaluation of the data was performed using One-way Analysis-of-variance (ANOVA) test, followed by Dunnett's post hoc test with the values $P < 0.05$ considered significant.

## 3. Results

*3.1. The Effect of FGFs on Hair Growth.* The black pigmentation was taken as evidence for transition of hair follicles from telogen to anagen phase. To evaluate the hair growth activity of FGFs, we topically applied FGF-1, FGF-2, and FGF-10 on the shaved dorsal skin of telogenic C57BL/6 mice for 14 d. Each week, we evaluated the degree of hair growth by observing the skin color. At 2 weeks, three FGFs induced black coloration in the shaved skin of C57BL/6 mice significantly; FGF-10 group showed the most black coloration, while very less visible hair growth and black coloration were observed in control group (Figure 1(a)). At 3 weeks, the FGFs group showed markedly hair growth, and FGF-10 stimulated hair growth over 1/2 area on the shaved

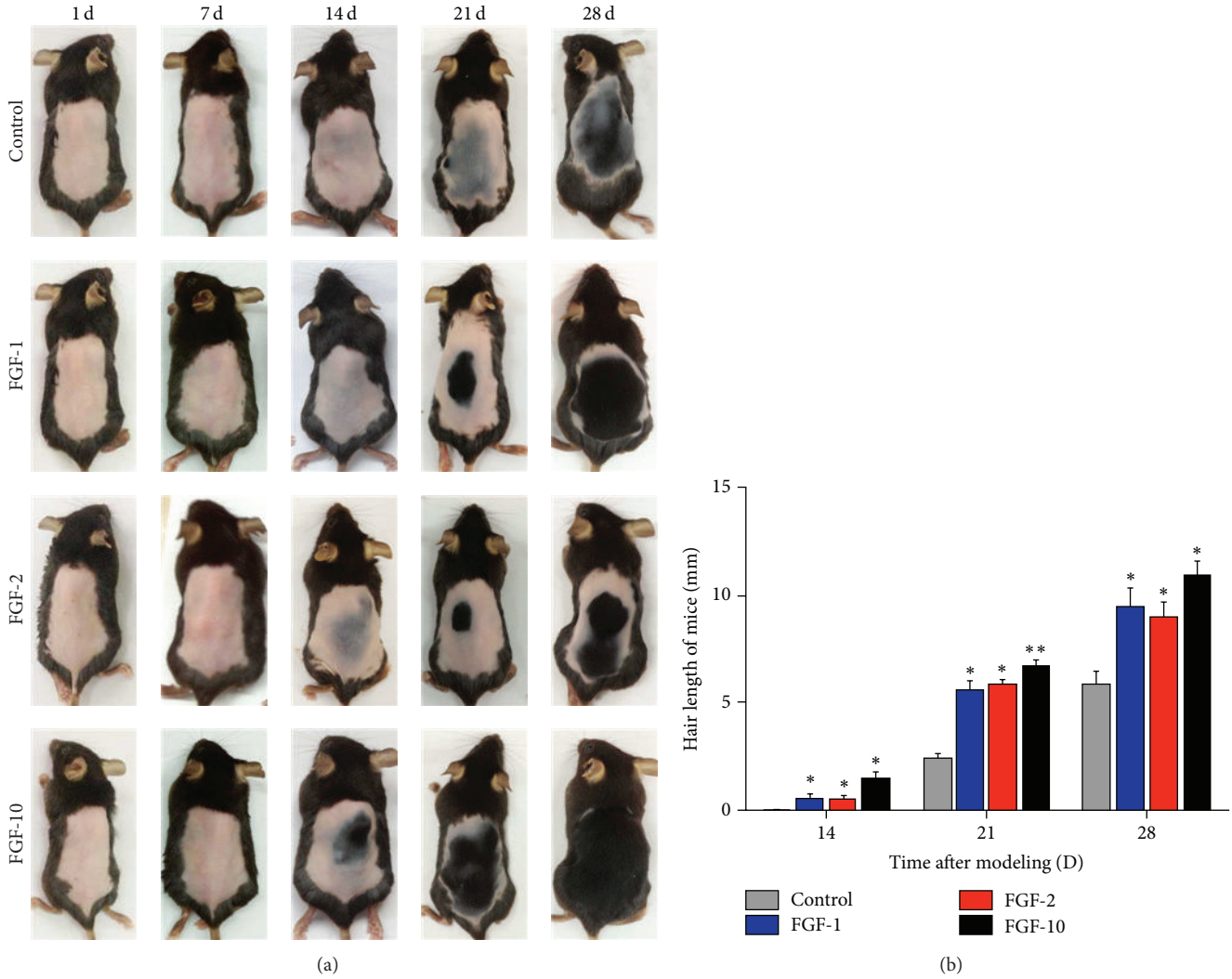
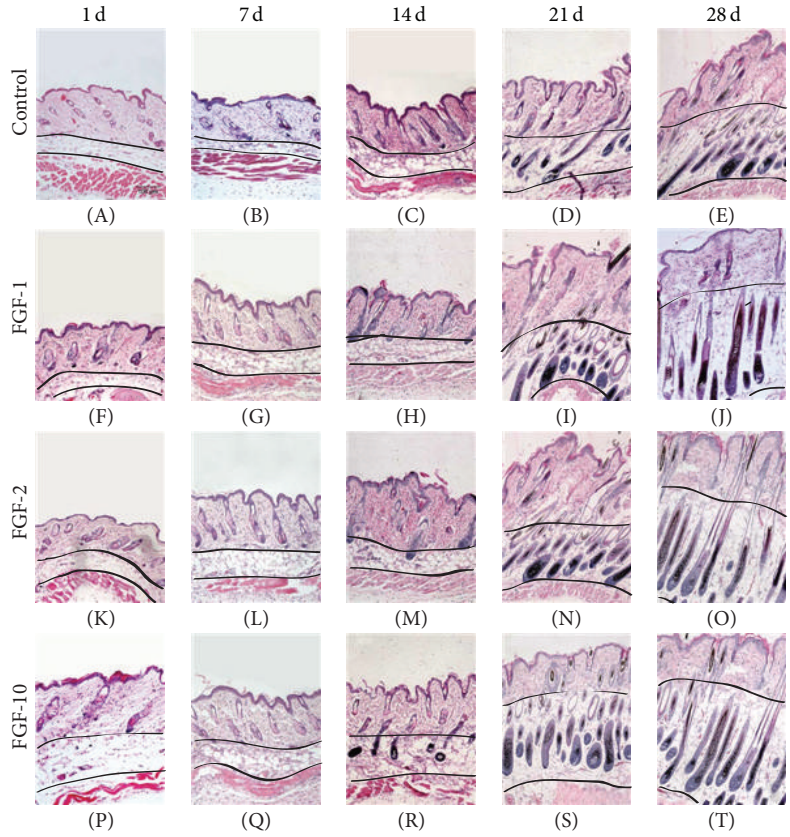


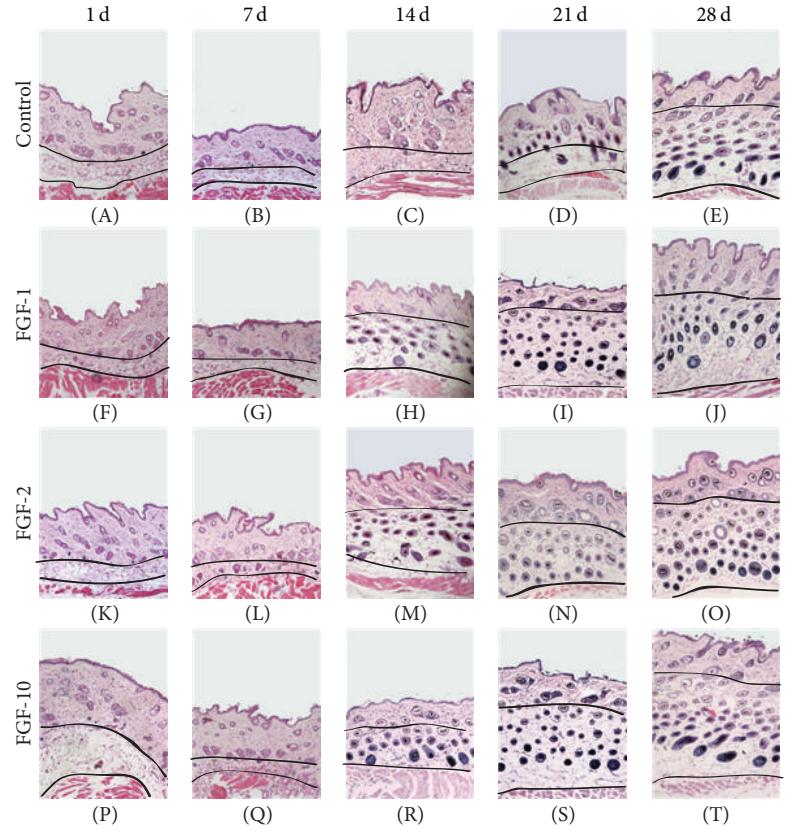

Figure 1: Hair growth promoting effect of FGFs. (a) 6-week-old C57BL6/N mice were shaved and topically applied with vehicle, FGF-1, FGF-2, and FGF-10. Photographs were taken every week after applying FGFs or vehicle on the shaved dorsal skin. (b) Hair length was measured after topical application of FGFs. The hair length of randomly plucked hairs ($n = 10$) was measured at 14, 21, and 28 days after topical application of FGFs. Data shown represent means ± S.D, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

dorsal skin. At 4 weeks, we observed that hair growth from FGF-1 and FGF-2 was confined to the proximal parts of epidermis; FGF-10 treated group showed overall hair growth which was not confined to the proximal parts. However, the control group only showed less hair growth (Figure 1(a)). To confirm whether FGFs promoted hair growth, we measured the length of 10 hairs plucked from the dorsal skin of each mouse at 2, 3, and 4 weeks. Since visible hair shaft was observed after 2 weeks, we measured length of 2-, 3-, and 4-week-old hairs. As shown in Figure 1(b), the length of hairs in FGF-10, FGF-1, and FGF-2 treated group was remarkably longer than that of control group, and FGF-10 exists with the strongest activity of hair growth. Taken together, these results indicated commercialized drugs, FGF-10, FGF-1, and FGF-2, promote hair growth and FGF-10 appears with the highest efficiency in the three protein drugs.

*3.2. Effect of FGFs on Hair Follicle Number.* It has reported increasing in the number and the size of hair follicles during anagen phase induction [15]. An increase in the density of hair follicles is an indicator for the transition of hair growth from the telogen to anagen phases [16, 17]. To investigate the progression of hair follicles in the hair cycle, HE staining was performed. In the representative longitudinal and transverse sections, the hair follicles in FGF-10, FGF-1, and FGF-2 treated group appeared earlier than those in the control group (Figures 2(a) and 2(b)). Meanwhile, the number of hair follicles of the relative area in FGFs treated group was higher than in the control group, consistent with the above results, and topical application of FGF-10 showed the maximum amount of hair follicles as compared to FGF-1 and FGF-2 group (Figure 2(c)). These data suggested that FGFs including FGF-10, FGF-1, and FGF-2 stimulate hair growth by inducing anagen phase of hair follicles.

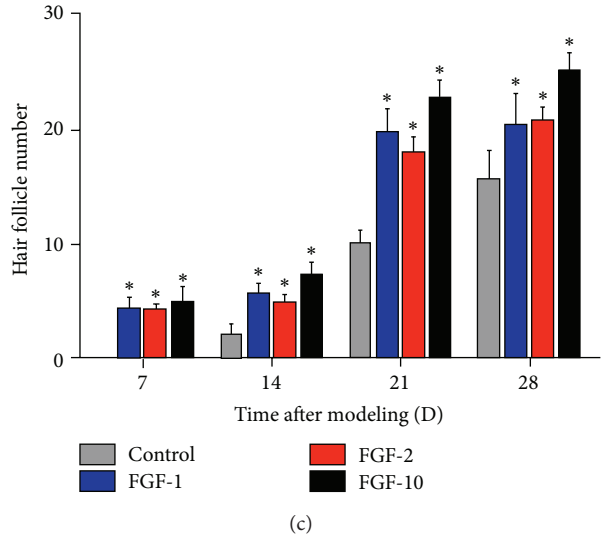



(a)




(b)

Figure 2: Continued.




(c)

Figure 2: The effect of FGFs on the hair follicles was analyzed by H&E staining. (a) Longitudinal sections of the dorsal skins. (b) Transverse sections of the dorsal skins. (c) The number of hair follicles in deep subcutis. Data shown represent means ± S.D, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

*3.3. FGFs Induced the Expression of β-Catenin and Sonic hedgehog (Shh).* Evidence showed that β-catenin induced the transition of the hair growth cycle from the telogen to anagen phases [18, 19]. To elucidate the mechanism of the early events of anagen induction by FGFs, immunohistochemistry analysis was performed to detect the expression of β-catenin. We observed that β-catenin protein expression appears in FGF-10, FGF-1, and FGF-2 at 1 week, and the levels of β-catenin were higher in FGFs group than in control group at 2 weeks (Figure 3). It should be noted that, at 3 weeks, the expression of β-catenin was remarkable in control group, while it is decreased in FGF-10 group compared to the appearance at 2 weeks. At 4 weeks, only the control group showed significant expression of β-catenin.

The secreted signaling molecule Sonic hedgehog (Shh) plays an important role in both embryonic and adult hair development. In adult mice, Shh expression is upregulated in early anagen, and ectopic application of Shh can prematurely induce anagen in resting telogen follicles [20]. Immunohistochemical analysis result showed that Shh expression was upregulated in FGF-10, FGF-1, and FGF-2 treated group compared to that in control group at 2 weeks (Figure 4). Taking together, these data indicated that the three FGFs drugs promote hair growth partly through upregulating β-catenin and Shh.

## 4. Discussion

Hair loss disorders are not life-threatening, but it may make afflicte the people vulnerable and lower their quality of life [21]. The estimated annual market value for hair growth promoting agents is multibillion dollars in the all world. Minoxidil is a widely used hair growth promoting drug for androgenic alopecia patients by inducing hair follicles in the telogen stage to undergo transition into the anagen stages [22]; however, it would also cause adverse dermatological effects, such as dryness, scaling, local irritation, and dermatitis [23, 24]. Finasteride has been reported to be efficacious for androgenic alopecia patients, but it is not recommended for female patients [25]. Therefore, developing new drugs for promoting hair growth is urgently.

Several growth factors (e.g., FGF-1, FGF-2, FGF-7, FGF-10, IGF-1, IGF-2, and EGF) can promote cell cycle and proliferation and have the potential to rescue hair loss and facilitate hair cell regeneration *in vivo* and *in vitro*. It has been shown that EGF and transforming growth factor-α (TGF-α) are contributed to hair cell proliferation and regeneration in avian utricles [26]. Regeneration of lost hair cells has also been found in rat utricular after treatment with FGF-2 and IGF-1 [27]. KGF protects hair follicles from cell death induced by UV irradiation, chemotherapeutic, or cytotoxic agents [28]. EGF, FGF-1, or FGF-2 maintains high proliferation and multipotent potential of human hair follicle-derived mesenchymal stem cells [29]. All of this literature demonstrated that growth factors may be potential for treating hair loss. Therefore, we searched all of the growth factors which proved by SFDA (China) and we found that FGF-1, FGF-2, and KGF have been applicated in clinical for wound healing, so we purchased these three drugs and investigated the hair growth promoting activity *in vivo*.

C57BL/6 mice are useful models for screening hair growth promoting agents, as their truncal pigmentation is dependent on their follicular melanocytes, producing pigment only during anagen [30]. The shaved back skins of C57BL6/N were treated with topical application of FGFs for 1, 2, 3, and 4 weeks. At 2 weeks, FGF-1, FGF-2, and FGF-10 induced hair growth in the telogenic C57BL/6 mice, while no less visible hair growth was observed in the control group. To further investigate the hair growth promoting effect, we plucked 10 hairs per mouse randomly from the

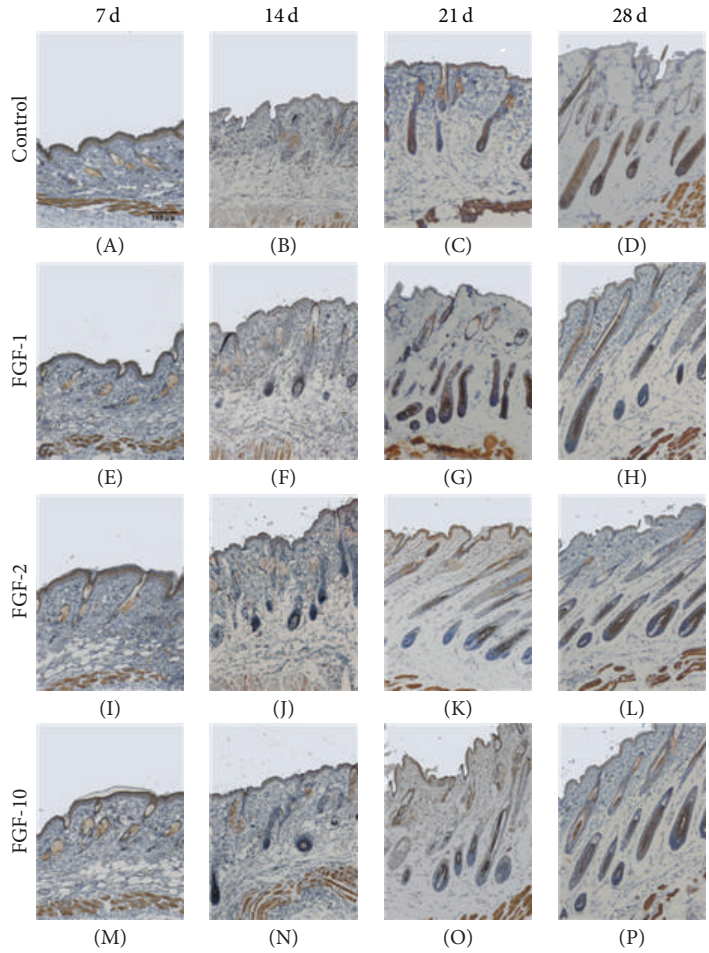



Figure 3: The expression of $\beta$-catenin after topical application of FGFs. Longitudinal sections of the dorsal skins from each group were stained for $\beta$-catenin by immunohistochemistry (brown staining). Digital photomicrographs were taken from representative areas at a fixed magnification of 100x.

treated area and measured the hair length. The hair length of FGFs treated mice was significantly longer than that of control group. Although FGF-2 is one of the most well-known mitogenic cytokine, interestingly, FGF-2 is not the strongest mitogenic cytokine for hair growth and FGF-10 exerts more potential hair growth promoting effect than FGF-1 and FGF-2. As we know, hair-follicle morphogenesis is governed by epithelial-mesenchymal interactions, between hair placode keratinocytes and fibroblasts of underlying mesenchymal condensations [31]. FGF-10 is found in the dermal papilla fibroblasts and its receptor FGFR2IIIb is found in the neighboring outer root sheath of the keratinocytes [32], suggesting that FGF-10 is a mesenchymally derived stimulator of hair-follicle cells, which contribute to the hair promoting activity.

The Wnt/$\beta$-catenin pathway plays an important role in the initiation, development, and growth of hair follicles. The transient activation of $\beta$-catenin results in hair regrowth in mice, while ablation of $\beta$-catenin results in dramatic hair shortening and abnormal regeneration of hair in the dermal papilla of mouse hair follicles [19, 33]. The levels of $\beta$-catenin in the dermal papilla are high in the anagen phase but low in the catagen and the telogen phases [18, 34]. Furthermore, the interaction between $\beta$-catenin, androgen receptors, and keratinocyte growth inhibition through modification of Wnt signaling contributes to androgenic alopecia, a common form of hair loss [35, 36]. Like $\beta$-catenin, Sonic hedgehog (Shh) also plays a vital role in the morphogenesis of hair follicles and acts as anagen-inducing signaling molecules. Mice lacking Shh activity exhibits follicles arrested at the hair germ stage of development [37, 38]. In the adult, Shh serves as a key regulator to induce the transition from the resting (telogen) to the growth stage (anagen) of the hair follicle cycle [20, 39]. Conversely, antibodies that block the activity of Shh are able to prevent hair growth in adult mice [40]. To elucidate the molecular mechanism underlying the ability of FGFs to induce anagen hair follicles, we examined the protein levels of $\beta$-catenin and Shh and in the shaved dorsal skin. Our immunohistochemical analysis results showed that the expression levels of $\beta$-catenin and Shh were upregulated in FGF-10, FGF-1, and FGF-2 treated group compared to that in the control group at 14 days. Its reported continuous $\beta$-catenin signaling is required to maintain hair follicle tumors [41]; we observed that Shh and $\beta$-catenin expression levels

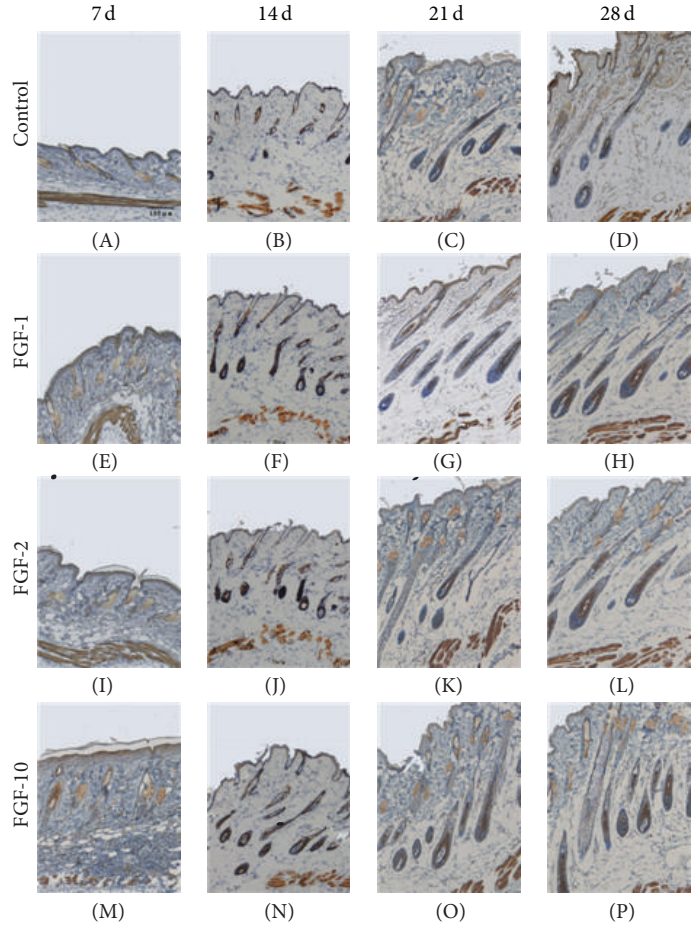



Figure 4: The expression of Shh after topical application of FGFs. Longitudinal sections of the dorsal skins from each group were stained for Shh by immunohistochemistry (brown staining). Digital photomicrographs were taken from representative areas at a fixed magnification of 100x.

gradually began to reduce in both groups at 3 and 4 weeks, indicating that anagen phase of hair follicles was ceased.

However, there is no doubt that the limitations of this study still need further investigations and improvements. For example, FGFs are not stable enough which is easy to be degradated by various enzymes *in vitro*, resulting in the loss of biological activity. So the combination with delivery systems to increase its stability may contribute to the functions of promoting hair growth. Moreover, mixtures of several growth factors might to some extent promote cooperation between growth factors and their receptors and contribute together for the protection of hair cells in a sequencing manner or at multiple steps. Further study also should consider the hair growth effect and the mechanism of these FGFs during wound healing. Nevertheless, the effect of FGF-1, FGF-2, and FGF-10 in the therapy of hair loss is confirmative and feasible.

Collectively, our study demonstrated that the commercialized FGF drugs promoted hair growth by inducing anagen in telogenic C57BL6/N mice. FGFs showed significant increase in the number and the size of hair follicles that is considered evidence for anagen phase induction. Immunohistochemical analysis revealed that $\beta$-catenin and Shh were expressed earlier in FGFs treated group than that in control group. Taken together, these results strongly suggest that FGF-1, FGF-2, and FGF-10 promote hair growth by inducing anagen phase of hair follicles, which is beneficial for the clinical therapy.

## Conflict of Interests

The authors have declared that no competing interests exist.

## Authors' Contribution

Wei-hong Lin, Li-Jun Xiang, and Hong-Xue Shi contributed equally to this work.

## Acknowledgments

This work was supported by Zhejiang Provincial Program for the Cultivation of High-level Innovative Health talents (to Jian Xiao), National Natural Science Funding of China (81372112 and 81372064), Zhejiang Provincial Natural Science Foundation (Y14H150023), Wenzhou Project of Science and Technology (Y20130246), State Key Basic Research Development Program (2012CB518105), and Ningbo City Natural

Science Foundation (2012A610254). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

## References


[1] H. Wosicka and K. Cal, "Targeting to the hair follicles: current status and potential," *Journal of Dermatological Science*, vol. 57, no. 2, pp. 83–89, 2010.

[2] G. Cotsarelis, "Epithelial stem cells: a folliculocentric view," *Journal of Investigative Dermatology*, vol. 126, no. 7, pp. 1459–1468, 2006.

[3] R. Jain and W. De-Eknamkul, "Potential targets in the discovery of new hair growth promoters for androgenic alopecia," *Expert Opinion on Therapeutic Targets*, vol. 18, no. 7, pp. 787–806, 2014.

[4] H. X. Shi, C. Lin, B. B. Lin et al., "The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo," *PLoS ONE*, vol. 8, no. 4, Article ID e59966, 2013.

[5] D. L. Coutu and J. Galipeau, "Roles of FGF signaling in stem cell self-renewal, senescence and aging," *Aging*, vol. 3, no. 10, pp. 920–933, 2011.

[6] D. L. du Cros, "Fibroblast growth factor and epidermal growth factor in hair development," *Journal of Investigative Dermatology*, vol. 101, no. 1, pp. 106S–113S, 1993.

[7] D. L. du Cros, "Fibroblast growth factor influences the development and cycling of murine hair follicles," *Developmental Biology*, vol. 156, no. 2, pp. 444–453, 1993.

[8] K. Katsuoka, H. Schell, B. Wessel, and O. P. Hornstein, "Effects of epidermal growth factor, fibroblast growth factor, minoxidil and hydrocortisone on growth kinetics in human hair bulb papilla cells and root sheath fibroblasts cultured in vitro," *Archives of Dermatological Research*, vol. 279, no. 4, pp. 247–250, 1987.

[9] K. Katsuoka, H. Schell, O. P. Hornstein, and B. Wessel, "Epidermal growth factor and fibroblast growth factor accelerate proliferation of human hair bulb papilla cells and root sheath tibroblasts cultured *in vitro*," *British Journal of Dermatology*, vol. 116, no. 3, pp. 464–465, 1987.

[10] M. Ozeki and Y. Tabata, "Promoted growth of murine hair follicles through controlled release of basic fibroblast growth factor," *Tissue Engineering*, vol. 8, no. 3, pp. 359–366, 2002.

[11] M. Ozeki and Y. Tabata, "In vivo promoted growth of mice hair follicles by the controlled release of growth factors," *Biomaterials*, vol. 24, no. 13, pp. 2387–2394, 2003.

[12] J.-H. Jang, "Stimulation of human hair growth by the recombinant human keratinocyte growth factor-2 (KGF-2)," *Biotechnology Letters*, vol. 27, no. 11, pp. 749–752, 2005.

[13] D. M. Danilenko, B. D. Ring, D. Yanagihara et al., "Keratinocyte growth factor is an important endogenous mediator of hair follicle growth, development, and differentiation: normalization of the nu/nu follicular differentiation defect amelioration of chemotherapy-induced alopecia," *The American Journal of Pathology*, vol. 147, no. 1, pp. 145–154, 1995.

[14] L. Guo, L. Degenstein, and E. Fuchs, "Keratinocyte growth factor is required for hair development but not for wound healing," *Genes and Development*, vol. 10, no. 2, pp. 165–175, 1996.

[15] M. Nakamura, M. R. Schneider, R. Schmidt-Ullrich, and R. Paus, "Mutant laboratory mice with abnormalities in hair follicle morphogenesis, cycling, and/or structure: an update," *Journal of Dermatological Science*, vol. 69, no. 1, pp. 6–29, 2013.

[16] H.-S. Shin, J.-M. Lee, S.-Y. Park, J.-E. Yang, J.-H. Kim, and T.-H. Yi, "Hair growth activity of Crataegus pinnatifida on C57BL/6 mouse model," *Phytotherapy Research*, vol. 27, no. 9, pp. 1352–1357, 2013.

[17] H.-J. Park, N. Zhang, and D. K. Park, "Topical application of *Polygonum multiflorum* extract induces hair growth of resting hair follicles through upregulating Shh and $\beta$-catenin expression in C57BL/6 mice," *Journal of Ethnopharmacology*, vol. 135, no. 2, pp. 369–375, 2011.

[18] B. Bierie, M. Nozawa, J.-P. Renou et al., "Activation of $\beta$-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation," *Oncogene*, vol. 22, no. 25, pp. 3875–3887, 2003.

[19] D. van Mater, F. T. Kolligs, A. A. Dlugosz, and E. R. Fearon, "Transient activation of $\beta$-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice," *Genes & Development*, vol. 17, no. 10, pp. 1219–1224, 2003.

[20] N. Sato, P. L. Leopold, and R. G. Crystal, "Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog," *Journal of Clinical Investigation*, vol. 104, no. 7, pp. 855–864, 1999.

[21] M. Patel, S. Harrison, and R. Sinclair, "Drugs and hair loss," *Dermatologic Clinics*, vol. 31, no. 1, pp. 67–73, 2013.

[22] S. Gregoriou, D. Papafragkaki, G. Kontochristopoulos, E. Rallis, D. Kalogeromitros, and D. Rigopoulos, "Cytokines and other mediators in alopecia areata," *Mediators of Inflammation*, vol. 2010, Article ID 928030, 5 pages, 2010.

[23] G. M. Gelfuso, T. Gratieri, M. B. Delgado-Charro, R. H. Guy, and R. F. Vianna Lopez, "Iontophoresis-targeted, follicular delivery of minoxidil sulfate for the treatment of alopecia," *Journal of Pharmaceutical Sciences*, vol. 102, no. 5, pp. 1488–1494, 2013.

[24] J. K. Tarlow, F. E. Clay, M. J. Cork et al., "Severity of alopecia areata is associated with a polymorphism in the interleukin-1 receptor antagonist gene," *Journal of Investigative Dermatology*, vol. 103, no. 3, pp. 387–390, 1994.

[25] A. V. D'Amico and C. G. Roehrborn, "Effect of 1 mg/day finasteride on concentrations of serum prostate-specific antigen in men with androgenic alopecia: a randomised controlled trial," *The Lancet Oncology*, vol. 8, no. 1, pp. 21–25, 2007.

[26] A. Zine and F. de Ribaupierre, "Replacement of mammalian auditory hair cells," *NeuroReport*, vol. 9, no. 2, pp. 263–268, 1998.

[27] J. L. Zheng, C. Helbig, and W.-Q. Gao, "Induction of cell proliferation by fibroblast and insulin-like growth factors in pure rat inner ear epithelial cell cultures," *Journal of Neuroscience*, vol. 17, no. 1, pp. 216–226, 1997.

[28] S. Braun, M. Krampert, E. Bodo et al., "Keratinocyte growth factor protects epedermis and hair follicles from cell death induced by UV irradiation, chemotherapeutic or cytotoxic agents," *Journal of Cell Science*, vol. 119, no. 23, pp. 4841–4849, 2006.

[29] X. Zhang, Y. Wang, Y. Gao et al., "Maintenance of high proliferation and multipotent potential of human hair follicle-derived mesenchymal stem cells by growth factors," *International Journal of Molecular Medicine*, vol. 31, no. 4, pp. 913–921, 2013.

[30] P. M. Plonka, D. Michalczyk, M. Popik, B. Handjiski, A. Slominski, and R. Paus, "Splenic eumelanin differs from hair eumelanin in C57BL/6 mice," *Acta Biochimica Polonica*, vol. 52, no. 2, pp. 433–441, 2005.

[31] A. Osada and K. Kobayashi, "Appearance of hair follicle-inducible mesenchymal cells in the rat embryo," *Development Growth and Differentiation*, vol. 42, no. 1, pp. 19–27, 2000.

[32] S. Saksena, S. Priyamvada, A. Kumar et al., "Keratinocyte growth factor-2 stimulates P-glycoprotein expression and function in intestinal epithelial cells," *The American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 304, no. 6, pp. G615–G622, 2013.

[33] D. Enshell-Seijffers, C. Lindon, M. Kashiwagi, and B. A. Morgan, "$\beta$-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair," *Developmental Cell*, vol. 18, no. 4, pp. 633–642, 2010.

[34] Y. Ouji, M. Yoshikawa, A. Shiroi, and S. Ishizaka, "Wnt-10b promotes differentiation of skin epithelial cells in vitro," *Biochemical and Biophysical Research Communications*, vol. 342, no. 1, pp. 28–35, 2006.

[35] R. Singh, J. N. Artaza, W. E. Taylor et al., "Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with $\beta$-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors," *Endocrinology*, vol. 147, no. 1, pp. 141–154, 2006.

[36] M. Ito, Z. Yang, T. Andl et al., "Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding," *Nature*, vol. 447, no. 7142, pp. 316–320, 2007.

[37] C. Chiang, R. Z. Swan, M. Grachtchouk et al., "Essential role for Sonic hedgehog during hair follicle morphogenesis," *Developmental Biology*, vol. 205, no. 1, pp. 1–9, 1999.

[38] B. St-Jacques, H. R. Dassule, I. Karavanova et al., "Sonic hedgehog signaling is essential for hair development," *Current Biology*, vol. 8, no. 19, pp. 1058–1068, 1998.

[39] K. S. Stenn and R. Paus, "Controls of hair follicle cycling," *Physiological Reviews*, vol. 81, no. 1, pp. 449–494, 2001.

[40] L. C. Wang, Z.-Y. Liu, L. Gambardella et al., "Conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration," *Journal of Investigative Dermatology*, vol. 114, no. 5, pp. 901–908, 2000.

[41] C. L. Celso, D. M. Prowse, and F. M. Watt, "Transient activaton of $\beta$-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours," *Development*, vol. 131, no. 8, pp. 1787–1799, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 154702, 16 pages
http://dx.doi.org/10.1155/2014/154702

*Review Article*

# Targeting Mast Cells Tryptase in Tumor Microenvironment: A Potential Antiangiogenetic Strategy

**Michele Ammendola,[1] Christian Leporini,[2] Ilaria Marech,[3] Cosmo Damiano Gadaleta,[3] Giovanni Scognamillo,[4] Rosario Sacco,[1] Giuseppe Sammarco,[1] Giovambattista De Sarro,[2] Emilio Russo,[2] and Girolamo Ranieri[3]**

[1] *Department of Medical and Surgery Sciences, Clinical Surgery Unit, University "Magna Graecia" Medical School, Viale Europa, Germaneto, 88100 Catanzaro, Italy*
[2] *Department of Health Science, Clinical Pharmacology and Pharmacovigilance Unit and Pharmacovigilance's Centre Calabria Region, University of Catanzaro "Magna Graecia" Medical School, Viale Europa, Germaneto, 88100 Catanzaro, Italy*
[3] *Diagnostic and Interventional Radiology Unit with Integrated Section of Translational Medical Oncology, Istituto Tumori "Giovanni Paolo II," Viale Orazio Flacco 65, 70124 Bari, Italy*
[4] *Radiotherapy Unit, Istituto Tumori "Giovanni Paolo II," Viale Orazio Flacco 65, 70124 Bari, Italy*

Correspondence should be addressed to Girolamo Ranieri; giroran@tiscalinet.it

Received 13 February 2014; Revised 14 August 2014; Accepted 19 August 2014; Published 11 September 2014

Academic Editor: Xiaokun Li



Angiogenesis is a complex process finely regulated by the balance between angiogenesis stimulators and inhibitors. As a result of proangiogenic factors overexpression, it plays a crucial role in cancer development. Although initially mast cells (MCs) role has been defined in hypersensitivity reactions and in immunity, it has been discovered that MCs have a crucial interplay on the regulatory function between inflammatory and tumor cells through the release of classical proangiogenic factors (e.g., vascular endothelial growth factor) and nonclassical proangiogenic mediators granule-associated (mainly tryptase). In fact, in several animal and human malignancies, MCs density is highly correlated with tumor angiogenesis. In particular, tryptase, an agonist of the proteinase-activated receptor-2 (PAR-2), represents one of the most powerful angiogenic mediators released by human MCs after c-Kit receptor activation. This protease, acting on PAR-2 by its proteolytic activity, has angiogenic activity stimulating both human vascular endothelial and tumor cell proliferation in paracrine manner, helping tumor cell invasion and metastasis. Based on literature data it is shown that tryptase may represent a promising target in cancer treatment due to its proangiogenic activity. Here we focused on molecular mechanisms of three tryptase inhibitors (gabexate mesylate, nafamostat mesylate, and tranilast) in order to consider their prospective role in cancer therapy.

## 1. Introduction

Angiogenesis is a complex process, mainly mediated by endothelial cells, consisting in the formation of new blood capillaries from existing vessels [1–4]. It is finely regulated by the balance between several angiogenesis stimulators, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF), angiopoietins, tryptase, and some angiogenesis inhibitors, including thrombospondin, angiostatin, and endostatin [5–11]. Angiogenesis, further than being involved in normal physiological processes, has been demonstrated to play a crucial role in cancer development inducing tumor growth, invasion, and metastasis [12, 13].

Mast cells (MCs) intervene in tissue angiogenesis through several classical proangiogenic factors such as VEGF, FGF-2, PDGF, interleukin-6 (IL-6), and nonclassical proangiogenic factors, such as tryptase and chymase, stored in their secretory granules [14–18]. In fact, MCs density is highly correlated with the extent of tumor angiogenesis both in benign tumors (e.g., in keloids) and in animal and human malignancies (systemic mastocytosis, head and neck, colorectal,

lung, and cutaneous cancer) [19–24]. Tryptase and chymase stimulate angiogenesis and the response is similar to that obtained with VEGF [16]. This evidence confirms even more the angiogenic activity of these two proteases stored in MCs granules [16].

## 2. Role of Mast Cell Tryptase in Angiogenesis and Tumor Growth

MCs are tissue leukocytes originating from hematopoietic stem cells in bone marrow. Generally, these precursor cells circulate in blood as agranular cells; then, MCs migrate into different tissues completing their maturation into granulated cells under the influence of several microenvironmental growth factors. One of these crucial factors is the stem cell factor (SCF), the ligand of c-Kit receptor (c-KitR) secreted by fibroblasts and stromal and endothelial cells. SCF is critically involved in MCs activation [25, 26]. MCs can be naturally found in association with connective tissue structures (i.e., blood vessels, lymphatic vessels, and nerves) and in the proximity of skin and mucosa of the gastrointestinal, respiratory, and genitourinary tracts [27], which represent common portals of infections [26, 28]. Accordingly, for many years, MCs have been implicated in the pathogenesis of IgE-associated allergic reactions and certain protective responses to parasites, bacteria, viruses, and fungi [29–31]. However, increasing evidence suggests the involvement of these cells in several biological settings, such as inflammation, immunomodulation, angiogenesis, wound healing, tissue remodeling, and cancer [17, 32–41]. Specifically, the multiple functions of MCs depend on their capability to release panoply of biologically active products upon suitable immunological and nonimmunological stimulation [42]. These mediators are either preformed in their secretory granules (biogenic amines, neutral serine proteases) or synthesized *de novo* (metabolites of arachidonic acid, cytokines) [43, 44]. MCs granules represent key functional elements, whose content can be released by two distinct secretory mechanisms: exocytosis (*anaphylactic degranulation*) or *piecemeal degranulation* [25]. Interestingly, the latter process is the most frequent secretory mechanism observed in chronic inflammatory settings, such as cancer [31, 45].

A possible causal relationship between MCs, chronic inflammation, and cancer has long been suggested. Accordingly, as most tumors contain inflammatory cell infiltrates, often including abundant MCs, the question about the possible contribution of MCs to tumor development has progressively been emerging [31, 39]. MCs have been recognized as one of the earliest cell types to infiltrate many developing tumors, particularly malignant melanoma and breast and colorectal cancer (CRC) [8, 17, 21, 23, 40, 70, 71]. Ample evidence highlights that MCs accumulate predominantly around several types of tumors, at the boundary between malignant and healthy tissues [8, 17]. In particular, these cells are often strategically located in proximity of blood vessels within the tumor microenvironment, suggesting an early role of MCs in angiogenesis and tumor growth; in fact angiogenesis generates a new vascular supply that delivers oxygen and nutrients to the rapidly proliferating malignant tissue [25, 39, 72]. In agreement with this role, MCs are an abundant source of potent proangiogenic factors, which represent a major issue linking these cells to cancer [26, 73]. In many experimental tumor settings, MCs promote angiogenesis by releasing preformed mediators or by activating proteolytic release of extracellular matrix-bound angiogenic molecules [25, 32, 72]. *In vitro* studies have demonstrated that MC granular components can induce vascularization [25]. Indeed, the addition of either human recombinant tryptase or chymase is able to stimulate neovascularization in the chick embryo chorioallantoic membrane assay (CAM) [32, 72]. Based on these results, treatment with cromolyn, an inhibitor of MCs degranulation, has been shown to restrain expansion and survival of pancreatic cancer and endothelial cells [15].

Tryptase and chymase are preformed active serine proteases and are stored in large amounts in MCs secretory granules [74], whose angiogenic role has been established [16, 75]. In particular, tryptase represents one of the most powerful angiogenic mediators released by human MCs upon c-KitR activation, and it may be angiogenic via several mechanisms [24]. This protease directly stimulates human vascular endothelial cell proliferation acting on protease-activated receptor-2 (PAR-2) by its proteolytic activity [24, 75, 76], leading to direct angiogenic effect (Figure 1). This particular proliferative pathway has been showed by Yoshii et al. [50] who have demonstrated that tryptase induces PAR-2-mediated proliferative effects on a human colon carcinoma cell line (DLD-1 cells) in a mitogen-activated protein kinase (MAP) kinase- and cyclooxygenase- (COX-) dependent manner. PAR-2 activation also leads to the release of IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF), which, in turn, act as angiogenic factors [77]. The important role of tryptase in neovascularization is also shown by its ability to degrade connective tissue matrix in order to provide rooms for neovascular growth. Tryptase may also contribute indirectly to tissue neovascularization by activating latent matrix-metalloproteinases (MMPs) and plasminogen activator, which, in turn, degrade extracellular matrix (ECM) with consequent release of ECM-bound angiogenic factors, such as VEGF and FGF-2 [25, 26, 52]. The disruption of local ECM leads also to release of SCF. Interestingly, tumor-derived SCF has been recently implicated both in MCs recruitment into the tumor environment as well as in increased MCs release and production of VEGF and FGF-2 [78, 79].

With reference to the above-described mechanisms that link tryptase to tumor angiogenesis and cancer progression, several studies have reported a linear correlation between mast cells density positive to tryptase (MCDPT) and angiogenesis in solid tumors, such as human malignant melanoma [80, 81], endometrial carcinoma [41], breast cancer [8, 82], uterine leiomyomas [83], gastric cancer [23, 24, 40], and CRC [21, 84]. Regarding hematological tumors, angiogenesis has been shown to increase with the MCDPT in B cell non-Hodgkin's lymphomas [85] as well as in the bone marrow of

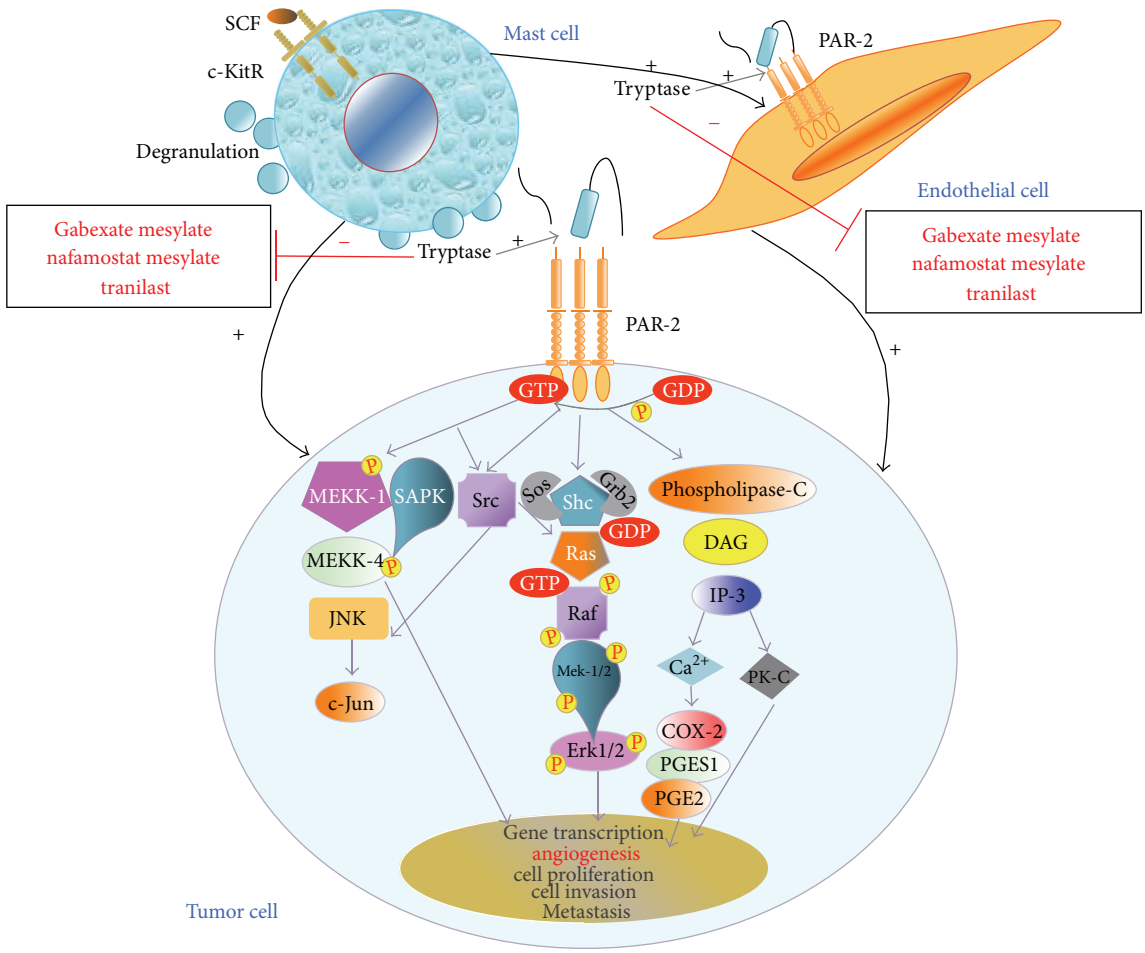



Figure 1: Tryptase, released after MCs activation of c-KitR/SCF-mediated, acting on PAR-2 by its proteolytic activity, has angiogenic activity stimulating both human vascular endothelial and tumor cell proliferation in paracrine manner, helping tumor cell invasion and metastasis. In cancer treatment, tryptase may represent a promising target by tryptase inhibitors (gabexate mesylate, nafamostat mesylate, tranilast) due to their potential antiangiogenic activity. c-KitR, c-Kit receptor; PAR-2, proteinase-activated receptor-2; VEGFR, vascular endothelial growth factor receptor; SCF, stem cell factor, VEGF, vascular endothelial growth factor; NHERF-1, Na+/H+ exchanger regulatory factor-1; MEKK-1, mitogen-activated protein kinase/extracellular signal-related kinase-1; MEKK-4, mitogen-activated protein kinase/extracellular signal-related kinase-4; JNK, c-Jun N-terminal kinase; c-Jun, Jun protooncogene; SAPK, mitogen-activated protein kinase-9; GEF, rho/rac guanine nucleotide exchange factor; Rho, rhodopsin transcription termination factor; SOS, SOn of sevenless protein; Grb2, growth factor receptor-bound protein 2; Shc, Shc transforming protein kinase; Ras, Ras protein kinase; Raf, Raf protein kinase; mitogen-activated protein kinase/extracellular signal-related kinase-1/2; Erk, Elk-related tyrosine kinase; DAG, Diacylglycerol; IP-3, inositol triphosphate; PK-C, protein kinase-C; COX-2, cyclooxygenase-2; PGE2, prostaglandin E2; PGES-1, prostaglandin E synthase-1; PK-A, protein kinase-A.

patients with multiple myeloma, monoclonal gammopathies of undetermined significance [86], myelodysplastic syndrome [87], and B-cell chronic lymphocytic leukemia [85]. In the majority of studies, MCDPT correlates with angiogenesis, tumor aggressiveness, and poor prognosis [25], even if some human studies have demonstrated a correlation between high mast cells density (MCD) and improved overall survival [88–91], suggesting that MCs effects on tumor fate may depend on some *bias* related to cancer (e.g., type of surgical treatment with relative lymph node collection, histology, stage tumor, small sample size) and different methods of MCs evaluation (e.g., histochemistry with toluidine blue, Giemsa stain, primary antibody antitryptase or antichymase for immunohistochemistry, standardization of MCs count with reference to magnification, MCs location, and microscopic field of evaluation).

Overall, despite conflicting reports on the role of MCD- and MCDPT-mediated angiogenesis in tumor development, literature data indicate that tryptase may represent a promising target in adjuvant cancer treatment [25, 26], leading to considering the therapeutic use of drugs which specifically inhibit its angiogenic activity. Therefore, tryptase inhibitors, such as gabexate mesylate and nafamostat mesylate [92–94], might be evaluated in clinical trials as new antiangiogenic agents in combination with chemotherapy in the treatment of cancer.

## 3. Potential Role of Mast Cells Tryptase Inhibitors in Cancer

In the light of the aforementioned complex relationship between MCs tryptase and angiogenesis in tumor development, we have described the possible molecular mechanisms of three drugs targeting tryptase functions, such as gabexate mesylate, nafamostat mesylate, and tranilast, in order to discuss their prospective role in cancer therapy.

*3.1. Gabexate Mesylate.* Gabexate mesylate (GM) is a synthetic inhibitor of trypsin-like serine proteases [95–97] that shows an antiproteinase activity on various kinds of plasma proteases, such as thrombin, plasmin, trypsin, kallikrein, C1 esterase in the complement system, and factor Xa in the coagulation cascade. Accordingly, GM has been therapeutically used for disseminated intravascular coagulation (DIC) and acute pancreatitis [95] in Japan, Italy, Korea, and Taiwan. In addition, several recent studies have reported that this protease inhibitor exerts a significant antitumorigenic effect, both *in vitro* and *in vivo* [46, 47, 94].

Proteolytic degradation of ECM components is a crucial step for tumor cell invasion and metastasis. Among several classes of degrading ECM proteinases, MMPs (MMP-2 and MMP-9) and urokinase-type plasminogen activator (uPA) have been closely associated with the metastatic phenotype of cancer cells [98–102]. These enzymes are also implicated in tumor angiogenesis [103]. Therefore, inhibitors of MMPs and uPA are able to inhibit invasion and metastasis [104, 105] by reducing angiogenesis *in vitro* and *in vivo* [106–109]. Furthermore, serine plasma proteases, such as thrombin and plasmin, are closely associated with activation pathways of certain MMPs (MMP-2, MMP-3, and MMP-9) [110, 111], indicating that multispecific protease inhibitors could be useful tools for an antimetastatic and antiangiogenic strategy. Based on these findings, GM has been shown to inhibit proliferation, invasion, and metastasis of human colon cancer cell lines through the inhibition of both MMPs and uPA-plasmin system, consequentially limiting angiogenesis [46]. Although the inhibition of the uPA system may be involved in downregulation of MMP activity, the results of this study have suggested that GM has a direct inhibitory effect on MMPs, whose related-mechanism is unknown [46].

Interestingly, the inhibition of MMPs by GM and, in general, its anti-invasive, antimetastatic, and antiangiogenic properties could also be explained through its potent and selective inhibition of human tryptase [92]. Indeed, as above described, in the early stages of tumor development several tumor-derived factors (i.e., SCF, adrenomedullin) recruit and activate MCs in tumor microenvironment, leading to the release of tryptase [25, 26], which, in turn, can indirectly stimulate tumor angiogenesis by activating latent MMPs and uPA [23, 24, 75]. Accordingly, Yoshii et al. [50] demonstrated the specific localization of MCDPT in the invasive front of tumor tissues by examining 30 cases of human colon adenocarcinoma. A previous study [49] has found the proliferation of DLD-1 colon cancer cells expressing PAR-2 in response to PAR-2 activating peptide (AP). Moreover, tryptase also enhanced DLD-1 cell proliferation by means of a specific stimulation of PAR-2 via MAPK- and COX-dependent manners. Furthermore, these proliferative effects were concentration-dependently inhibited by nafamostat mesylate, a very potent inhibitor of human tryptase [93, 112], suggesting that PAR-2 activation was dependent on tryptase proteolytic activity. In the same study, PAR-2 density in tumor tissues was higher than that in the normal tissues, as revealed by the immunohistochemical analysis. This suggests that tryptase released by MCs surrounding tumor tissues may induce the PAR-2-mediated proliferation of colon cancer cells in a paracrine way [49]. Similarly, tryptase has been reported to stimulate angiogenesis directly [75] via PAR-2 activation on vascular endothelial cells [24, 76]. Moreover, increasing evidences support that MCs tryptase is involved in angiogenesis through the direct degradation of connective tissue matrix [25, 26, 75], with consequent release of matrix-associated angiogenic substances, such as VEGF or FGF-2 [40, 72, 113–116]. These findings as a whole suggest that MCs tryptase may sustain colon cancer cell growth in two ways: direct proliferative effect via PAR-2 stimulation and indirect support through angiogenesis stimulation. Thus, tryptase may be considered a novel target of colon cancer therapy. Taken together, all the reported evidences suggest that the inhibition of colon cancer growth, invasion, and metastasis by GM may be also due to its selective inhibition of MC tryptase. Therefore, GM could be potentially useful for antimetastatic and antiangiogenic treatment of colon cancers. Noteworthy, this assumption is corroborated by a recent study by Brandi et al. [47] aimed to investigate the antitumor efficacy of GM, alone, and in combination with the antiepidermal growth factor receptor (EGFR) monoclonal antibody cetuximab, in a group of human CRC cell lines with a different expression pattern of wild-type/mutated V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS), protooncogene B-Raf murine sarcoma viral oncogene homolog B1 (BRAF), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha oncogene (PIK3CA). Besides confirming the lack of response to cetuximab in CRC cells bearing such mutations [117–119], results demonstrated that GM significantly inhibited the growth, invasiveness, and tumor-induced angiogenesis in all CRC cells tested in this study [47]. In particular, the antiangiogenic effect of GM in combination with the anti-EGFR antibody was found to be not superior than that observed with GM as single agent, suggesting that the inhibition of tumor angiogenesis may be largely related to GM mechanism of action, most notably the inhibition of MCs tryptase. Therefore, also considering its good toxicological profile, these findings indicate that GM could represent a valuable therapeutic option for patients with EGFR-expressing metastatic CRC (mCRC), particularly for those ones bearing KRAS, BRAF, and PIK3CA mutations, either as monotherapy or in combination with standard chemotherapy [47].

The antimetastatic and antiangiogenic mechanisms of GM have also been investigated in pancreatic cancer cell lines. As in colon cancer, MMPs and uPA play a crucial role also in the progression of pancreatic cancer [48]. In addition, a previous study by Uchima et al. [48] reported the involvement of tumor-associated trypsinogen (TAT) and

Table 1: All preclinical studies mentioned above that have considered gabexate mesylate.

| Author, reference, year | Drug/s | Tumor target | Molecular mechanisms of action | Results |
|---|---|---|---|---|
| Yoon et al. [46] 2004 | *gabexate mesylate* | several human colon cancer cell lines | (1) down-regulation of MMPs (2) inhibition of uPA-plasmin system | inhibition of angiogenesis, tumor cell growth, invasion, metastasis |
| Brandi et al. [47] 2012 | (1) *gabexate mesylate* (2) *gabexate mesylate plus cetuximab* | several human colorectal cancer cell lines (wt/mut KRAS, BRAF, PIK3CA) | not analyzed | (1) inhibition of tumor cell growth, angiogenesis, invasion, metastasis (2) antitumoral efficacy of the combination therapy was not superior than gabexate mesylate alone |
| Uchima et al. [48] 2003 | *gabexate mesylate* | several human pancreatic cancer cell lines | down-regulation of uPA, TAT, PAT, MMPs, TGF-$\beta$1, VEGF | inhibition of angiogenesis, cell growth, invasion, metastasis |

MMPs, Metalloproteinases; uPA, urokinase-type plasminogen activator; wt, wild-type; mut, mutated; TAT, Tumor-associated trypsinogen; PAT, Pancreatic acinar trypsinogen; TGF-$\beta$1, Tumor growth factor-beta1; VEGF, Vascular endothelial growth factor.

pancreatic acinar trypsinogen (PAT) in pancreatic cancer invasion and metastasis. Both these serine proteases can be activated by uPA, which is produced by pancreatic cancer. Following, they are able to degrade ECM components and can also directly activate TAT, PAT, pro-MMPs, and pro-uPA, leading to further ECM breakdown. The resulting *vicious cycle* would activate latent ECM-degrading proteases, thereby promoting tumor cell invasion and metastasis. In particular, PAT and TAT had been shown to continuously stimulate pancreatic cancer cell proliferation by activating PAR-2 [120]. Furthermore, several investigations reported that transforming growth factor-beta 1 (TGF-$\beta$1), produced in the tumor microenvironment, could be a strong mediator of pancreatic cancer cell invasion, metastasis, and angiogenesis by upregulating VEGF, MMP-2, and uPA secretion [121–123]. High uPA levels, in turn, could activate latent TGF-beta1, resulting in a positive feedback loop on tumor progression [123]. Starting from these data, Uchima et al. [94] suggested that GM inhibited the invasiveness, proliferation, and potential liver metastatic of pancreatic cancer cell lines by downregulating TAT and uPA activities, reducing PAR-2 activation, and inhibiting the production of TGF-$\beta$1 and VEGF. Moreover, in pancreatic cancer the inhibitory effects of GM may be, in part, associated with tryptase inhibition. In fact, similarly to TAT, MCs tryptase may be responsible for PAR-2-mediated pancreatic cell proliferation, since tryptase is a natural agonist of this receptor [124]. Moreover, tryptase-mediated activation of latent MMPs and uPA [25, 26, 75] may induce further TAT, MMPs, and uPA activation and ECM degradation, thus triggering an ECM-protease network responsible for tumor cell invasion and metastasis [48, 94]. In this context, tryptase inhibition by GM may downregulate TAT and uPA enzymatic activities. The resulting downregulation of uPA levels may decrease the activation of latent TGF-$\beta$1, thereby impairing the abovementioned *cycle vicious* of uPA and TGF-$\beta$1 and downregulating VEGF production. On the other hand, tryptase inhibition may also directly suppress the production of TGF-$\beta$1 and VEGF involved in tumor growth and angiogenesis. In agreement with this proposed mechanism, tryptase has been reported to increase the production of TGF-$\beta$1 in other pathophysiological settings [125, 126]. The findings about the GM mechanism of action in pancreatic cancer cells, together with our considerations, indicate that this protease inhibitor could be a useful therapeutic option for antimetastatic and antiangiogenic treatment of pancreatic cancer.

The above studies are summarized in Table 1.

*3.2. Nafamostat Mesylate.* Similarly to GM, nafamostat mesylate (NM) is able to inhibit a variety of trypsin-like serine proteases and some proteases implicated in the coagulation cascade [127, 128]. Interestingly, Mori et al. [93] have demonstrated that NM inhibits human tryptase with potency 1000 times higher than that of GM, concluding that NM is an extremely potent and selective inhibitor when employed at relatively low concentration. They have also suggested that such inhibitory action on tryptase activity can account for some therapeutic effects of NM in specific clinical conditions. Indeed, human tryptase may be involved in the pathogenesis of several MCs-mediated allergic and inflammatory diseases, such as rhinitis and asthma. It is also implicated in specific gastrointestinal, dermatological, and cardiovascular disorders [129–131]. Therefore, NM has been widely used for the treatment of acute pancreatitis and DIC in Japan [132, 133].

The antitumor potential of NM is suggested by Yoshii et al.'s study previously described [50]. In fact, the *in vitro* analysis showed that NM concentration-dependently inhibited the tryptase-induced enhancement of proliferation of DLD-1 cells, thus suggesting that tryptase inhibition may mediate the anticancer effect of NM. It has also been reported that NM inhibits liver metastases of colon cancer cells in mice [134]. Moreover, previous studies showed that NM inhibited the proliferation and invasion of pancreatic cancer cells by antagonizing TAT-induced activation of PAR-2 *in vitro*, in the same fashion of GM [51, 52]. Indeed, several studies have recently revealed that NM exerts antiproliferative, antiangiogenic, and antimetastatic effects also in pancreatic cancer, proposing the use of this serine protease inhibitor

in combination with standard chemotherapy regimens for pancreatic cancer management [53–56]. In particular, the blockade of nuclear factor kappa-B (NF-$\kappa$B) activation has been reported to underlie antitumor effects of NM [55]. In this regard, Karin and Lin have demonstrated that NF-$\kappa$B plays an important role in the modulation of inflammatory responses, cell proliferation, apoptosis, and oncogenesis including invasion and angiogenesis [55]. Typically, inactive NF-$\kappa$B is sequestered in the cytoplasm by nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IkB$\alpha$); however, a specific activation signaling leads to IkB$\alpha$ phosphorylation and consequent release of NF-$\kappa$B protein, which translocates into the nucleus, where it induces the transcription of target genes [135]. Most notably, constitutive activation of NF-$\kappa$B has been identified in a variety of tumors including pancreatic cancer [135] and it is known to contribute to the aggressive phenotype [136] and chemoresistance [137]. The resulting overexpression of downstream target genes of NF-$\kappa$B, such as intercellular adhesion molecule-1 (ICAM-1) [138], IL-8 [136, 139], VEGF [136, 139, 140], MMP-9 [140], and uPA [141], promotes cell adhesion, angiogenesis, invasion, and metastasis. Interestingly, some cancer chemotherapy drugs, such as oxaliplatin and gemcitabine, have been shown to activate NF-$\kappa$B by themselves, thereby reducing their antitumor efficacy [142–144]. Based on these findings, a NF-$\kappa$B inhibitor like NM may be able to suppress proliferation, angiogenesis, and metastasis both in pancreatic cancer and in other malignancies, opening an avenue for novel therapeutic approaches. In the study by Fujiwara et al. [55], NM has been shown to downregulate activities of phosphorylated IkB$\alpha$, NF-$\kappa$B, and its target genes, resulting in inhibition of cell adhesion, invasion, and increase of a particular programmed cell death (*anoikis*) in human pancreatic tumor cell lines. *In vivo*, intraperitoneal administration of pancreatic cancer cells, pretreated with NM, in nude mice revealed reduced peritoneal metastasis and neovascularization and increased survival compared with controls. This suggests that NM may potentially reduce the incidence of postoperative recurrences due to peritoneal dissemination in pancreatic cancer patients [145]. In accordance with these findings, the authors have already reported the ability of NM to inhibit NF-$\kappa$B activation and induce caspase-8-mediated apoptosis when this serine protease inhibitor was used as monotherapy or with gemcitabine, *in vitro* and *in vivo* [53, 146, 147]. Most notably, they reported a better clinical outcome of combination therapy of gemcitabine or paclitaxel with NM in comparison with gemcitabine or paclitaxel alone in pancreatic cancer-bearing mice through the inhibition of chemotherapeutic drug-induced NF-$\kappa$B activation [53, 55]. It was also demonstrated the clinical usefulness of intra-arterial NM administration combined with gemcitabine in patients with unresectable pancreatic cancer [54, 148]. Accordingly, Gocho and colleagues [56] have recently proven that NM enhances the antitumor effect of oxaliplatin by inhibiting oxaliplatin-induced NF-$\kappa$B activation. This leads to downregulation of the cellular inhibitor of apoptosis proteins, c-IAP1 and c-IAP2, resulting in cleavage of poly ADP-ribose polymerase (PARP) and caspase-8-mediated apoptosis *in vitro* and *in vivo*: the inhibition of NF-$\kappa$B activity results in chemosensitization of pancreatic cancer. Therefore, combination chemotherapy with NM and oxaliplatin exerts a synergistic cytotoxic effect in pancreatic cancer both *in vitro* and *in vivo*.

Taking into account the above-illustrated pathophysiological pathways, we propose that the potent inhibition of MCs tryptase may also be involved in the antitumor activities of NM. Firstly, this hypothesis is supported by the ability of tryptase to stimulate cell proliferation and invasion of cancer cells *in vitro* through the activation of PAR-2. We herein report the evidence of these tryptase-mediated proliferative effects only in colon cancer cells [50]; however, tryptase, being a natural agonist of PAR-2 [124], may be potentially able to activate this receptor class expressed also in the gastrointestinal tract, pancreas, liver, kidney, and sensory neurons [149–151], triggering a proliferative response. Moreover, the above mentioned antiproliferative effect of NM in pancreatic cancer cells by blocking TAT-induced PAR-2 stimulation [51, 52] may be indirectly related to tryptase inhibition. In fact, we have previously reported that tryptase can activate the uPA system [25, 26, 75], which, in turn, activates TAT leading to stimulation of PAR-2 on the surface of pancreatic cancer cells [94]. On the other hand, the inhibition of tryptase-mediated activation of PAR-2 on vascular endothelial cells could contribute to antiangiogenic effects of NM.

MCs tryptase may contribute to cancer pathways triggered by the constitutive activation of NF-$\kappa$B. In particular, tryptase may upregulate the levels of several target genes overexpressed owing to the pathological NF-$\kappa$B activation, such as VEGF, IL-8, MMP-9, and uPA, thereby contributing to promote angiogenesis, invasion, and metastasis in a variety of tumors. Interestingly, several studies have reported that PAR-2 is able to mediate some important tryptase-induced inflammatory processes, such as microglia activation and skin inflammation [152, 153]. In particular, it has been shown that MC tryptase, via PAR-2, may induce the upregulation/release of proinflammatory cytokines (i.e., IL-6, IL-8, TNF-$\alpha$) and activate important inflammatory signaling cascades such as NF-$\kappa$B pathway in human dermal microvascular endothelial cells and microglia: MAPK signaling pathways are involved in NF-$\kappa$B activation and consequent production/release of proinflammatory cytokines by tryptase [152, 153]. Furthermore, according to Ma et al. [154] tryptase could phosphorylate protein-kinase B (PKB, also known as AKT) through PAR-2, activate phosphoinositol-3-kinase (PI3K)/PKB pathway, and upregulate the expression of NF-$\kappa$B in inflammatory settings. Most notably, PKB/AKT is involved in cellular survival pathways by inhibiting apoptotic processes [155]; hence, it has been implicated as a major factor in many types of cancers [156].

In the light of these last findings, MCs tryptase may probably contribute to the aggressive behavior and chemoresistance of pancreatic cancer cells, by activating NF-$\kappa$B. Therefore, the inhibitory effect of NM on NF-$\kappa$B activities may also indirectly depend on the selective tryptase inhibition. On the other hand, tryptase inhibition could also justify the apoptotic effect of NM through the downregulation of PI3K/protein kinase B (PKB) signaling pathway. As a whole, the above detailed findings and mechanisms suggest

Table 2: All studies mentioned above that have considered nafamostat mesylate.

| Author, reference, year | Drug/s | Tumor target | Molecular mechanisms of action | Results |
|---|---|---|---|---|
| Jikuhara et al. [49] 2003 | *nafamostat mesylate* | human colon cancer cell line (DLD-1) | (1) inhibition of PAR-2 stimulation via MAPK- and COX-dependent manner (2) inhibition of VEGF and FGF-2 levels | inhibition of tumor cell growth, angiogenesis, invasion, metastasis |
| Yoshii et al. [50] 2005 | *nafamostat mesylate* | human colon cancer cell line (DLD-1) | (1) Inhibition of PAR-2 stimulation via MAPK- and COX-dependent manner (2) Inhibition of the release of IL-6 and GM-CSF | inhibition of angiogenesis, cell growth, invasion, metastasis |
| Tajima et al. [51] 2001 | *nafamostat mesylate* | several human pancreatic cancer cell lines | antagonizing TAT-induced activation of PAR-2 | inhibition of tumor cell growth and invasion |
| Ohta et al. [52] 2003 | *nafamostat mesylate* | several human pancreatic cancer cell lines | antagonizing TAT-induced activation of PAR-2 | inhibition of tumor cell growth and invasion |
| Uwagawa et al. [53] 2009 | (1) *nafamostat mesylate* (2) *nafamostat mesylate plus gemcitabine* | human pancreatic cancer cell line (Panc-1) | Down-regulation of NF-$\kappa$B with reduction of ICAM-1, IL-8, VEGF, MMP-9, uPA, RRM1 | (1) inhibition of tumor cell adhesion and growth, angiogenesis, invasion metastasis (2) increase of apoptosis (3) increase of body weight loss of mice |
| Uwagawa et al. [54] 2009 | *nafamostat mesylate plus intra-arterial gemcitabine* | unresectable locally advanced or metastatic pancreatic cancer (20 pts) | not analyzed | (1) CBR of 60% (2) reduction of CA19-9 serum level in 90% of pts (3) improvement in health-related quality of life |
| Fujiwara et al. [55] 2011 | *nafamostat mesylate* | human pancreatic cancer cell lines (AsPC-1, BxPC-3, PANC-1) | down-regulation of IkB$\alpha$, NF-$\kappa$B with reduction of ICAM-1, IL-8, VEGF, MMP-9, uPA | (1) increase of cell adhesion, programmed cell death (2) inhibition of angiogenesis, invasion, metastasis in peritoneal dissemination |
| Gocho et al. [56] 2013 | (1) *nafamostat mesylate* (2) *nafamostat mesylate plus oxaliplatin* | human pancreatic cancer cell line (Panc-1) and pancreatic cancer mouse model | down-regulation of NF-$\kappa$B with reduction of ICAM-1, IL-8, VEGF, MMP-9, uPA, c-IAP1, c-IAP2 | (1) increase of cell adhesion, caspase-8-mediated apoptosis (2) inhibition of PARP, angiogenesis, invasion and metastasis, (3) synergistic cytotoxic effect |

PAR-2, Protease-activated receptor-2; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; IL, Interleukin; GM-CSF, Granulocyte-macrophage colony stimulating factor; TAT, Tumor-associated trypsinogen; IkB, Inhibitor of NF-$\kappa$B; NF-$\kappa$B, Nuclear factor-kappaB; MMPs, metalloproteinases; uPA, urokinase-type plasminogen activator; ICAM-1, Intercellular Adhesion Molecule-1, VEGF, Vascular endothelial growth factor, IAP, Inhibitors of apoptosis.

a potential usefulness of NM in preoperative management of pancreatic cancer patients, because its use may reduce postoperative recurrences and improve survival by inhibition of metastasis induced by surgical resection [157]. Moreover, taking into account the improved outcomes and relatively low toxicity of preclinical and clinical studies of the combination therapy with traditional chemotherapeutic agents and NM, these combination chemotherapy regimens could represent a novel promising strategy for pancreatic cancer treatment.

The above studies are summarized in Table 2.

*3.3. Tranilast.* Among pharmacological agents that affect several inflammatory and allergic pathways mediated by MCs tryptase, also tranilast (TN) has progressively attracted considerable attention because of its antitumor potential. Since 1982, this drug has been approved in Japan and Korea for the systemic and topical treatment of bronchial asthma, atopic dermatitis, and allergic conjunctivitis, with indications for keloids and hypertrophic scar added in 1993 [158]. Follow-up studies have revealed that clinical effectiveness of TN in such applications depends on inhibition of the release of biologically active mediators from MCs [158, 159]. Moreover, tranilast was reported to inhibit the VEGF-induced angiogenesis both *in vitro* and *in vivo*, and most notably, these antiangiogenic activities have been shown to be concomitant with inhibitory effects on MCs degranulation [160].

TN was also reported to inhibit the release of TGF-beta, IL-1beta, prostaglandin (PG) $E_2$, and IL-2 from human

monocytes and macrophages [161, 162]. In the late 1980s, Isaji et al. [160] discovered the antiproliferative properties of TN. In particular, it was found that this agent inhibited fibroblast proliferation *in vitro*, resulting in suppression of proliferative inflammation *in vivo*. Subsequent studies confirmed the ability of TN in inhibiting tumor cell growth and proliferation in various models of cancer [59, 163, 164]. Overall, data from *in vitro* and *in vivo* models for proliferative disorders, clinical studies, and case reports have corroborated the antiproliferative and antitumor potential of TN [165], providing important insights into its mechanisms of action. Two studies, addressing antiproliferative activity of TN in several breast cancer cell lines, revealed that TN inhibits cell proliferation, by arresting cell cycle progression, and downregulates TGF-$\beta$ signaling pathway [57, 58]. Moreover, Chakrabarti et al. [57] demonstrated that TN is able to inhibit MAPK signaling pathway.

TN was also reported to suppress the proliferation of cultured human leiomyoma cells by inhibiting cell cycle modulators, such as cyclin-dependent kinase 2 (CDK-2) [164].

As concerns pancreatic cancer, Hiroi et al. [59] reported that TN significantly inhibited proliferation of PGHAM-1, a hamster pancreatic cancer cell line. Moreover, TN was able to inhibit tumor angiogenesis in response to VEGF. Interestingly, in another study by Mitsuno et al. [60] TN was found to enhance chemotherapeutic effect of gemcitabine, as above reported for NM [53, 54]. However, unlike NM-induced effect, this chemosensitization was associated with the downregulation of ribonucleotide reductase M1 (RRM1) [53, 54].

Further experiments revealed that TN treatment inhibited prostate cancer cell proliferation *in vitro* by promoting apoptosis. In addition, it was reported the ability of TN to downregulate TGF-beta production from bone stromal cells and other different cell types, thereby suppressing TGF-$\beta$-stimulated osteoclast differentiation which underlies, in part, osteoblastic bone metastasis [61, 166]. Noguchi et al. [62] demonstrated that three weeks of TN treatment significantly reduced the tumor growth and metastasis, when administered daily by intraperitoneal injection (4 mg/animal), in a mouse model of oral squamous cell carcinoma. TN has also been reported to exert antitumor effects in gastric cancer [63] and malignant glioma [64] through different mechanisms. Izumi et al. [61] have reported that the treatment with oral TN (300 mg/day) promoted a reduction of prostate-specific antigen (PSA) levels in 4 out of 16 patients with advanced castration-resistant prostate cancer (CRPC). Accordingly, in the subsequent follow-up pilot study, oral treatment with TN (300 mg/day) for a median period of five months documented a continuous PSA inhibition in 3 out of 21 patients with advanced CRPC. Overall survival rates at 12 and 24 months were 74.5% and 61.5%, respectively [167]. As a whole, these results suggest that TN could be used to improve the prognosis of patients with advanced CRPC. However, the two clinical investigations had some limitations: (1) open-label studies with one arm; (2) short follow-up period; (3) small sample size; (4) all patients were Japanese. Therefore, the reported findings need further confirmation. Finally, several case studies have reported that transdermal application of TN was able to relieve both itching and pain associated with hypertrophic, keloid scars [168].

Several important pathways have been recognized as potential targets of TN antitumor activity. In particular, the TN inhibitory effects on cell proliferation depend mainly on its ability to interfere with TGF-$\beta$ signaling and also reduce TGF-$\beta$ secretion [57, 61, 63, 64]. Also, TN-mediated inhibition of cell proliferation has been markedly associated with blockade of cell cycle progression and consequent cell arrest in the $G_0/G_1$ transition [58, 59, 164, 169]. Probably, TN can induce cell cycle arrest also through the inhibition of calcium influx, which is crucial for $G_1/S$ transition, as demonstrated by Nie et al. [65]. After TN treatment, the induction of apoptosis has been reported in several breast and prostate cancer cell lines [61, 66]. In particular, Subramaniam et al. [58] showed that TN induced p53 upregulation, enhanced RAC-alpha serine/threonine-protein kinase (AKT1) phosphorylation, and reduced phosphorylation of extracellular regulated kinase 2 (ERK2). Another work by Subramaniam et al. [66] detected an increased level of a PARP-cleavage product in human cancer cell lines treated with TN.

TN also acts as a nontoxic agonist of the aryl hydrocarbon receptor (ARH) [58, 170], whose function is involved in anticancer effects [67, 68]: ARH presence in the cell is critical for TN-mediated cell cycle arrest. Interestingly, the AHR also antagonizes TGF-$\beta$ activity [171] and exerts ligand-dependent inhibitory effects on NF-$\kappa$B signaling [172]. These AHR-mediated activities may contribute to the antiproliferative, antiangiogenic, and antimetastatic effects of TN.

The ability of TN to inhibit MAPK signaling pathway could also explain its antimetastatic potential, because this pathway is known to be implicated during the epithelial to mesenchymal transition (EMT), which is important for tumor cell invasion [57]. Moreover, the downregulation of certain MMPs, such as MMP-9, contributes to TN-mediated inhibition of tumor cell invasion during metastasis: such reduction of MMP-9 levels has been also linked to inhibition of TGF-$\beta$ signaling [58].

In addition to the above detailed potential targets, the antitumor action of TN relies on the blocking of the release of chemical mediators from MCs [57, 61, 64, 159], which is also the mechanism responsible for its antiallergic and anti-inflammatory efficacy [158]. In agreement with this correlation, Yamamoto et al. [158] have recently documented that TN downregulated neurofibroma cell (NF1 cells) proliferation through not only suppression of cell-growth promoting pathways but also the inhibition of biologically active mediators by MCs. Interestingly, this study supports the involvement of tryptase in the antitumor activities of TN. Indeed, following its addition to NF1 cells cocultured with MCs, this agent was reported to significantly inhibit NF1 cell proliferation and lower the levels of TGF-$\beta$, SCF, and tryptase. These findings suggest that TN inhibits tumor proliferation also through the downregulation of MC tryptase, whose PAR-2-mediated proliferative and angiogenic effects have been previously described [50, 76]. Furthermore, tryptase has been reported to activate PI3K/PKB pathway via PAR-2 cleavage/activation and subsequently upregulate

Table 3: All studies mentioned above that have considered tranilast.

| Author, reference, year | Drug/s | Tumor target | Molecular mechanisms of action | Results |
|---|---|---|---|---|
| Chakrabarti et al. [57] 2009 | *tranilast* | several mouse, rat and human breast cancer cell lines | (1) down-regulation of TGF-$\beta$ pathway<br>(2) inhibition of MAPK pathway | inhibition of tumor cell proliferation, angiogenesis, apoptosis, migration |
| Subramaniam et al. [58] 2010 | *tranilast* | mouse breast cancer cell line (4T1) | (1) down-regulation of TGF-$\beta$ pathway<br>(2) induction cell arrest in the $G_0/G_1$ transition, PARP cleavage, AKT1 phosphorylation<br>(3) up-regulation of p53<br>(4) reduction of ERK1/2 phosphorylation | inhibition of tumor cell proliferation, angiogenesis, apoptosis, migration |
| Hiroi et al. [59] 2002 | *tranilast* | hamster pancreatic cancer cell line (PGHAM-1) | (1) down-regulation of TGF-$\beta$ pathway with reduction of MMP-9 and VEGF levels<br>(2) induction cell arrest in the $G_0/G_1$ transition | inhibition of tumor cell proliferation, angiogenesis |
| Mitsuno et al. [60] 2010 | (1) *tranilast plus gemcitabine*<br>(2) *gemcitabine* | human pancreatic cancer cell line (KP4) | decrease of RRM1 expression | (1) inhibition of tumor cell proliferation, angiogenesis, apoptosis<br>(2) synergistic cytotoxic effect of combination therapy |
| Izumi et al. [61] 2009 | *tranilast* | (1) prostate cancer cell lines and bone-derived stromal cells<br>(2) SCID mice<br>(3) advanced hormone-refractory prostaste cancer (21 pts) | down-regulation of TGF-$\beta$1 pathway | (1) induction of apoptosis<br>(2) reduction of invasion and bone metastasis, PSA levels, improve prognosis |
| Noguchi et al. [62] 2003 | *tranilast* | mouse model of oral squamous cell carcinoma | not analyzed | decrease of tumor growth, angiogenesis, cervical lymph node metastases |
| Yashiro et al. [63] 2003 | *tranilast* | human gastric carcinoma cell line (OCUM-2D) and gastric fibroblast cell line (NF-10) | down-regulation of TGF-$\beta$ pathway | decrease of tumor growth, angiogenesis, invasion |
| Platten et al. [64] 2001 | *tranilast* | human malignant glioma cell line | down-regulation of TGF-$\beta$1-2 pathway | decrease of tumor growth, angiogenesis, migration, invasion |
| Nie et al. [65] 1997 | *tranilast* | breast cancer cell lines (MCF-7) | induction cell arrest in the $G_0/G_1$ transition | decrease of tumor growth |
| Subramaniam et al. [66] 2011 | *tranilast* | human breast cancer cell lines (triple positive-BT-474, triple negative-MDA-MB-231) | (1) up-regulation of p53<br>(2) induction cell arrest in the $G_0/G_1$ transition, AKT1 and ERK2 phosphorylation, PARP-cleavage product | induction of apoptosis, tumor growth, migration |
| Zhang et al. [67] 2009 | *tranilast* | several ER negative human breast cancer cell lines | agonizing ARH with down-regulation of TGF-$\beta$ and NF-$\kappa$B pathways | (1) induction of apoptosis<br>(2) inhibition of angiogenesis, cell growth, invasion and metastasis |
| Hall et al. [68] 2010 | *tranilast* | several human breast cancer cell lines | agonizing ARH with down-regulation of TGF-$\beta$ and NF-$\kappa$B pathways | (1) induction of apoptosis<br>(2) inhibition of angiogenesis, cell growth, invasion and metastasis |

Table 3: Continued.

| Author, reference, year | Drug/s | Tumor target | Molecular mechanisms of action | Results |
|---|---|---|---|---|
| Isaji et al. [69] 1997 | *tranilast* | human pancreatic cancer cell lines | decrease of VEGF and MMPs levels | inhibition of angiogenesis, cell growth, migration |

TGF-$\beta$1, Tumor growth factor-beta1, MMPs, metalloproteinases; MAPK, mitogen-activated protein kinase uPA, PARP, poly ADP-ribose polymerase; urokinase-type plasminogen activator; AKT1, RAC-alpha serine/threonine-protein kinase; ERK, Extracellular regulated kinase 2; VEGF, Vascular endothelial growth factor; RRM1, Ribonucleotide reductase M1.

NF-$\kappa$B expression [154], promoting tumor cell survival and chemoresistance [56, 155, 156]. Thus, the inhibition of tryptase release may represent a further molecular mechanism involved in the induction of apoptosis and cell cycle arrest upon TN treatment. Because tryptase-mediated PAR-2 activation triggers the MAPK signaling pathway, which is involved in the EMT process [57], tryptase inhibition by TN may also mediate its anti-invasion and antimetastatic properties.

The inhibitory effect on tryptase release could contribute to the ability of TN treatment to target TGF-beta-regulated signaling cascade and reduce TGF-$\beta$ production. As above described, indeed, in the tumor microenvironment, tryptase may upregulate uPA levels, [25, 26, 75] thereby activating latent TGF-$\beta$ which, in turn, upregulates the production of uPA, MMP-2, and VEGF. This *vicious cycle* has been implicated in angiogenesis, tumor cell invasion, and metastasis [94]. By the way, tryptase can also participate to the neovascular growth by activating latent MMPs, which, in turn, promote tumor invasiveness and release of angiogenic factors (VEGF or FGF-2) from their matrix-bound state [25, 26, 75]. Therefore, also taking into account the previously reported study by Isaji et al. [69], the downregulation of tryptase release may probably contribute to the TN-induced inhibition of tumor angiogenesis in response to VEGF, as observed in experimental pancreatic cancer [59].

In the light of the exposed considerations, we suggest that the inhibition of tryptase functions may underlie the anti-invasion, antimetastatic, and antiangiogenic effects of TN treatment. As concerns safety, TN shows relatively low toxicity in [61, 69, 173], making it a promising candidate for further clinical investigations. Based on the encouraging *in vitro* and *in vivo* research data, TN seems to be a safe and effective agent for the treatment of several proliferative and angiogenic diseases.

The above studies are summarized in Table 3.

## 4. Concluding Remarks

Several literature data support a potential implication of MCs tryptase in three pivotal processes involved in cancer development and metastasization: cell growth, tumor-induced angiogenesis, and invasion [174, 175]. Therefore, this serine protease may be considered a novel promising target for the adjuvant treatment of tumors through the selective inhibition of angiogenesis, proliferation, and tissue remodelling. In agreement with these considerations, compounds targeting tryptase functions, although designed as antiallergic drugs, could exert a useful antitumor activity as well. In this regard, it is of interest to underline that many new anticancer drugs used in clinical field, such as sorafenib [18], sunitinib [176], pazopanib [177] axitinib [178], and masitinib [179] are all targeted against c-KitR, whose activation leads to the release of tryptase by MCs [24].

In particular, we herein discuss the antitumor and antiangiogenic potential of three agents which are able to inhibit the functions of MCs tryptase: gabexate mesylate, nafamostat mesylate, and tranilast. Although no definitive experimental data are available to confirm the role that tryptase released from mast cells stimulate tumor angiogenesis, the above hypothesis is supported by a pilot study in the *in vivo* chorioallantoic membrane assay [16]. In this study an angiogenic activity of human recombinant tryptase comparable to the angiogenic activity induced by the VEGF has been demonstrated. Data from this study suggest that the inhibition of tryptase is intriguing hypothesis worthy to further investigation.

The new antiangiogenic approach here reviewed should be substantially strengthened by future awaited clinical studies having the aim to evaluate the truly efficacy of the tryptase inhibitors as a novel tumor antiangiogenic therapy.

## Disclosure

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence their work.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

[1] J. Folkman and Y. Shing, "Angiogenesis," *Journal of Biological Chemistry*, vol. 267, no. 16, pp. 10931–10934, 1992.

[2] G. Breier, "Angiogenesis in embryonic development—a review," *Placenta*, vol. 21, supplement A, pp. S11–S15, 2000.

[3] M. L. Iruela-Arispe and H. F. Dvorak, "Angiogenesis: a dynamic balance of stimulators and inhibitors," *Thrombosis and Haemostasis*, vol. 78, no. 1, pp. 672–677, 1997.

[4] T. O. Daniel and D. Abrahamson, "Endothelial signal integration in vascular assembly," *Annual Review of Physiology*, vol. 62, pp. 649–671, 2000.

[5] T. V. Petrova, T. Makinen, and K. Alitalo, "Signaling via vascular endothelial growth factor receptors," *Experimental Cell Research*, vol. 253, no. 1, pp. 117–130, 1999.

[6] M. A. Nugent and R. V. Iozzo, "Fibroblast growth factor-2," *The International Journal of Biochemistry and Cell Biology*, vol. 32, no. 2, pp. 115–120, 2000.

[7] C. D. Gadaleta and G. Ranieri, "Trans-arterial chemoembolization as a therapy for liver tumours: new clinical developments and suggestions for combination with angiogenesis inhibitors," *Critical Reviews in Oncology/Hematology*, vol. 80, no. 1, pp. 40–53, 2011.

[8] G. Ranieri, M. Ammendola, R. Patruno et al., "Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients," *International Journal of Oncology*, vol. 35, no. 1, pp. 115–120, 2009.

[9] D. D. Roberts, "Regulation of tumor growth and metastasis by thrombospondin-1," *FASEB Journal*, vol. 10, no. 10, pp. 1183–1191, 1996.

[10] M. S. O'Reilly, L. Holmgren, Y. Shing et al., "Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma," *Cell*, vol. 79, no. 2, pp. 315–328, 1994.

[11] M. S. O'Reilly, T. Boehm, Y. Shing et al., "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth," *Cell*, vol. 88, no. 2, pp. 277–285, 1997.

[12] G. Ranieri, M. Pantaleo, M. Piccinno et al., "Tyrosine kinase inhibitors (TKIs) in human and pet tumours with special reference to breast cancer: a comparative review," *Critical Reviews in Oncology/Hematology*, vol. 88, no. 2, pp. 293–308, 2013.

[13] G. Ranieri, C. D. Gadaleta, R. Patruno et al., "A model of study for human cancer: spontaneous occurring tumors in dogs: biological features and translation for new anticancer therapies," *Critical Reviews in Oncology/Hematology*, vol. 88, no. 1, pp. 187–197, 2013.

[14] J. P. Kankkunen, I. T. Harvima, and A. Naukkarinen, "Quantitative analysis of tryptase and chymase containing mast cells in benign and malignant breast lesions," *International Journal of Cancer*, vol. 72, no. 3, pp. 385–338, 1997.

[15] L. Soucek, E. R. Lawlor, D. Soto, K. Shchors, L. B. Swigart, and G. I. Evan, "Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors," *Nature Medicine*, vol. 13, no. 10, pp. 1211–1218, 2007.

[16] D. Ribatti, G. Ranieri, B. Nico, V. Benagiano, and E. Crivellato, "Tryptase and chymase are angiogenic in vivo in the chorioallantoic membrane assay," *International Journal of Developmental Biology*, vol. 55, no. 1, pp. 99–102, 2011.

[17] A. Mangia, A. Malfettone, R. Rossi et al., "Tissue remodelling in breast cancer: human mast cell tryptase as an initiator of myofibroblast differentiation," *Histopathology*, vol. 58, no. 7, pp. 1096–1106, 2011.

[18] G. Ranieri, G. Gadaleta-Caldarola, V. Goffredo et al., "Sorafenib (BAY 43-9006) in hepatocellular carcinoma patients: from discovery to clinical development," *Current Medicinal Chemistry*, vol. 19, no. 7, pp. 938–944, 2012.

[19] G. Ranieri, L. Passantino, R. Patruno et al., "The dog mast cell tumour as a model to study the relationship between angiogenesis, mast cell density and tumour malignancy," *Oncology Reports*, vol. 10, no. 5, pp. 1189–1193, 2003.

[20] G. Ranieri, A. Labriola, G. Achille et al., "Microvessel density, mast cell density and thymidine phosphorylase expression in oral squamous carcinoma," *International Journal of Oncology*, vol. 21, no. 6, pp. 1317–1323, 2002.

[21] M. Gulubova and T. Vlaykova, "Prognostic significance of mast cell number and microvascular density for the survival of patients with primary colorectal cancer," *Journal of Gastroenterology and Hepatology*, vol. 24, no. 7, pp. 1265–1275, 2009.

[22] H. Yano, M. Kinuta, H. Tateishi et al., "Mast cell infiltration around gastric cancer cells correlates with tumour angiogenesis and metastasis," *Gastric Cancer*, vol. 2, no. 1, pp. 26–32, 1999.

[23] M. Ammendola, R. Sacco, G. Donato et al., "Mast cell positivity to tryptase correlates with metastatic lymph nodes in gastrointestinal cancer patients treated surgically," *Oncology*, vol. 85, no. 2, pp. 111–116, 2013.

[24] T. C. Moon, E. Lee, S.-H. Baek et al., "Degranulation and cytokine expression in human cord blood-derived mast cells cultured in serum-free medium with recombinant human stem cell factor," *Molecules and Cells*, vol. 16, no. 2, pp. 154–160, 2003.

[25] W. R. Sperr, K. Czerwenka, G. Mundigler et al., "Specific activation of human mast cells by the ligand for c-Kit: comparison between lung, uterus and heart mast cells," *International Archives of Allergy and Immunology*, vol. 102, no. 2, pp. 170–175, 1993.

[26] A. Iemura, M. Tsai, A. Ando, B. K. Wershil, and S. J. Galli, "The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis," *The American Journal of Pathology*, vol. 144, no. 2, pp. 321–328, 1994.

[27] M. F. Gurish and K. F. Austen, "The diverse roles of mast cells," *The Journal of Experimental Medicine*, vol. 194, no. 1, pp. 1–5, 2001.

[28] Y. B. Shaik-Dasthagirisaheb, G. Varvara, G. Murmura et al., "Vascular endothelial growth factor (VEGF), mast cells and inflammation," *International Journal of Immunopathology and Pharmacology*, vol. 26, no. 2, pp. 327–335, 2013.

[29] I. Puxeddu, D. Ribatti, E. Crivellato, and F. Levi-Schaffer, "Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases," *Journal of Allergy and Clinical Immunology*, vol. 116, no. 3, pp. 531–536, 2005.

[30] J. S. Marshall, "Mast-cell responses to pathogens," *Nature Reviews Immunology*, vol. 4, no. 10, pp. 787–799, 2004.

[31] E. Crivellato and D. Ribatti, "The mast cell: an evolutionary perspective," *Biological Reviews of the Cambridge Philosophical Society*, vol. 85, no. 2, pp. 347–360, 2010.

[32] D. Ribatti, E. Crivellato, L. Candussio et al., "Mast cells and their secretory granules are angiogenic in the chick embryo chorioallantoic membrane," *Clinical and Experimental Allergy*, vol. 31, no. 4, pp. 602–608, 2001.

[33] S. J. Galli, J. Kalesnikoff, M. A. Grimbaldeston, A. M. Piliponsky, C. M. M. Williams, and M. Tsai, "Mast cells as "tunable" effector and immunoregulatory cells: recent advances," *Annual Review of Immunology*, vol. 23, pp. 749–786, 2005.

[34] M. A. Beaven, "Our perception of the mast cell from Paul Ehrlich to now," *European Journal of Immunology*, vol. 39, no. 1, pp. 11–25, 2009.

[35] K. Oda and H. Kitano, "A comprehensive map of the toll-like receptor signaling network," *Molecular Systems Biology*, vol. 2, Article ID 2006.0015, 2006.

[36] L. M. Coussens, W. W. Raymond, G. Bergers et al., "Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis," *Genes and Development*, vol. 13, no. 11, pp. 1382–1397, 1999.

[37] T. Nakayama, L. Yao, and G. Tosato, "Mast cell-derived angiopoietin-1 plays a critical role in the growth of plasma cell tumors," *The Journal of Clinical Investigation*, vol. 114, no. 9, pp. 1317–1325, 2004.

[38] T. C. Theoharides and P. Conti, "Mast cells: the JEKYLL and HYDE of tumor growth," *Trends in Immunology*, vol. 25, no. 5, pp. 235–241, 2004.

[39] D. Ribatti and E. Crivellato, "Mast cells, angiogenesis and cancer," *Advances in Experimental Medicine and Biology*, vol. 716, pp. 270–288, 2011.

[40] D. Ribatti, D. Guidolin, A. Marzullo et al., "Mast cells and angiogenesis in gastric carcinoma," *International Journal of Experimental Pathology*, vol. 91, no. 4, pp. 350–356, 2010.

[41] D. Ribatti, N. Finato, E. Crivellato et al., "Neovascularization and mast cells with tryptase activity increase simultaneously with pathologic progression in human endometrial cancer," *The American Journal of Obstetrics and Gynecology*, vol. 193, no. 6, pp. 1961–1965, 2005.

[42] S. J. Galli and M. Tsai, "Mast cells: versatile regulators of inflammation, tissue remodeling, host defense and homeostasis," *Journal of Dermatological Science*, vol. 49, no. 1, pp. 7–19, 2008.

[43] A. M. Gilfillan and C. Tkaczyk, "Integrated signalling pathways for mast-cell activation," *Nature Reviews Immunology*, vol. 6, no. 3, pp. 218–230, 2006.

[44] S. C. Bischoff, "Physiological and pathophysiological functions of intestinal mast cells," *Seminars in Immunopathology*, vol. 31, no. 2, pp. 185–205, 2009.

[45] T. C. Theoharides, D. Kempuraj, M. Tagen, P. Conti, and D. Kalogeromitros, "Differential release of mast cell mediators and the pathogenesis of inflammation," *Immunological Reviews*, vol. 217, no. 1, pp. 65–78, 2007.

[46] W.-H. Yoon, Y.-J. Jung, T.-D. Kim et al., "Gabexate mesilate inhibits colon cancer growth, invasion, and metastasis by reducing matrix metalloproteinases and angiogenesis," *Clinical Cancer Research*, vol. 10, no. 13, pp. 4517–4526, 2004.

[47] G. Brandi, S. Tavolari, F. de Rosa et al., "Antitumoral efficacy of the protease inhibitor gabexate mesilate in colon cancer cells harbouring KRAS, BRAF and PIK3CA mutations," *PLoS ONE*, vol. 7, no. 7, Article ID e41347, 2012.

[48] Y. Uchima, T. Sawada, T. Nishihara et al., "Identification of a trypsinogen activity stimulating factor produced by pancreatic cancer cells: its role in tumor invasion and metastasis," *International Journal of Molecular Medicine*, vol. 12, no. 6, pp. 871–878, 2003.

[49] A. Jikuhara, M. Yoshii, H. Iwagaki, S. Mori, M. Nishibori, and N. Tanaka, "MAP kinase-mediated proliferation of DLD-1 carcinoma by the stimulation of protease-activated receptor 2," *Life Sciences*, vol. 73, no. 22, pp. 2817–2829, 2003.

[50] M. Yoshii, A. Jikuhara, S. Mori et al., "Mast cell tryptase stimulates DLD-1 carcinoma through prostaglandin-and MAP kinase-dependent manners," *Journal of Pharmacological Sciences*, vol. 98, no. 4, pp. 450–458, 2005.

[51] H. Tajima, T. Ohta, A. Elnemr et al., "Enhanced invasiveness of pancreatic adenocarcinoma cells stably transfected with cationic trypsinogen cDNA," *International Journal of Cancer*, vol. 94, no. 5, pp. 699–704, 2001.

[52] T. Ohta, K. Shimizu, S. Yi et al., "Protease-activated receptor-2 expression and the role of trypsin in cell proliferation in human pancreatic cancers," *International Journal of Oncology*, vol. 23, no. 1, pp. 61–66, 2003.

[53] T. Uwagawa, P. J. Chiao, T. Gocho, S. Hirohara, T. Misawa, and K. Yanaga, "Combination chemotherapy of nafamostat mesilate with gemcitabine for pancreatic cancer targeting NF-$\kappa$B activation," *Anticancer Research*, vol. 29, no. 8, pp. 3173–3178, 2009.

[54] T. Uwagawa, T. Misawa, T. Sakamoto et al., "A phase I study of full-dose gemcitabine and regional arterial infusion of nafamostat mesilate for advanced pancreatic cancer," *Annals of Oncology*, vol. 20, no. 2, pp. 239–243, 2009.

[55] Y. Fujiwara, K. Furukawa, K. Haruki et al., "Nafamostat mesilate can prevent adhesion, invasion and peritoneal dissemination of pancreatic cancer thorough nuclear factor kappa-B inhibition," *Journal of Hepato-Biliary-Pancreatic Sciences*, vol. 18, no. 5, pp. 731–739, 2011.

[56] T. Gocho, T. Uwagawa, K. Furukawa et al., "Combination chemotherapy of serine protease inhibitor nafamostat mesilate with oxaliplatin targeting NF-$\kappa$B activation for pancreatic cancer," *Cancer Letters*, vol. 333, no. 1, pp. 89–95, 2013.

[57] R. Chakrabarti, V. Subramaniam, S. Abdalla, S. Jothy, and G. J. Prud'homme, "Tranilast inhibits the growth and metastasis of mammary carcinoma," *Anti-Cancer Drugs*, vol. 20, no. 5, pp. 334–345, 2009.

[58] V. Subramaniam, R. Chakrabarti, G. J. Prud'Homme, and S. Jothy, "Tranilast inhibits cell proliferation and migration and promotes apoptosis in murine breast cancer," *Anti-Cancer Drugs*, vol. 21, no. 4, pp. 351–361, 2010.

[59] M. Hiroi, M. Onda, E. Uchida, and T. Aimoto, "Anti-tumor effect of N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) on experimental pancreatic cancer," *Journal of Nippon Medical School*, vol. 69, no. 3, pp. 224–234, 2002.

[60] M. Mitsuno, Y. Kitajima, K. Ohtaka et al., "Tranilast strongly sensitizes pancreatic cancer cells to gemcitabine via decreasing protein expression of ribonucleotide reductase 1," *International Journal of Oncology*, vol. 21, no. 4, pp. 351–361, 2010.

[61] K. Izumi, A. Mizokami, Q. L. You et al., "Tranilast inhibits hormone refractory prostate cancer cell proliferation and suppresses transforming growth factor $\beta$1-associated osteoblastic changes," *Prostate*, vol. 69, no. 11, pp. 1222–1234, 2009.

[62] N. Noguchi, S. Kawashiri, A. Tanaka, K. Kato, and H. Nakaya, "Effects of fibroblast growth inhibitor on proliferation and metastasis of oral squamous cell carcinoma," *Oral Oncology*, vol. 39, no. 3, pp. 240–247, 2003.

[63] M. Yashiro, K. Murahashi, T. Matsuoka et al., "Tranilast (N-3 ,4 -dimethoxycinamoyl anthranilic acid): a novel inhibitor of invasion-stimulating interaction between gastric cancer cells and orthotopic fibroblasts," *Anticancer Research*, vol. 23, no. 5A, pp. 3899–3904, 2003.

[64] M. Platten, C. Wild-Bode, W. Wick, J. Leitlein, J. Dichgans, and M. Weller, "N-[3,4-dimethoxycinnamoyl]-anthranilic acid (tranilast) inhibits transforming growth factor-$\beta$ release and reduces migration and invasiveness of human malignant glioma cells," *International Journal of Cancer*, vol. 93, no. 1, pp. 53–61, 2001.

[65] L. Nie, Y. Oishi, I. Doi, H. Shibata, and I. Kojima, "Inhibition of proliferation of MCF-7 breast cancer cells by a blocker of $Ca^{2+}$-permeable channel," *Cell Calcium*, vol. 22, no. 2, pp. 75–82, 1997.

[66] V. Subramaniam, O. Ace, G. J. Prud'homme, and S. Jothy, "Tranilast treatment decreases cell growth, migration and inhibits colony formation of human breast cancer cells," *Experimental and Molecular Pathology*, vol. 90, no. 1, pp. 116–122, 2011.

[67] S. Zhang, P. Lei, X. Liu et al., "The aryl hydrocarbon receptor as a target for estrogen receptor-negative breast cancer chemotherapy," *Endocrine-Related Cancer*, vol. 16, no. 3, pp. 835–844, 2009.

[68] J. M. Hall, M. A. Barhoover, D. Kazmin, D. P. McDonnell, W. F. Greenlee, and R. S. Thomas, "Activation of the aryl-hydrocarbon receptor inhibits invasive and metastatic features

of human breast cancer cells and promotes breast cancer cell differentiation," *Molecular Endocrinology*, vol. 24, no. 2, pp. 359–369, 2010.

[69] M. Isaji, H. Miyata, Y. Ajisawa, Y. Takehana, and N. Yoshimura, "Tranilast inhibits the proliferation, chemotaxis and tube formation of human microvascular endothelial cells in vitro and angiogenesis in vivo," *British Journal of Pharmacology*, vol. 122, no. 6, pp. 1061–1066, 1997.

[70] S. Ch'ng, R. A. Wallis, L. Yuan, P. F. Davis, and S. T. Tan, "Mast cells and cutaneous malignancies," *Modern Pathology*, vol. 19, no. 1, pp. 149–159, 2006.

[71] R.-M. Amini, K. Aaltonen, H. Nevanlinna et al., "Mast cells and eosinophils in invasive breast carcinoma," *BMC Cancer*, vol. 7, article 165, 2007.

[72] S. Maltby, K. Khazaie, and K. M. McNagny, "Mast cells in tumor growth: angiogenesis, tissue remodelling and immune-modulation," *Biochimica et Biophysica Acta—Reviews on Cancer*, vol. 1796, no. 1, pp. 19–26, 2009.

[73] K. Norrby, "Mast cells and angiogenesis," *APMIS*, vol. 110, no. 5, pp. 355–371, 2002.

[74] D. D. Metcalfe, D. Baram, and Y. A. Mekori, "Mast cells," *Physiological Reviews*, vol. 77, no. 4, pp. 1033–1079, 1997.

[75] R. J. Blair, H. Meng, M. J. Marchese et al., "Human mast cells stimulate vascular tube formation. Tryptase is a novel, potent angiogenic factor," *The Journal of Clinical Investigation*, vol. 99, no. 11, pp. 2691–2700, 1997.

[76] A. F. Milia, M. B. Salis, T. Stacca et al., "Protease-activated receptor-2 stimulates angiogenesis and accelerates hemodynamic recovery in a mouse model of hindlimb ischemia," *Circulation Research*, vol. 91, no. 4, pp. 346–352, 2002.

[77] Y. Liu and B. M. Mueller, "Protease-activated receptor-2 regulates vascular endothelial growth factor expression in MDA-MB-231 cells via MAPK pathways," *Biochemical and Biophysical Research Communications*, vol. 344, no. 4, pp. 1263–1270, 2006.

[78] W. Zhang, G. Stoica, S. I. Tasca, K. A. Kelly, and C. J. Meininger, "Modulation of tumor angiogenesis by stem cell factor," *Cancer Research*, vol. 60, no. 23, pp. 6757–6762, 2000.

[79] B. Huang, Z. Lei, G.-M. Zhang et al., "SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment," *Blood*, vol. 112, no. 4, pp. 1269–1279, 2008.

[80] D. Ribatti, A. Vacca, R. Ria et al., "Neovascularisation, expression of fibroblast growth factor-2, and mast cells with tryptase activity increase simultaneously with pathological progression in human malignant melanoma," *European Journal of Cancer*, vol. 39, no. 5, pp. 666–674, 2003.

[81] D. Ribatti, M. G. Ennas, A. Vacca et al., "Tumor vascularity and tryptase-positive mast cells correlate with a poor prognosis in melanoma," *European Journal of Clinical Investigation*, vol. 33, no. 5, pp. 420–425, 2003.

[82] D. Ribatti, N. Finato, E. Crivellato et al., "Angiogenesis and mast cells in human breast cancer sentinel lymph nodes with and without micrometastases," *Histopathology*, vol. 51, no. 6, pp. 837–842, 2007.

[83] D. Ribatti, A. S. Belloni, B. Nico et al., "Tryptase- and leptin-positive mast cells correlate with vascular density in uterine leiomyomas," *The American Journal of Obstetrics and Gynecology*, vol. 196, no. 5, pp. 470.e1–470.e7, 2007.

[84] S. Yodavudh, S. Tangjitgamol, and S. Puangsa-art, "Prognostic significance of microvessel density and mast cell density for the survival of Thai patients with primary colorectal cancer," *Journal of the Medical Association of Thailand*, vol. 91, no. 5, pp. 723–732, 2008.

[85] D. Ribatti, S. Molica, A. Vacca et al., "Tryptase-positive mast cells correlate positively with bone marrow angiogenesis in B-cell chronic lymphocytic leukemia," *Leukemia*, vol. 17, no. 7, pp. 1428–1430, 2003.

[86] D. Ribatti, A. Vacca, B. Nico et al., "Bone marrow angiogenesis and mast cell density increase simultaneously with progression of human multiple myeloma," *British Journal of Cancer*, vol. 79, no. 3-4, pp. 451–455, 1999.

[87] D. Ribatti, G. Polimeno, A. Vacca et al., "Correlation of bone marrow angiogenesis and mast cells with tryptase activity in myelodysplastic syndromes," *Leukemia*, vol. 16, no. 9, pp. 1680–1684, 2002.

[88] H. J. Nielsen, U. Hansen, I. J. Christensen, C. M. Reimert, N. Brünner, and F. Moesgaard, "Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue," *The Journal of Pathology*, vol. 189, no. 4, pp. 487–495, 1999.

[89] S. Aaltomaa, P. Lipponen, S. Papinaho, and V.-M. Kosma, "Mast cells in breast cancer," *Anticancer Research*, vol. 13, no. 3, pp. 785–788, 1993.

[90] S. Dabiri, D. Huntsman, N. Makretsov et al., "The presence of stromal mast cells identifies a subset of invasive breast cancers with a favorable prognosis," *Modern Pathology*, vol. 17, no. 6, pp. 690–695, 2004.

[91] T. J. Welsh, R. H. Green, D. Richardson, D. A. Waller, K. J. O'Byrne, and P. Bradding, "Macrophage and mast-cell invasion of tumor cell islets confers a marked survival advantage in non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 23, no. 35, pp. 8959–8967, 2005.

[92] F. Erba, L. Fiorucci, S. Pascarella, E. Menegatti, P. Ascenzi, and F. Ascoli, "Selective inhibition of human mast cell tryptase by gabexate mesylate, an antiproteinase drug," *Biochemical Pharmacology*, vol. 61, no. 3, pp. 271–276, 2001.

[93] S. Mori, Y. Itoh, R. Shinohata, T. Sendo, R. Oishi, and M. Nishibiro, "Nafamostat mesilate is an extremely potent inhibitor of human tryptase," *Journal of Pharmacological Sciences*, vol. 92, no. 4, pp. 420–423, 2003.

[94] Y. Uchima, T. Sawada, and K. Hirakawa, "Action of antiproteases on pancreatic cancer cells," *Journal of the Pancreas*, vol. 8, supplement 4, pp. 479–487, 2007.

[95] J. J. Martindale, *The Extra Pharmacopeia*, The Royal Pharmaceutical Society, London, UK, 31st edition, 1996.

[96] E. Menegatti, M. Bolognesi, S. Scalia, F. Bortolotti, M. Guarneri, and P. Ascenzi, "Gabexate mesylate inhibition of serine proteases: thermodynamic and computer-graphics analysis," *Journal of Pharmaceutical Sciences*, vol. 75, no. 12, pp. 1171–1174, 1986.

[97] R. Cortes, P. Ascenzi, M. Colasanti et al., "Cross-enzyme inhibition by gabexate mesylate: formulation and reactivity study," *Journal of Pharmaceutical Sciences*, vol. 87, no. 11, pp. 1335–1340, 1998.

[98] A.-P. Sappino, N. Busso, D. Belin, and J.-D. Vassalli, "Increase of urokinase-type plasminogen activator gene expression in human lung and breast carcinomas," *Cancer Research*, vol. 47, no. 15, pp. 4043–4046, 1987.

[99] M. Gottesman, "The role of proteases in cancer," *Seminars in Cancer Biology*, vol. 1, pp. 97–160, 1990.

[100] L. A. Liotta and W. G. Stetler-Stevenson, "Tumor invasion and metastasis: an imbalance of positive and negative regulation," *Cancer Research*, vol. 51, supplement 18, pp. 5045–5059, 1991.

[101] R. Dahiya, W.-H. Yoon, R. Boyle, T. Schoenberg, T.-S. B. Yen, and P. Narayan, "Biochemical,cytogenetic, and morphological characteristics of human primary and metastatic prostate cancer cell lines," *Biochemistry International*, vol. 27, no. 4, pp. 567–577, 1992.

[102] W. G. Stetler-Stevenson, "Progelatinase A activation during tumor cell invasion," *Invasion and Metastasis*, vol. 95, no. 14, pp. 259–268, 1994.

[103] M. S. Pepper, "Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 21, no. 7, pp. 1104–1117, 2001.

[104] K. Naito, N. Kanbayashi, S. Nakajima et al., "Inhibition of growth of human tumor cells in nude mice by a metalloproteinase inhibitor," *International Journal of Cancer*, vol. 58, no. 5, pp. 730–735, 1994.

[105] S. A. Watson, T. M. Morris, G. Robinson, M. J. Crimmin, P. D. Brown, and J. D. Hardcastle, "Inhibition of organ invasion by the matrix metalloproteinase inhibitor batimastat (BB-94) in two human colon carcinoma metastasis models," *Cancer Research*, vol. 55, no. 16, pp. 3629–3633, 1995.

[106] R. Reich, E. W. Thompson, Y. Iwamoto et al., "Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells," *Cancer Research*, vol. 48, no. 12, pp. 3307–3312, 1988.

[107] R. Benelli, R. Adatia, B. Ensoli, W. G. Stetler-Stevenson, L. Santi, and A. Albini, "Inhibition of AIDS-Kaposi's sarcoma cell induced endothelial cell invasion by TIMP-2 and a synthetic peptide from the metalloproteinase propeptide: Implications for an anti-angiogenic therapy," *Oncology Research*, vol. 6, no. 6, pp. 251–257, 1994.

[108] B. Anand-Apte, M. S. Pepper, E. Voest et al., "Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3," *Investigative Ophthalmology and Visual Science*, vol. 38, no. 5, pp. 817–823, 1997.

[109] N. Hiraoka, E. Allen, I. J. Apel, M. R. Gyetko, and S. J. Weiss, "Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins," *Cell*, vol. 95, no. 3, pp. 365–377, 1998.

[110] M. Nguyen, J. Arkell, and C. J. Jackson, "Thrombin rapidly and efficiently activates gelatinase A in human microvascular endothelial cells via a mechanism independent of active MT1 matrix metalloproteinase," *Laboratory Investigation*, vol. 79, no. 4, pp. 467–475, 1999.

[111] N. Ramos-DeSimone, E. Hahn-Dantona, J. Sipley, H. Nagase, D. L. French, and J. P. Quigley, "Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion," *The Journal of Biological Chemistry*, vol. 274, no. 19, pp. 13066–13076, 1999.

[112] T. Sendo, T. Sumimura, Y. Itoh et al., "Involvement of proteinase-activated receptor-2 in mast cell tryptase-induced barrier dysfunction in bovine aortic endothelial cells," *Cellular Signalling*, vol. 15, no. 8, pp. 773–781, 2003.

[113] C. J. Meininger, "Mast cells and tumor-associated angiogenesis," *Chemical Immunology*, vol. 61, no. 1, pp. 239–257, 1995.

[114] L. F. Brown, M. Detmar, K. Claffey et al., "Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine.," *EXS*, vol. 79, pp. 233–269, 1997.

[115] A. Friedl, Z. Chang, A. Tierney, and A. C. Rapraeger, "Differential binding of fibroblast growth factor-2 and -7 to basement membrane heparan sulfate: comparison of normal and abnormal human tissues," *The American Journal of Pathology*, vol. 150, no. 4, pp. 1443–1455, 1997.

[116] Z. Poltorak, T. Cohen, R. Sivan et al., "VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix," *The Journal of Biological Chemistry*, vol. 272, no. 11, pp. 7151–7158, 1997.

[117] C. S. Karapetis, S. Khambata-Ford, D. J. Jonker et al., "K-ras mutations and benefit from cetuximab in advanced colorectal cancer," *The New England Journal of Medicine*, vol. 359, no. 17, pp. 1757–1765, 2008.

[118] N. Normanno, S. Tejpar, F. Morgillo, A. de Luca, E. van Cutsem, and F. Ciardiello, "Implications for KRAS status and EGFR-targeted therapies in metastatic CRC," *Nature Reviews Clinical Oncology*, vol. 6, no. 9, pp. 519–527, 2009.

[119] C. Leporini, F. Saullo, G. Filippelli et al., "Management of dermatologic toxicities associated with monoclonal antibody epidermal growth factor receptor inhibitors: a case review," *Journal of Pharmacology & Pharmacotherapeutics*, vol. 4, supplement 1, pp. S78–S85, 2013.

[120] R. Shimamoto, T. Sawada, Y. Uchima et al., "A role for protease-activated receptor-2 in pancreatic cancer cell proliferation," *International Journal of Oncology*, vol. 24, no. 6, pp. 1401–1406, 2004.

[121] V. Ellenrieder, S. F. Hendler, C. Ruhland, W. Boeck, G. Adler, and T. M. Gress, "TGF-$\beta$-induced invasiveness of pancreatic cancer cells is mediated by matrix metalloproteinase-2 and the urokinase plasminogen activator system," *International Journal of Cancer*, vol. 93, no. 2, pp. 204–211, 2001.

[122] H. Teraoka, T. Sawada, Y. Yamashita et al., "TGF-beta1 promotes liver metastasis of pancreatic cancer by modulating the capacity of cellular invasion," *International Journal of Oncology*, vol. 19, no. 4, pp. 709–715, 2001.

[123] H. Teraoka, T. Sawada, T. Nishihara et al., "Enhanced VEGF production and decreased immunogenicity induced by TGF-$\beta$ 1 promote liver metastasis of pancreatic cancer," *British Journal of Cancer*, vol. 85, no. 4, pp. 612–617, 2001.

[124] V. Malamud, A. Vaaknin, O. Abramsky et al., "Tryptase activates peripheral blood mononuclear cells causing the synthesis and release of TNF-$\alpha$, IL-6 and IL-1$\beta$: possible relevance to multiple sclerosis," *Journal of Neuroimmunology*, vol. 138, no. 1-2, pp. 115–122, 2003.

[125] L. Woodman, S. Siddiqui, G. Cruse et al., "Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-$\beta$1," *The Journal of Immunology*, vol. 181, no. 7, pp. 5001–5007, 2008.

[126] X. Zeng, S. Zhang, L. Xu, H. Yang, and S. He, "Activation of protease-activated receptor 2-mediated signaling by mast cell tryptase modulates cytokine production in primary cultured astrocytes," *Mediators of Inflammation*, vol. 2013, Article ID 140812, 10 pages, 2013.

[127] T. Aoyama, Y. Ino, M. Ozeki et al., "Pharmacological studies of FUT-175, nafamstat mesilate. I. Inhibition of protease activity in in vitro and in vivo experiments," *Japanese Journal of Pharmacology*, vol. 35, no. 3, pp. 203–227, 1984.

[128] M. Uchiba, K. Okajima, H. Abe, H. Okabe, and K. Takatsuki, "Effect of nafamostat mesilate, a synthetic protease inhibitor, on tissue factor-factor VIIa complex activity," *Thrombosis Research*, vol. 74, no. 2, pp. 155–161, 1994.

[129] L. B. Schwartz, "Mast cell tryptase: properties and roles in human allergic responses," in *Mast Cell Proteases in Immunology*

*and Biology*, G. M. Caughey, Ed., pp. 9–23, Marcel Dekker, New York, NY, USA, 1995.

[130] J. M. Clark, W. R. Moore, and R. D. Tanaka, "Tryptase inhibitors: a new class of antiinflammatory drugs," *Drugs Future*, vol. 21, no. 8, pp. 811–816, 1996.

[131] C. P. Sommerhoff, W. Bode, G. Matschiner, A. Bergner, and H. Fritz, "The human mast cell tryptase tetramer: a fascinating riddle solved by structure," *Biochimica et Biophysica Acta*, vol. 1477, no. 1-2, pp. 75–89, 2000.

[132] T. Yoshikawa, M. Murakami, Y. Furukawa, H. Kato, S. Takemura, and M. Kondo, "Effects of FUT-175, a new synthetic protease inhibitor on endotoxin-induced disseminated intravascular coagulation in rats," *Haemostasis*, vol. 13, no. 6, pp. 374–378, 1983.

[133] H. Takahashi, S. Takizawa, W. Tatewaki et al., "Nafamostat mesilate (FUT-175) in the treatment of patients with disseminated intravascular coagulation," *Thrombosis and Haemostasis*, vol. 62, p. 372, 1989.

[134] T. Kimura, S. Fuchimoto, H. Iwagaki, A. Hizuta, and K. Orita, "Inhibitory effect of nafamostat mesilate on metastasis into the livers of mice and on invasion of the extracellular matrix by cancer cells," *Journal of International Medical Research*, vol. 20, no. 4, pp. 343–352, 1992.

[135] W. Wang, J. L. Abbruzzese, D. B. Evans, L. Larry, K. R. Cleary, and P. J. Chiao, "The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells," *Clinical Cancer Research*, vol. 5, no. 1, pp. 119–127, 1999.

[136] S. Fujioka, G. M. Sclabas, C. Schmidt et al., "Function of nuclear factor kappaB in pancreatic cancer metastasis," *Clinical Cancer Research*, vol. 9, no. 1, pp. 346–354, 2003.

[137] A. Arlt and H. Schäfer, "NFκB-dependent chemoresistance in solid tumors," *International Journal of Clinical Pharmacology and Therapeutics*, vol. 40, no. 8, pp. 336–347, 2002.

[138] C. Rosette, R. B. Roth, P. Oeth et al., "Role of ICAM1 in invasion of human breast cancer cells," *Carcinogenesis*, vol. 26, no. 5, pp. 943–950, 2005.

[139] S. Huang, J. B. Robinson, A. DeGuzman, C. D. Bucana, and I. J. Fidler, "Blockade of nuclear factor-κb signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8," *Cancer Research*, vol. 60, no. 19, pp. 5334–5339, 2000.

[140] Y. Nagakawa, T. Aoki, K. Kasuya, A. Tsuchida, and Y. Koyanagi, "Histologic features of venous invasion, expression of vascular endothelial growth factor and matrix metalloproteinase-2 and matrix metalloproteinase-9, and the relation with liver metastasis in pancreatic cancer," *Pancreas*, vol. 24, no. 2, pp. 169–178, 2002.

[141] W. Wang, J. L. Abbruzzese, D. B. Evans, and P. J. Chiao, "Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA," *Oncogene*, vol. 18, no. 32, pp. 4554–4563, 1999.

[142] T. V. Rakitina, I. A. Vasilevskaya, and P. J. O'Dwyer, "Additive interaction of oxaliplatin and 17-allylamino-17-demethoxygeldanamycin in colon cancer cell lines results from inhibition of nuclear factor κB signaling," *Cancer Research*, vol. 63, no. 24, pp. 8600–8605, 2003.

[143] C. Wilson, C. Purcell, A. Seaton et al., "Chemotherapy-induced CXC-chemokine/CXC-chemokine receptor signaling in metastatic prostate cancer cells confers resistance to oxaliplatin through potentiation of nuclear factor-κB transcription and evasion of apoptosis," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 327, no. 3, pp. 746–759, 2008.

[144] S. Banerjee, A. O. Kaseb, Z. Wang et al., "Antitumor activity of gemcitabine and oxaliplatin is augmented by thymoquinone in pancreatic cancer," *Cancer Research*, vol. 69, no. 13, pp. 5575–5583, 2009.

[145] C. Sperti, C. Pasquali, A. Piccoli, and S. Pedrazzoli, "Recurrence after resection for ductal adenocarcinoma of the pancreas," *World Journal of Surgery*, vol. 21, no. 2, pp. 195–200, 1997.

[146] T. Uwagawa, Z. Li, Z. Chang et al., "Mechanisms of synthetic serine protease inhibitor (FUT-175)-mediated cell death," *Cancer*, vol. 109, no. 10, pp. 2142–2153, 2007.

[147] K. Furukawa, T. Iida, H. Shiba et al., "Anti-tumor effect by inhibition of NF-κB activation using nafamostat mesilate for pancreatic cancer in a mouse model," *Oncology Reports*, vol. 24, no. 4, pp. 843–850, 2010.

[148] T. Uwagawa, T. Misawa, N. Tsutsui et al., "Phase II study of gemcitabine in combination with regional arterial infusion of nafamostat mesilate for advanced pancreatic cancer," *American Journal of Clinical Oncology: Cancer Clinical Trials*, vol. 36, no. 1, pp. 44–48, 2013.

[149] S. Nystedt, K. Emilsson, C. Wahlestedt, and J. Sundelin, "Molecular cloning of a potential proteinase activated receptor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 20, pp. 9208–9212, 1994.

[150] S. K. Böhm, W. Kong, D. Brömme et al., "Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2," *Biochemical Journal*, vol. 314, no. 3, part 3, pp. 1009–1016, 1996.

[151] A. Kawabata, R. Kuroda, M. Nishida et al., "Protease-activated receptor-2 (PAR-2) in the pancreas and parotid gland: immunolocalization and involvement of nitric oxide in the evoked amylase secretion," *Life Sciences*, vol. 71, no. 20, pp. 2435–2446, 2002.

[152] V. M. Shpacovitch, T. Brzoska, J. Buddenkotte et al., "Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor κB in human dermal microvascular endothelial cells," *Journal of Investigative Dermatology*, vol. 118, no. 2, pp. 380–385, 2002.

[153] S. Zhang, X. Zeng, H. Yang, G. Hu, and S. He, "Mast cell tryptase induces microglia activation via protease-activated receptor 2 signaling," *Cellular Physiology and Biochemistry*, vol. 29, no. 5-6, pp. 931–940, 2012.

[154] Y. Ma, B. Zhang, R. Qian, C. Lu, F. Zhao, and L. Yin, "Tryptase activates PKB in inflammatory reaction in ECV304 cells," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1763, no. 3, pp. 313–321, 2006.

[155] W. S. Chen, P.-Z. Xu, K. Gottlob et al., "Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene," *Genes & Development*, vol. 15, no. 17, pp. 2203–2208, 2001.

[156] M. Cheung and J. R. Testa, "Diverse mechanisms of AKT pathway activation in human malignancy," *Current Cancer Drug Targets*, vol. 13, no. 3, pp. 234–244, 2013.

[157] S. Kobayashi, T. Asano, and T. Ochiai, "A proposal of no-touch isolation technique in pancreatoduodenectomy for periampullary carcinomas," *Hepato-Gastroenterology*, vol. 48, no. 38, pp. 372–374, 2001.

[158] H. Azuma, K. Banno, and T. Yoshimura, "Pharmacological properties of N-(3′, 4′ dimethoxycinnamoyl) anthranilic acid (N-5′), a new anti atopic agent," *British Journal of Pharmacology*, vol. 58, no. 4, pp. 483–488, 1976.

[159] H. Komatsu, M. Kojima, N. Tsutsumi et al., "Study of the mechanism of inhibitory action of tranilast on chemical mediator release," *Japanese Journal of Pharmacology*, vol. 46, no. 1, pp. 43–51, 1988.

[160] M. Isaji, M. Nakajoh, and J. Naito, "Selective inhibition of collagen accumulation by N-(3,4-dimethoxycinnamoyl)anthranilic acid (N-5') in granulation tissue," *Biochemical Pharmacology*, vol. 36, no. 4, pp. 469–474, 1987.

[161] T. Yanagi, M. Watanabe, S. Fukuda, and Y. Tsuji, "Suppressive effects of tranilast (TN) on human mononuclear cells," *Japanese Journal of Inflammation*, vol. 7, pp. 169–173, 1987.

[162] H. Suzawa, S. Kikuchi, N. Arai, and A. Koda, "The mechanism involved in the inhibitory action of tranilast on collagen biosynthesis of keloid fibroblasts," *Japanese Journal of Pharmacology*, vol. 60, no. 2, pp. 91–96, 1992.

[163] M. Yashiro, Y.-S. Chung, and M. Sowa, "Tranilast (N-(3,4-dimethoxycinnamoyl) anthranilic acid) down-regulates the growth of scirrhous gastric cancer," *Anticancer Research A*, vol. 17, no. 2, pp. 895–900, 1997.

[164] H. Shime, M. Kariya, A. Orii et al., "Tranilast inhibits the proliferation of uterine leiomyoma cells in vitro through G1 arrest associated with the induction of p21waf1 and p53," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 12, pp. 5610–5617, 2002.

[165] M. Rogosnitzky, R. Danks, and E. Kardash, "Therapeutic potential of tranilast, an anti-allergy drug, in proliferative disorders," *Anticancer Research*, vol. 32, no. 7, pp. 2471–2478, 2012.

[166] D. Padua and J. Massagué, "Roles of TGFbeta in metastasis," *Cell Research*, vol. 19, no. 1, pp. 89–102, 2009.

[167] K. Izumi, A. Mizokami, T. Shima et al., "Preliminary results of tranilast treatment for patients with advanced castration-resistant prostate cancer," *Anticancer Research*, vol. 30, no. 7, pp. 3077–3081, 2010.

[168] S. Shigeki, T. Murakami, N. Yata, and Y. Ikuta, "Treatment of keloid and hypertrophic scars by iontophoretic transdermal delivery of tranilast," *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery*, vol. 31, no. 2, pp. 151–158, 1997.

[169] M. Isaji, H. Miyata, and Y. Ajisawa, "Tranilast: a new application in the cardiovascular field as an antiproliferative drug," *Cardiovascular Drug Reviews*, vol. 16, no. 3, pp. 288–299, 1998.

[170] N. I. Kerkvliet, "AHR-mediated immunomodulation: the role of altered gene transcription," *Biochemical Pharmacology*, vol. 77, no. 4, pp. 746–760, 2009.

[171] A. Gomez-Duran, J. M. Carvajal-Gonzalez, S. Mulero-Navarro, B. Santiago-Josefat, A. Puga, and P. M. Fernandez-Salguero, "Fitting a xenobiotic receptor into cell homeostasis: How the dioxin receptor interacts with TGF$\beta$ signaling," *Biochemical Pharmacology*, vol. 77, no. 4, pp. 700–712, 2009.

[172] C. E. Ruby, M. Leid, and N. I. Kerkvliet, "2,3,7,8-Tetrachlorodibenzo-*p*-dioxin suppresses tumor necrosis factor-$\alpha$ and anti-CD40-induced activation of NF-$\kappa$B/Rel in dendritic cells: p50 homodimer activation is not affected," *Molecular Pharmacology*, vol. 62, no. 3, pp. 722–728, 2002.

[173] M. Ammendola, R. Sacco, G. Sammarco et al., "Correlation between serum tryptase, mast cells positive to tryptase and microvascular density in colo-rectal cancer patients: possible biological-clinical significance," *Plos ONE*, vol. 9, no. 6, Article ID e99512, 2014.

[174] I. Marech, M. Ammendola, R. Sacco et al., "Serum tryptase, mast cells positive to tryptase and microvascular density evaluation in early breast cancer patients: possible translational significance," *BMC Cancer*, vol. 14, article 534, 2014.

[175] K. Nanba, T. Oura, S. Soeda, N. Sioya, S. Tsukada, and K. Hanaoka, "Clinical evaluation of tranilast for keloid and hypertrophic scarring. Optimal dose finding study in a double blind study," *Nessho*, vol. 18, no. 30–45, 1992.

[176] L. K. Ashman and R. Griffith, "Therapeutic targeting of c-KIT in cancer," *Expert Opinion on Investigational Drugs*, vol. 22, no. 1, pp. 103–115, 2013.

[177] G. Ranieri, M. Mammì, E. Donato Di Paola et al., "Pazopanib a tyrosine kinase inhibitor with strong anti-angiogenetic activity: a new treatment for metastatic soft tissue sarcoma," *Critical Review in Oncology/Hematology*, vol. 89, no. 2, pp. 322–329, 2014.

[178] E. R. Kessler, D. W. Bowles, T. W. Flaig, E. T. Lam, and A. Jimeno, "Axitinib, a new therapeutic option in renal cell carcinoma," *Drugs of Today*, vol. 48, no. 10, pp. 633–644, 2012.

[179] I. Marech, R. Patruno, N. Zizzo et al., "Masitinib (AB1010), from canine tumor model to human clinical development: where we are?" *Critical Reviews in Oncology/Hematology*, vol. 91, no. 1, pp. 98–111, 2014.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 759473, 10 pages
http://dx.doi.org/10.1155/2014/759473

*Review Article*

# The Nerve Growth Factor Signaling and Its Potential as Therapeutic Target for Glaucoma

**Haitao Wang,[1] Rikang Wang,[1] Thilini Thrimawithana,[2] Peter J. Little,[2] Jiangping Xu,[3] Zhong-Ping Feng,[4] and Wenhua Zheng[1]**

[1] *State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong 510006, China*
[2] *Discipline of Pharmacy, School of Medical Sciences and Diabetes Complications Group, Health Innovations Research Institute, RMIT University, Bundoora, VIC 3083, Australia*
[3] *School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*
[4] *Department of Physiology, Faculty of Medicine, University of Toronto, 1 King's College Circle, Toronto, ON, Canada M5S 1A8*

Correspondence should be addressed to Wenhua Zheng; whzheng1231@gmail.com

Received 8 March 2014; Accepted 12 August 2014; Published 31 August 2014

Academic Editor: Jian Xiao



Neuroprotective therapies which focus on factors leading to retinal ganglion cells (RGCs) degeneration have been drawing more and more attention. The beneficial effects of nerve growth factor (NGF) on the glaucoma have been recently suggested, but its effects on eye tissue are complex and controversial in various studies. Recent clinical trials of systemically and topically administrated NGF demonstrate that NGF is effective in treating several ocular diseases, including glaucoma. NGF has two receptors named high affinity NGF tyrosine kinase receptor TrkA and low affinity receptor p75NTR. Both receptors exist in cells in retina like RGC (expressing TrkA) and glia cells (expressing p75NTR). NGF functions by binding to TrkA or p75NTR alone or both together. The binding of NGF to TrkA alone in RGC promotes RGC's survival and proliferation through activation of TrkA and several prosurvival pathways. In contrast, the binding of NGF to p75NTR leads to apoptosis although it also promotes survival in some cases. Binding of NGF to both TrkA and p75NTR at the same time leads to survival in which p75NTR functions as a TrkA helping receptor. This review discusses the current understanding of the NGF signaling in retina and the therapeutic implications in the treatment of glaucoma.

## 1. Introduction

Glaucoma is one of the leading causes of blindness worldwide. Glaucoma is characterized by retinal ganglion cell (RGC) degeneration and loss of visual field and it occurs with or without elevated intraocular pressure (IOP) [1]. Apoptosis or programmed cell death of RGCs and optic nerve degeneration may be the cause of blindness and it may occur in the state of elevated intraocular pressure; however, both RGC apoptosis and optic nerve atrophy, due to glaucoma, can occur independently of elevated IOP. Clinically, in addition to the lowering of IOP, one of the main targets of glaucoma therapy is to delay the apoptosis and promote the survival of RGC. Up to now, there is considerable evidence showing that attenuation of RGC degeneration is potentially an effective therapeutic strategy for treatment of glaucoma [2, 3]. Thus, therapeutic neuroprotection of RGCs aims to prevent or delay cell death and maintaining normal neuronal functions is an important alternative approach for the treatment of glaucoma.

Nerve growth factor (NGF) was discovered in 1948. It prevents neuronal apoptosis in primary cultured neurons and reduces neuronal degeneration in animal models of neurodegenerative diseases [4]. These results in animals have led to several clinical trials [5, 6]. In clinical studies, treatment with NGF was accompanied by beneficial effects on cognitive performance, but it also led to back pain [7]. Positive results from the use of NGF in the treatment of classical

neurodegenerative diseases lead researchers to investigate the role of NGF of the treatment of glaucoma based on glaucoma being a neurodegenerative disease related to the damage of optic nerves. RGCs are special neurons which receive visual information from photoreceptors and transmit signals to several brain regions including the thalamus, hypothalamus, and mesencephalon and midbrain [8]. Although NGF treatment is effective in the treatment of glaucoma, in some studies, there are also some negative reports; one example is the proapoptotic effect of p75 neurotrophin receptor (p75NTR) in glia cells; binding of NGF to p75NTR is associated with retinal ganglion cell apoptosis [9]. Thus, an enhanced understanding of the molecular pathways and mechanisms is required to better appreciate and potentially exploit the therapeutic potential NGF and its signaling pathways for the treatment of glaucoma. In this review, we will examine the current understanding of the NGF signaling pathway and its potential as a therapeutic target for the treatment of glaucoma.

## 2. Nerve Growth Factor Receptors and Their Signaling Pathways

*2.1. General Features of NGF and Its Similarity with Other Growth Factors.* Growth factors are produced by our body and they have a very extensive role in the regulation of many cellular processes. The binding of growth factors to their receptors on the cell surface affects cellular survival, proliferation, and/or differentiation [10, 11]. For example, platelet-derived growth factor promotes the proliferation of glioblastoma cells through downregulation of miR-21 [12]; the breadth of actions of such an agent is apparent from the observation that it can also enhance glycosaminoglycan elongation on the proteoglycan biglycan and thereby plays a role in the initiation of atherosclerosis [13]. The pleiotropic growth factor, transforming growth factor-$\beta$, promotes wound healing, inhibits macrophage proliferation, and protects against nerve-injury-induced neuropathic pain [14].

NGF is another important polypeptide growth factor which functions to regulate the growth and survival of nerve cells; it was discovered by Rita Levi-Montalcini and Stanley Cohen in the 1950s [15]. NGF belongs to a family of factors also known as neurotrophins. Other members of the neurotrophin family include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5); all of them are known for regulating brain development and functions [16]. NGF is formed by cleavage from Pro-NGF, which is the precursor protein form of NGF; however, the roles of Pro-NGF and NGF are not consistent; treatment with Pro-NGF in cervical ganglia neurons which express both of the NGF receptors p75NTR and tyrosine receptor kinase A (TrkA) leads to programmed cell death, whereas NGF treatment of these same neurons results in survival and axonal growth [17]. Free NGF displays multiple physiological actions, in the central nerve system. NGF possesses neurotrophic effects and is critical for the neurite outgrowth and survival and maintenance of neurons. Studies, *in vitro* and *in vivo*, have shown that NGF stimulates neurite outgrowth and axonal branching and extension [18–20]. Most importantly, NGF has strong antiapoptotic effect and, with deprivation of NGF, neurons exhibit a series of morphological changes and eventually undergo apoptosis [21].

The clinical significance of NGF has been widely studied and it is recognized that NGF profoundly affects the development of both the young and the adult nervous systems [22]. In the central nervous system, NGF is a key neurotrophin and its dysregulation could be involved in various neuronal degeneration diseases such as Alzheimer's disease and multiple sclerosis [23, 24]. Dysfunction of NGF may also be linked to mental or psychiatric disorders, such as schizophrenia, depression, and autism [25–27]. Low levels of NGF in cerebrospinal fluid and a deficit of NGF signaling might provide the basis for the occurrence of these neurological diseases [27]. Besides its role in the CNS, there is evidence that NGF circulates throughout the body and plays roles in many organs [28]. For example, variability in NGF levels is associated with atherosclerosis and hence cardiovascular disease and also metabolic disorders such as diabetes and obesity [29, 30]. Specifically, NGF levels are decreased in atherosclerotic coronary vascular tissue and a decrease in plasma NGF could be detected in metabolic syndrome patients [31]. NGF deficits are the main cause of these diseases mentioned above, so, to some extent, supply of NGF to the target region may reverse the pathology of the diseases or alleviate the symptoms. However, the obstacle is the drug delivery technology and pharmacokinetic properties since the apparent need is to deliver NGF to the target region and specifically the target regions in order to reduce the adverse effects at other sites [32]. This obstacle will not occur if NGF is used topically, for example, in the application of NGF in the treatment of ocular disease, and we will illustrate these possibilities in the following sections.

NGF plays its role by binding to its receptors located in the surface of cells. TrkA is the high affinity catalytically active receptor for NGF. NGF binding to TrkA leads to the phosphorylation of TrkA and activation of its downstream targets, such as protein kinase B (Akt) or extracellular signal-regulated protein kinase 1/2 (ERK1/2), which eventually cause neural differentiation and prevention of apoptosis [33]. The other NGF receptor, p75NTR, is a low affinity receptor [34]. The precise role of p75NTR is complicated, and, depending upon the cellular context, it can promote cell survival, cell death, or growth inhibition; for example, treatment of normal eyes with an NGF mutant-selective p75NTR agonist causes progressive RGC death [35]; in contrast, p75NTR overexpression in breast tumor cells favors tumor survival and contributes to tumor resistance to drugs [36].

*2.2. Signaling Pathway of NGF and Its Receptors.* The affinity of NGF binding to p75NTR receptor is weaker than NGF binding to TrkA, but the cell type distribution of p75NTR is wider than that of TrkA; TrkA receptor is mainly expressed at neurons responsive to NGF: peripheral sensory neurons, sympathetic neurons, and basal forebrain cholinergic neurons [37], while the p75NTR receptor is more widely distributed. In addition to cells expressing the TrkA receptor,

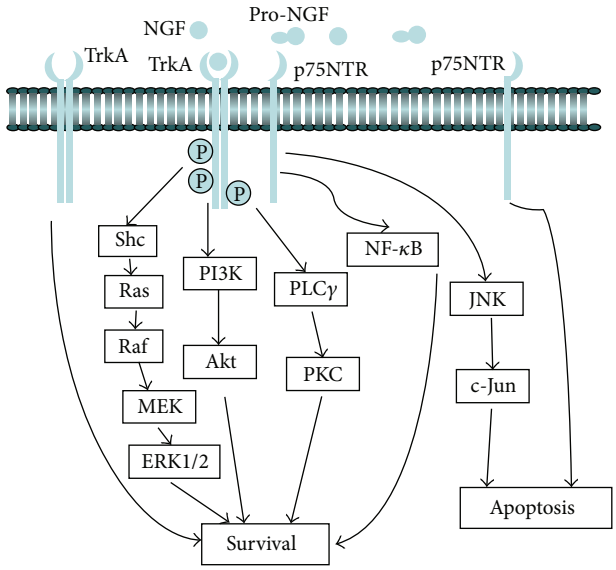



Figure 1: Signaling of NGF receptors. NGF is formed by cleavage from Pro-NGF, which is the precursor protein form of NGF. TrkA receptor is the high affinity receptor for NGF; NGF binding to TrkA causes the phosphorylation of TrkA and activation of multiple signaling pathways such as the PI3K/Akt, Ras/Raf/MEK/ERK1/2, or PLC$\gamma$/PKC signaling pathways. Activation of these pathways eventually leads to different biological functions including the prevention of apoptosis. The other NGF receptor, p75NTR, is a low affinity receptor. The precise role of p75NTR depends upon the cellular context; it can enhance cell survival through NF-$\kappa$B pathway or promote cell death through JNK/c-Jun signal pathway.

the p75NTR receptor could be detected in motor neurons, Schwann cells, and cerebellar Purkinje cells [38]. Signaling of NGF receptors is shown in Figure 1.

TrkA is the high affinity catalytic receptor for the NGF and it mediates the main effects of NGF, which include cell growth, the formation and regeneration of neurites, and avoidance of programmed cell death [39]. Binding of NGF to TrkA receptor facilitates receptor dimerization and autophosphorylation of its tyrosine residues. Activation (phosphorylation) of the TrkA receptor provides docking sites for effector molecules such as Shc which in turn induces the recruitment of a complex of Shc/Grb2, subsequent to which several downstream signaling cascades are initiated and propagated [40].

Phosphorylation of the TrkA receptor leads to the interaction of TrkA and phosphatidylinositiol-3 kinase (PI3K). PI3K is activated and recruited to the plasma membrane and leads to the production of phosphoinositide 3,4,5-trisphosphate and membrane translocation of the serine/threonine-protein kinases Akt and Akt activation [20]. Although there are other downstream targets of PI3K, the PI3K/Akt signaling pathway is particularly important for neuronal survival and the synthesis of many new cellular proteins. In one exemplar, pathway Akt phosphorylates the proapoptotic proteins, such as forkhead box-O transcription factors (FoxO) and B-cell lymphoma 2 family members, thereby inhibiting neuronal apoptosis [41–43]. FoxO is a classic target of growth factor Trk receptor signaling [44, 45]; FoxO proteins are expressed in the detached retina [46]; therefore, phosphorylation of FoxO mediated by Akt may play a role in neurotrophins-mediated cell survival.

Another NGF-activated signaling pathway is the Ras-mediated activation of the mitogen-activated protein kinase (MAPK) pathway, which is initiated through recruitment and phosphorylation of Shc [47]. Ras is a membrane-associated G protein; the active Ras protein binds to and phosphorylates several proteins, including the protooncogene Raf. Raf, in turn, activates MAPK kinase (MEK) and phosphorylated MEK activates ERK1/2 [48]. Phosphorylated ERK1/2 may enter into nucleus and regulate the activity of many transcription factors including ETS domain-containing protein ELK1 [49]. ERK1/2 may also phosphorylate ribosomal S6 kinase (S6K), which leads to the phosphorylation of cyclic adenosine monophosphate response element binding protein, eventually affecting the regulation of the expression of NGF-inducible genes and, thus, contributing to neuronal differentiation or neurite outgrowth [50]. Besides the two pathways mentioned above, TrkA activation also leads to the survival and growth of neuronal cells through Phospholipase C gamma1 (PLC$\gamma$1) [51]. PLC$\gamma$1 supports activation of PKC signaling pathway and is thus involved in antimitogenic/mitogenic signaling.

The other receptor for NGF is p75NTR, but it is not a specific receptor for NGF as it also binds other neurotrophins, such as NT-3, NT-4/5, and BDNF [52]. The role of the p75NTR receptor is complicated. Binding of NGF with p75NTR with low affinity leads to apoptosis or cell survival in different cellular contexts [35, 36]. p75NTR can induce apoptosis both *in vitro* and *in vivo*. p75NTR activates Rac GTPase and activated c-jun N-terminal kinase (JNK), including an injury-specific isoform, JNK3 [53]. JNKs stimulate the expression of proapoptotic genes via the transactivation of specific transcription factors [54], so p75NTR can promote cell death. However, it also enhances cell survival; NGF treatment activates nuclear factor $\kappa$B (NF-$\kappa$B) through p75NTR and during this process, p75NTR-mediated NF-$\kappa$B activation enhances the survival response of developing sensory neurons to nerve growth factor [55]. NF-$\kappa$B is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of cell survival. Thus, in summary, the p75NTR receptor activates apoptotic signaling through the JNK cascade or cell survival through NF-$\kappa$B pathway.

NGF binds to both TrkA and p75NTR receptors when they coexpressed on the outer cell membrane, even though the affinity of TrkA with NGF is much higher. Studies *in vitro* have shown that neurons coexpressing p75NTR and TrkA respond to lower concentrations of NGF [56, 57], which means that p75NTR increases the responsiveness of TrkA to NGF. When the two receptors are coexpressed, the rate of association of NGF with TrkA increases compared to cells expressing TrkA alone [58]. The result infers that this interaction leads to the formation of binding sites with higher affinity for NGF than that of either receptor alone. Structural and mechanistic insights into NGF interactions with the TrkA and p75NTR receptors indicate that NGF could dimerize

TrkA and p75NTR exists as a preformed oligomer that is not dissociated by NGF [59]. There is no evidence to show that TrkA and p75NTR interact directly, so they may interact indirectly through the convergence of downstream signaling pathways and/or share adaptor molecules, rather than through direct receptor-receptor interactions [59]. These data indicate that the final fate of cells coexpressing both TrkA and p75NTR is complicated with the functional response related to the abundance of each receptor and the different agonists.

## 3. Causes and Pathophysiology of Glaucoma

Glaucoma is a severe eye disease, which is usually associated with absolute or relative elevated fluid pressure within the eyes and gradually progressive visual field loss. One major risk factor for glaucoma is the raised IOP. However, other mechanisms must be involved in the pathology of glaucoma because glaucoma can develop in the absence of elevated IOP and, in the clinic, different individuals respond differently to the elevated pressure [60]. In some populations, patients may have high eye pressure for many years but this never leads to glaucoma. Molecular studies on the differential expression of human genes under conditions of elevated IOP revealed that MMP1, MMP10, CXCL2, and PDPN were general responders and were altered in almost all the patients with glaucoma whilst STATH, FBN2, TF, OGN, IL6, IGF1, CRYAB, and ELAM1 (marker for glaucoma) had very patient specific changes in expression [61].

Notwithstanding the above, among the several causes for glaucoma, elevated eye pressure is one of the most important and best recognized acceptable risk factors [62]. Besides elevated IOP, glaucoma is also thought to arise from a mutation in a single gene or a group of genes; for instance, it is well identified that MYOC mutations are a major cause of glaucoma [63, 64]. This notion is supported by the observation that people with a family history of glaucoma have higher risk of developing glaucoma. The relationship between glaucoma with gene mutations and phenotypes has been reviewed by Fan and Wiggs [65]. Other risk factors for glaucoma include severely restricted blood flow to the eye, prolonged use of steroids, and ethnic and gender factors. Figure 2 shows a model of insults and their involvement of glaucoma.

Optic nerve damage is a common optic neuropathy of glaucoma, which is characterized by loss of retinal ganglion cells [66]. Both the high pressure and relatively low pressure can develop nerve damage and lead to blindness if left untreated. The inconsistent relationship of glaucomatous optic neuropathy with ocular hypertension has provoked the generation of other hypotheses and investigations. Among these studies, excitatory neurotransmitter toxicity (such as excessive glutamate release), hypoperfusion, trophic factors, retinal ganglion cell/axon degeneration, and neuron loss have received attention [67–70]. Neuroprotection is an effective strategy to attenuate RGC degeneration and facilitate the survival of optic nerves through blocking these risk factors associated with RGC loss in glaucoma. NGF plays an effect by preventing neuronal degeneration in animal models of

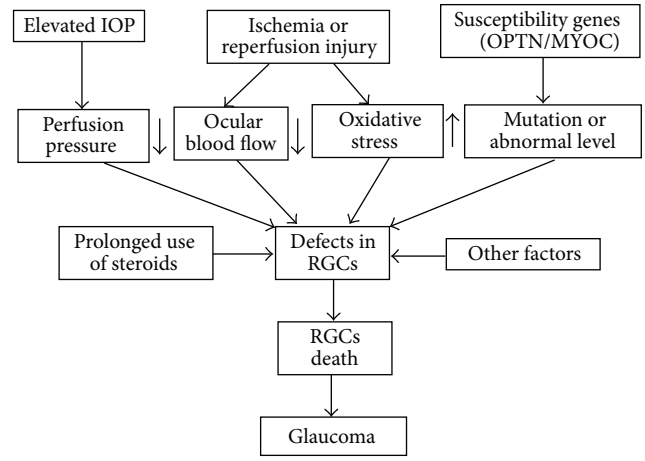



Figure 2: A model of insults and their involvement of glaucoma. Increased intraocular pressure (IOP) usually leads to abnormal pressure-flow relationship; periods of ischemia are then more likely to occur when ocular perfusion pressure is reduced leading to increased oxidative stress due to the reactive oxygen species. These insults lead to the impairment of RGCs and eventually lead to RGC's death and glaucoma. Functional defects caused by mutations in susceptibility genes, such as OPTN/MYOC, could also lead to defects in RGCs and contribute to the pathogenesis of glaucoma. Other factors, such as topical ocular administration of steroids, are the most likely to cause alteration of IOP and increase the risk of developing glaucoma.

neurodegenerative diseases [71, 72], so the application of NGF as a neuroprotective strategy for the medical treatment of glaucoma should be reasonable.

## 4. Interaction between NGF and Glaucoma

*4.1. Glaucoma Alters the Expression of NGF and Its Receptors in Retinal Ganglion Cells and Visual Cortex.* In normal conditions, primary cultured RGCs and transformed RGC-5 cells express various neurotrophins, such as brain-derived neurotrophic factor (BDNF), NGF, neurotrophin-3 (NT3), neurotrophin-4 (NT4), and also their relevant receptors, such as TrkA, p75NTR, or TrkB. RGC-5 cells also secrete NT3, BDNF, NGF, and NT4 into the cultured media [73]. In the visual system, the secretion of neurotrophins and the expression of their receptors maintain the morphological development; for example, neurotrophins and their receptors have been shown to exert various influences on guiding the morphological differentiation of neurons and controlling the functional plasticity of visual circuits; they may also participate in visual connectivity and the transmission of images into brain through optic nerve fibers [74].

While, in pathological conditions, such as glaucoma, the increased IOP will alter the level of NGF and NGF receptor expression, glaucoma significantly reduces the content of NGF in the cerebrospinal fluid (CSF) and lateral geniculate nucleus (LGN), but serum NGF protein levels may not be affected; this finding suggests that the NGF present in the CSF is most likely taken up by damaged retinal or brain

neurons. Ongoing research shows that glaucoma increases the basal level of TrkA in the LGN and NGF administration further enhances this increase. However glaucoma had no effect on the expression of p75NTR at early stage, while NGF still enhanced the level of p75NTR; these results support the contention that glaucoma altered the basal level of NGF and NGF receptors in brain visual centers [75]. A relatively long term study showed that, after 7 days of ocular hypertension, the content of retinal NGF increased but it did so transiently. However, the TrkA receptor is upregulated and expression is sustained for a long period in RGCs [76]. After 28 days of ocular hypertension, the level of retinal BDNF increased significantly, but the hypertension had no effect on its receptor TrkB [76]. The TrkC receptor was also enhanced in Müller cells but not in retinal ganglion cells even though the level of NT-3 remained unchanged. Expression of retinal p75NTR increased late at day 28 [76]. These results are consistent with the observation in the neurodegeneration; transgenic mice of neurodegeneration showed a lower level of NGF and NGF deficits elicit a progressive neurodegeneration [77, 78]. In Alzheimer's disease, NGF has been shown to be effective in preventing the onset of the central cholinergic deficit, so the role of NGF in RGC degeneration caused by glaucoma is certainly worthy of further investigation.

*4.2. NGF Affects Synaptic Plasticity and Glaucoma-Associated Proteins in the Optic Nerve System.* In the CNS, NGF possesses obvious effects on modulating neuronal inputs and thereby synaptic plasticity. NGF augmentation significantly enhanced cholinergic neuronal markers and facilitated induction of hippocampal long-term potentiation (LTP); moreover, blockade of endogenous NGF significantly reduced hippocampal LTP and impaired retention of spatial memory [79]. These findings provide evidence that NGF is essential for NGF hippocampal plasticity and learning. NGF also plays a role in neuronal plasticity in the optical system or visual cortex where monocular deprivation leads to decreased cell size and impairment of synaptic plasticity in visual cortex, while intraventricular administration of NGF prevents the cell shrinkage and demonstrated a substantial recovery of functional binocular connections [80]. This information indicates that neurotrophic factors may contribute to the regulation of experience-dependent modifications of synaptic connectivity in the visual cortex. Intracortical infusion of NGF into adult cat visual cortex can recreate ocular dominance plasticity, suggesting that NGF is also involved in the activity-dependent modification of synaptic connectivity in the adult brain [81]. GAP-43 and synaptophysin are two presynaptic elements in the visual cortex, which are highly related to synaptic plasticity. NGF treatment stimulates phosphorylation of GAP-43 and increases the level of synaptophysin immunoreactivity in adult visual cortex [82]. NGF treatment of the adult visual cortex modulates presynaptic terminals, possibly by inducing axonal sprouting and formation of new synapses, and these changes may play a role in the NGF-induced functional plasticity.

Optineurin (OPTN) and myocilin (MYOC) are two genes linked to glaucoma [83]. Rezaie et al. identified that OPTN may be an adult-onset glaucoma gene and speculated that wild-type optineurin played a neuroprotective role in the eye and optic nerve through the TNF-$\alpha$ pathway; accordingly, when OPTN was defective, it may lead to visual loss and optic neuropathy as typically seen in normal and high-pressure glaucoma [84]. This speculation was supported by the results that NGF treatment enhanced the endogenous levels of both OPTN and MYOC genes in PC12 cells [85]. These results demonstrate that NGF affects the expression of glaucoma-associated genes and their protein products. The data also indicate that NGF treatment may be an effective way to intervene in the process of glaucoma. However, the roles of OPTN and MYOC are complicated. OPTN overexpression induces an upregulation of the endogenous MYOC in both RGC and PC12 cells, while overexpressing MYOC does not affect the OPTN level. MYOC and OPTN contribute to the development of neurodegenerative glaucoma through different mechanisms. Overexpression of MYOC inhibits NGF-induced neurite outgrowth in both PC12 cells and RGC-5 cells, while transfection of optineurin leads to increased apoptosis [86]. The acute role of NGF and the glaucoma-associated proteins still needs further investigation and a specific gene knock-out mouse may serve this purpose. So far, a conditional knockout mouse line of OPTN has been generated and is available at the Wellcome Trust Sanger Institute [87]; Myoc-null mice have also been produced by Kim and coworkers [88]; these genetically altered mice are useful tools for understanding the physiological function of glaucoma-associated proteins; they can also be used to model the phenotype of glaucoma. In addition, these mouse lines provide a foundation for future efforts aimed at deciphering the role of NGF in the treatment of glaucoma.

*4.3. Differential Effects Mediated by NGF Receptors in Glaucoma.* As discussed above, the role of the receptors, TrkA and p75NTR, is not consistent in the CNS. It is widely accepted that sympathetic neuron survival is regulated positively by TrkA and negatively by p75NTR. The apoptotic effect of p75NTR signaling occurs only under suboptimal survival conditions, while TrkA-mediated responses occur when survival conditions are optimal [89–91]. In fact, the survival response to NGF is mediated by competitive signaling between TrkA and p75NTR; as mentioned previously, p75NTR induces NF-$\kappa$B and JNK activation, whereas TrkA mediates MAPK and Akt activation and when the two receptors are expressed together, TrkA blocks the p75NTR-mediated JNK activation, but NF-$\kappa$B is unaffected [88, 90].

In the optical system, the two receptors may also function differently. In normal adult retinas, TrkA is expressed in RGCs, whereas P75 is expressed in glia. In contrast to results from *in vitro* studies, *in vivo* studies indicate that NGF binds to TrkA and p75NTR but fails to promote the survival of axotomized RGCs in the adult retina; however, TrkA agonists showed robust neuroprotective effects [92]. Pharmacological inhibition of p75NTR or in p75NTR knockout mice showed enhanced survival of axotomized RGCs. A combination of NGF or TrkA agonists with p75NTR antagonists further potentiated RGC neuroprotection *in vivo* [93]. Treatment

with a mutant NGF that only activates TrkA affords significant neuroprotection. Supporting results were obtained for a biological response modifier that prevents endogenous NGF and pro-NGF from binding to p75NTR. Treatment of normal eyes with an NGF mutant-selective p75NTR agonist causes progressive RGC death, and, in injured eyes, it accelerates RGC death [35]. In a model of experimental glaucoma, the loss of RGC is accompanied by increased retinal p75 and Bax expression [9]. In summary, much data support the idea that NGF can be neuroprotective when acting on neuronal TrkA receptors on RGCs but engagement of p75NTR on glial cells antagonizes this effect. However, p75NTR cannot be viewed simply as a proapoptotic factor as in some situations; p75NTR may be a neuroprotective molecule [94] and p75NTR has beneficial effects on myelin formation and regeneration [95]. Thus, it is apparent that the role of p75NTR is complex and we need to unravel the precise role of p75NTR under different conditions and its related molecules during retinal development and degeneration.

## 5. Application of NGF in Retina Neurodegeneration and Glaucoma

NGF is a widely studied growth factor which possesses significant neuroprotective effects on neurons against various insults. In the central nervous system, NGF deficiency is involved in age-related neurodegenerative diseases; a deficit in the signaling and/or transport of NGF also leads to neurodegeneration [96]. Treatment with NGF can attenuate cholinergic deficit and improve cognitive behavior in animals. Clinical studies with chronic NGF administration in patients with Alzheimer's disease normalized electroencephalograph patterns and improved performance in words recognition tests [97]. These researchers proposed that NGF or compounds that induce the expression of endogenous NGF may be useful in the treatment of Alzheimer's disease or other neurodegenerative diseases. The application of NGF in the treatment of neurodegeneration prompts us to explore whether NGF will produce similar role in the treatment of optic nerve degeneration.

Sustained elevated IOP causes RGC degeneration and results in cell death. In animal models, glaucoma is induced by the injection of hypertonic saline into the episcleral vein of the eyes. A rat model of glaucoma showed that glaucoma led to progressive degeneration of RGCs, with the loss of nearly 40% of these cells after 7 weeks of treatment. The RGC loss is associated with the downregulation of NGF and NGF receptor expression in the retina and ocular treatment with NGF significantly reduced the deficit induced by glaucoma [98]. Another study showed that using NGF as an eye drop can attenuate the optic nerve damage that accompanies glaucoma; these investigators induced glaucoma in rats and measured the survival of RGCs with and without NGF eye drops administered four times daily for 7 weeks; it was found that significantly more RGCs survived in the treated group. In three patients with advanced glaucoma, treatment with topical NGF produced an improvement in visual acuity, contrast sensitivity, and electrophysiological functions [99]. What we should keep in mind is that most of the growth factors, including NGF, have low bioactive stability in the body owing to their short half-lives and slow diffusion, thus limiting its use as a neuroprotective drug [100]; *ex vivo* gene delivery or biologically stable small molecules that could bind and activate the TrkA signaling pathway are alternative strategies [101, 102]. From the angle of tissue engineering, two distinct strategies could be used for delivering growth factors: growth factors can be chemically bound into or onto the matrix; growth factors may infiltrate the material and make them available to cells. On the other hand, growth factors could also be physically encapsulated in the delivery system, and they can be released from synthetic extracellular matrix to target specific cell populations [103]; however, none has been evaluated for ocular applications. The most apparent advantage of NGF for the application in the clinic is its ability to penetrate to the retina when administrated topically. The application of NGF may open therapeutic perspectives for glaucoma and other neurodegenerative diseases.

## 6. Conclusions and Future Research

The ultimate goal of glaucoma research is to find new compounds that will not only normalize IOP but also arrest or even reverse apoptotic damage to the optic nerve and RGCs and slow the rate of progression of the disease. This review discussed current knowledge of glaucoma, with an emphasis on the NGF and NGF receptor(s) signaling pathways, and further explored the possibility of targeting the NGF signaling pathway as a strategy for the treatment of glaucoma. NGF offers the promise of actually restoring visual function through acting on the TrkA receptor; however, we should be cautious regarding the future of NGF-dependent treatments in the armamentarium of glaucoma therapy as most of the present studies were in animal models and none has reached clinical success. After more research and deeper mechanistic understanding a randomized, controlled glaucoma, clinical trials need to be performed to evaluate the therapeutic effect of NGF in the treatment of glaucoma and this also will involve an evaluation of the magnitude and occurrence rate of adverse effects. In the future, developments of novel pharmacological interventions for glaucoma may include the development of small molecules that are specific for TrkA receptors in the optical nerves. With protein chemistry entering into a new phase of "peptide design," it is hoped that careful design of these small molecules may be able to limit biological activity to only the wanted effects and minimize the adverse effects. Other strategies such as chemical immobilization of NGF into or onto the matrix and physical encapsulation of NGF in the delivery system may also be promising in the future studies. Such novel ideas for trophic support are still developing and may provide future "smart drugs" for glaucoma.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

This research was financially supported by National Natural Science Foundation of China (no. 30670652; no. 30970935; and no. 31371088), the Guangdong Provincial Project of Science and Technology (nos. 2010A030100006 and 2011B050200005), and Director's Fund of State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China.

## References

[1] M. V. Boland, A.-M. Ervin, D. S. Friedman et al., "Comparative effectiveness of treatments for open-angle glaucoma: a systematic review for the U.S. preventive services task force," *Annals of Internal Medicine*, vol. 158, no. 4, pp. 271–279, 2013.

[2] L. Feng, Y. Zhao, M. Yoshida et al., "Sustained ocular hypertension induces dendritic degeneration of mouse retinal ganglion cells that depends on cell type and location," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 2, pp. 1106–1117, 2013.

[3] R. H. Foxton, A. Finkelstein, S. Vijay et al., "VEGF-A is necessary and sufficient for retinal neuroprotection in models of experimental glaucoma," *The American Journal of Pathology*, vol. 182, no. 4, pp. 1379–1390, 2013.

[4] M. V. Sofroniew, C. L. Howe, and W. C. Mobley, "Nerve growth factor signaling, neuroprotection, and neural repair," *Annual Review of Neuroscience*, vol. 24, pp. 1217–1281, 2001.

[5] S. J. Allen, J. J. Watson, D. K. Shoemark, N. U. Barua, and N. K. Patel, "GDNF, NGF and BDNF as therapeutic options for neurodegeneration," *Pharmacology and Therapeutics*, vol. 138, no. 2, pp. 155–175, 2013.

[6] L. U. Wahlberg, G. Lind, P. M. Almqvist et al., "Targeted delivery of nerve growth factor via encapsulated cell biodelivery in Alzheimer disease: a technology platform for restorative neurosurgery," *Journal of Neurosurgery*, vol. 117, no. 2, pp. 340–347, 2012.

[7] J. M. Eriksdotter, A. Nordberg, K. Amberla et al., "Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease," *Dementia and Geriatric Cognitive Disorders*, vol. 9, no. 5, pp. 246–257, 1998.

[8] L. A. Quina, W. Pak, J. Lanier et al., "Brn3a-expressing retinal ganglion cells project specifically to thalamocortical and collicular visual pathways," *The Journal of Neuroscience*, vol. 25, no. 50, pp. 11595–11604, 2005.

[9] M. Coassin, A. Lambiase, V. Sposato, A. Micera, S. Bonini, and L. Aloe, "Retinal p75 and bax overexpression is associated with retinal ganglion cells apoptosis in a rat model of glaucoma," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 246, no. 12, pp. 1743–1749, 2008.

[10] A. S. Felix, R. P. Edwards, R. A. Stone et al., "Associations between hepatocyte growth factor, c-Met, and basic fibroblast growth factor and survival in endometrial cancer patients," *The British Journal of Cancer*, vol. 106, no. 12, pp. 2004–2009, 2012.

[11] B. P. Madakashira, D. A. Kobrinski, A. D. Hancher et al., "Frs2$\alpha$ enhances fibroblast growth factor-mediated survival and differentiation in lens development," *Development*, vol. 139, no. 24, pp. 4601–4612, 2012.

[12] P. M. Costa, A. L. Cardoso, L. F. P. de almeida, J. N. Bruce, P. Canoll, and M. C. P. de lima, "PDGF-B-mediated downregulation of miR-21: new insights into PDGF signaling in glioblastoma," *Human Molecular Genetics*, vol. 21, no. 23, Article ID dds358, pp. 5118–5130, 2012.

[13] R. Getachew, M. L. Ballinger, M. L. Burch et al., "PDGF $\beta$-receptor kinase activity and ERK1/2 mediate glycosaminoglycan elongation on biglycan and increases binding to LDL," *Endocrinology*, vol. 151, no. 9, pp. 4356–4367, 2010.

[14] A. Lantero, M. Tramullas, A. Díaz, and M. A. Hurlé, "Transforming growth factor-$\beta$ in normal nociceptive processing and pathological pain models," *Molecular Neurobiology*, vol. 45, no. 1, pp. 76–86, 2012.

[15] W. M. Cowan, "Viktor Hamburger and Rita Levi-Montalcini: the path to the discovery of nerve growth factor," *Annual Review of Neuroscience*, vol. 24, pp. 551–600, 2001.

[16] C. Lanave, A. M. Colangelo, C. Saccone, and L. Alberghina, "Molecular evolution of the neurotrophin family members and their Trk receptors," *Gene*, vol. 394, no. 1-2, pp. 1–12, 2007.

[17] R. Lee, P. Kermani, K. K. Teng, and B. L. Hempstead, "Regulation of cell survival by secreted proneurotrophins," *Science*, vol. 294, no. 5548, pp. 1945–1948, 2001.

[18] B. A. Tucker, M. Rahimtula, and K. M. Mearow, "Src and FAK are key early signalling intermediates required for neurite growth in NGF-responsive adult DRG neurons," *Cellular Signalling*, vol. 20, no. 1, pp. 241–257, 2008.

[19] Z. Hu, M. Ulfendahl, and N. P. Olivius, "NGF stimulates extensive neurite outgrowth from implanted dorsal root ganglion neurons following transplantation into the adult rat inner ear," *Neurobiology of Disease*, vol. 18, no. 1, pp. 184–192, 2005.

[20] L. Xu, S. Zhou, G.-Y. Feng et al., "Neural stem cells enhance nerve regeneration after sciatic nerve injury in rats," *Molecular Neurobiology*, vol. 46, no. 2, pp. 265–274, 2012.

[21] D. J. Lomb, L. A. Desouza, J. L. Franklin, and R. S. Freeman, "Prolyl hydroxylase inhibitors depend on extracellular glucose and hypoxia-inducible factor (HIF)-2$\alpha$ to inhibit cell death caused by nerve growth factor (NGF) deprivation: evidence that HIF-2$\alpha$ has a role in NGF-promoted survival of sympathetic neurons," *Molecular Pharmacology*, vol. 75, no. 5, pp. 1198–1209, 2009.

[22] M. H. Tuszynski and A. Blesch, "Nerve growth factor: from animal models of cholinergic neuronal degeneration to gene therapy in Alzheimer's disease," *Progress in Brain Research*, vol. 146, pp. 441–449, 2004.

[23] A. Cattaneo and P. Calissano, "Nerve growth factor and Alzheimer's disease: new facts for an old hypothesis.," *Molecular neurobiology*, vol. 46, no. 3, pp. 588–604, 2012.

[24] K. Biernacki, J. P. Antel, M. Blain, S. Narayanan, D. L. Arnold, and A. Prat, "Interferon beta promotes nerve growth factor secretion early in the course of multiple sclerosis," *Archives of Neurology*, vol. 62, no. 4, pp. 563–568, 2005.

[25] J. E. McGeary, V. Gurel, V. S. Knopik, J. Spaulding, and J. McMichael, "Effects of nerve growth factor (NGF), fluoxetine, and amitriptyline on gene expression profiles in rat brain," *Neuropeptides*, vol. 45, no. 5, pp. 317–322, 2011.

[26] K. B. Nelson, J. K. Grether, L. A. Croen et al., "Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation," *Annals of Neurology*, vol. 49, no. 5, pp. 597–606, 2001.

[27] W. Zheng, H. Wang, Z. Zeng et al., "The possible role of the Akt signaling pathway in schizophrenia," *Brain Research*, vol. 1470, pp. 145–158, 2012.

[28] R. Levi-Montalcini, "The nerve growth factor and the neuroscience chess board," *Progress in brain research*, vol. 146, pp. 525–527, 2004.

[29] G. N. Chaldakov, I. S. Stankulov, M. Fiore, P. I. Ghenev, and L. Aloe, "Nerve growth factor levels and mast cell distribution in human coronary atherosclerosis," *Atherosclerosis*, vol. 159, no. 1, pp. 57–66, 2001.

[30] L. Manni, V. Nikolova, D. Vyagova, G. N. Chaldakov, and L. Aloe, "Reduced plasma levels of NGF and BDNF in patients with acute coronary syndromes," *International Journal of Cardiology*, vol. 102, no. 1, pp. 169–171, 2005.

[31] H. T. Cheng, J. R. Dauch, J. M. Hayes, B. M. Yanik, and E. L. Feldman, "Nerve growth factor/p38 signaling increases intraepidermal nerve fiber densities in painful neuropathy of type 2 diabetes," *Neurobiology of Disease*, vol. 45, no. 1, pp. 280–287, 2012.

[32] G. N. Chaldakov, M. Fiore, I. S. Stankulov et al., "Neurotrophin presence in human coronary atherosclerosis and metabolic syndrome: a role for NGF and BDNF in cardiovascular disease?" *Progress in Brain Research*, vol. 146, pp. 279–289, 2004.

[33] R. Romon, E. Adriaenssens, C. Lagadec, E. Germain, H. Hondermarck, and X. le Bourhis, "Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways," *Molecular Cancer*, vol. 9, article 157, 2010.

[34] D. Deponti, R. Buono, G. Catanzaro et al., "The low-affinity receptor for neurotrophins p75 NTR plays a key role for satellite cell function in muscle repair acting via RhoA," *Molecular Biology of the Cell*, vol. 20, no. 16, pp. 3620–3627, 2009.

[35] Y. Bai, P. Dergham, H. Nedev et al., "Chronic and acute models of retinal neurodegeneration TrkA activity are neuroprotective whereas p75NTR activity is neurotoxic through a paracrine mechanism," *The Journal of Biological Chemistry*, vol. 285, no. 50, pp. 39392–39400, 2010.

[36] S. Verbeke, S. Meignan, C. Lagadec et al., "Overexpression of p75NTR increases survival of breast cancer cells through p21waf1," *Cellular Signalling*, vol. 22, no. 12, pp. 1864–1873, 2010.

[37] K.-F. Lee, A. M. Davies, and R. Jaenisch, "p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF," *Development*, vol. 120, no. 4, pp. 1027–1033, 1994.

[38] M. Bothwell, "Tissue localization of nerve growth factor and nerve growth factor receptors," *Current Topics in Microbiology and Immunology*, vol. 165, pp. 55–70, 1991.

[39] G. Niewiadomska, A. Mietelska-Porowska, and M. Mazurkiewicz, "The cholinergic system, nerve growth factor and the cytoskeleton," *Behavioural Brain Research*, vol. 221, no. 2, pp. 515–526, 2011.

[40] S. K. Mahata, M. Mahata, H. Wu, R. J. Parmer, and D. T. O'Connor, "Neurotrophin activation of catecholamine storage vesicle protein gene expression: signaling to chromogranin a biosynthesis," *Neuroscience*, vol. 88, no. 2, pp. 405–424, 1999.

[41] W.-H. Zheng, S. Kar, and R. Quirion, "Insulin-like growth factor-1-induced phosphorylation of transcription factor FKHRL1 is mediated by phosphatidylinositol 3-kinase/Akt kinase and role of this pathway in insulin-like growth factor-1-induced survival of cultured hippocampal neurons," *Molecular Pharmacology*, vol. 62, no. 2, pp. 225–233, 2002.

[42] Y.-P. Xia, R.-L. Dai, Y.-N. Li et al., "The protective effect of sonic hedgehog is mediated by the phosphoinositide [corrected] 3-kinase/AKT/Bcl-2 pathway in cultured rat astrocytes under oxidative stress," *Neuroscience*, vol. 209, pp. 1–11, 2012.

[43] H. Wang, X. Zhou, J. Huang et al., "The role of Akt/FoxO3a in the protective effect of venlafaxine against corticosterone-induced cell death in PC12 cells," *Psychopharmacology*, vol. 228, no. 1, pp. 129–141, 2013.

[44] W.-H. Zheng, S. Kar, and R. Quirion, "FKHRL1 and its homologs are new targets of nerve growth factor Trk receptor signaling," *Journal of Neurochemistry*, vol. 80, no. 6, pp. 1049–1061, 2002.

[45] W.-H. Zheng and R. Quirion, "Glutamate acting on N-methyl-D-aspartate receptors attenuates insulin-like growth factor-1 receptor tyrosine phosphorylation and its survival signaling properties in rat hippocampal neurons," *The Journal of Biological Chemistry*, vol. 284, no. 2, pp. 855–861, 2009.

[46] W. Huang, G. Li, J. Qiu, P. Gonzalez, and P. Challa, "Protective effects of resveratrol in experimental retinal detachment," *PLoS ONE*, vol. 8, no. 9, Article ID e75735, 2013.

[47] W. Chen, J. L. Martindale, N. J. Holbrook, and Y. Liu, "Tumor promoter arsenite activates extracellular signal-regulated kinase through a signaling pathway mediated by epidermal growth factor receptor and Shc," *Molecular and Cellular Biology*, vol. 18, no. 9, pp. 5178–5188, 1998.

[48] Z. Wang, Y. Liu, M. Takahashi et al., "N terminus of ASPP2 binds to Ras and enhances Ras/Raf/MEK/ERK activation to promote oncogene-induced senescence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 1, pp. 312–317, 2013.

[49] Y.-T. Oh, P. Yue, W. Zhou et al., "Oncogenic Ras and B-Raf proteins positively regulate death receptor 5 expression through co-activation of ERK and JNK signaling," *The Journal of Biological Chemistry*, vol. 287, no. 1, pp. 257–267, 2012.

[50] H.-C. Cheng, H.-M. Shih, and Y. Chern, "Essential role of cAMP-response element-binding protein activation by $A_{2A}$ adenosine receptors in rescuing the nerve growth factor-induced neurite outgrowth impaired by blockage of the MAPK cascade," *The Journal of Biological Chemistry*, vol. 277, no. 37, pp. 33930–33942, 2002.

[51] C. Cabeza, A. Figueroa, O. M. Lazo et al., "Cholinergic abnormalities, endosomal alterations and up-regulation of nerve growth factor signaling in Niemann-Pick type C disease," *Molecular Neurodegeneration*, vol. 7, article 11, 2012.

[52] H. S. Je, F. Yang, Y. Ji, G. Nagappan, B. L. Hempstead, and B. Lu, "Role of pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF conversion in activity-dependent competition at developing neuromuscular synapses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 39, pp. 15924–15929, 2012.

[53] A. W. Harrington, J. Y. Kim, and S. O. Yoon, "Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis," *Journal of Neuroscience*, vol. 22, no. 1, pp. 156–166, 2002.

[54] J. Liu and A. Lin, "Role of JNK activation in apoptosis: a double-edged sword," *Cell Research*, vol. 15, no. 1, pp. 36–42, 2005.

[55] M. Hamanoue, G. Middleton, S. Wyatt, E. Jaffray, R. T. Hay, and A. M. Davies, "p75-Mediated NF-$\kappa$B activation enhances the survival response of developing sensory neurons to nerve growth factor," *Molecular and Cellular Neurosciences*, vol. 14, no. 1, pp. 28–40, 1999.

[56] P. A. Barker and E. M. Shooter, "Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells," *Neuron*, vol. 13, no. 1, pp. 203–215, 1994.

[57] P. A. Hantzopoulos, C. Suri, D. J. Glass, M. P. Goldfarb, and G. D. Yancopoulos, "The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins," *Neuron*, vol. 13, no. 1, pp. 187–201, 1994.

[58] D. Mahadeo, L. Kaplan, M. V. Chao, and B. L. Hempstead, "High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors," *Journal of Biological Chemistry*, vol. 269, no. 9, pp. 6884–6891, 1994.

[59] T. Wehrman, X. He, B. Raab, A. Dukipatti, H. Blau, and K. C. Garcia, "Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors," *Neuron*, vol. 53, no. 1, pp. 25–38, 2007.

[60] S. L. Mansberger, B. A. Hughes, M. O. Gordon et al., "Comparison of initial intraocular pressure response with topical $\beta$-adrenergic antagonists and prostaglandin analogues in African American and white individuals in the ocular hypertension treatment study," *Archives of Ophthalmology*, vol. 125, no. 4, pp. 454–459, 2007.

[61] N. Comes and T. Borrás, "Individual molecular response to elevated intraocular pressure in perfused postmortem human eyes," *Physiological Genomics*, vol. 38, no. 2, pp. 205–225, 2009.

[62] B. J. Frankfort, A. Kareem Khan, D. Y. Tse et al., "Elevated intraocular pressure causes inner retinal dysfunction before cell loss in a mouse model of experimental glaucoma," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 1, pp. 762–770, 2013.

[63] S.-P. Cai, P. Muhemaiti, Y. Yin et al., "A novel MYOC heterzygous mutation identified in a Chinese Uygur pedigree with primary open-angle glaucoma," *Molecular Vision*, vol. 18, pp. 1944–1951, 2012.

[64] V. Mendoza-Reinoso, T. S. Patil, M. L. Guevara-Fujita et al., "Novel and known *MYOC* exon 3 mutations in an admixed Peruvian primary open-angle glaucoma population," *Molecular Vision*, vol. 18, pp. 2067–2075, 2012.

[65] B. J. Fan and J. L. Wiggs, "Glaucoma: genes, phenotypes, and new directions for therapy," *The Journal of Clinical Investigation*, vol. 120, no. 9, pp. 3064–3072, 2010.

[66] J. B. Jonas, A. Bergua, P. Schmitz-Valckenberg, K. I. Papastathopoulos, and W. M. Budde, "Ranking of optic disc variables for detection of glaucomatous optic nerve damage," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 7, pp. 1764–1773, 2000.

[67] C. T. Fu and D. W. Sretavan, "Ectopic vesicular glutamate release at the optic nerve head and axon loss in mouse experimental glaucoma," *The Journal of Neuroscience*, vol. 32, no. 45, pp. 15859–15876, 2012.

[68] B. Junglas, S. Kuespert, A. A. Seleem et al., "Connective tissue growth factor causes glaucoma by modifying the actin cytoskeleton of the trabecular meshwork," *The American Journal of Pathology*, vol. 180, no. 6, pp. 2386–2403, 2012.

[69] J. T. Wang, Z. A. Medress, and B. A. Barres, "Axon degeneration: molecular mechanisms of a self-destruction pathway," *Journal of Cell Biology*, vol. 196, no. 1, pp. 7–18, 2012.

[70] Y. Lei, N. Garrahan, B. Hermann et al., "Topography of neuron loss in the retinal ganglion cell layer in human glaucoma," *British Journal of Ophthalmology*, vol. 93, no. 12, pp. 1676–1679, 2009.

[71] C. V. Borlongan, "Recent preclinical evidence advancing cell therapy for Alzheimer's disease," *Experimental Neurology*, vol. 237, no. 1, pp. 142–146, 2012.

[72] L.-Y. Li, J.-T. Li, Q.-Y. Wu et al., "Transplantation of NGF-gene-modified bone marrow stromal cells into a rat model of Alzheimer' disease," *Journal of Molecular Neuroscience*, vol. 34, no. 2, pp. 157–163, 2008.

[73] N. Agarwal, R. Agarwal, D. M. Kumar et al., "Comparison of expression profile of neurotrophins and their receptors in primary and transformed rat retinal ganglion cells," *Molecular Vision*, vol. 13, pp. 1311–1318, 2007.

[74] S. Cohen-Cory and B. Lom, "Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses," *International Journal of Developmental Biology*, vol. 48, no. 8-9, pp. 947–956, 2004.

[75] V. Sposato, V. Parisi, L. Manni et al., "Glaucoma alters the expression of NGF and NGF receptors in visual cortex and geniculate nucleus of rats: effect of eye NGF application," *Vision Research*, vol. 49, no. 1, pp. 54–63, 2009.

[76] M. Rudzinski, T.-P. Wong, and H. U. Saragovi, "Changes in retinal expression of neurotrophins and neurotrophin receptors induced by ocular hypertension," *Journal of Neurobiology*, vol. 58, no. 3, pp. 341–354, 2004.

[77] S. Capsoni, C. Tiveron, D. Vignone, G. Amato, and A. Cattaneo, "Dissecting the involvement of tropomyosin-related kinase A and p75 neurotrophin receptor signaling in NGF deficit-induced neurodegeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 27, pp. 12299–12304, 2010.

[78] S. Capsoni, C. Tiveron, G. Amato, D. Vignone, and A. Cattaneo, "Peripheral neutralization of nerve growth factor induces immunosympathectomy and central neurodegeneration in transgenic mice," *Journal of Alzheimer's Disease*, vol. 20, no. 2, pp. 527–546, 2010.

[79] J. M. Conner, K. M. Franks, A. K. Titterness et al., "NGF is essential for hippocampal plasticity and learning," *The Journal of Neuroscience*, vol. 29, no. 35, pp. 10883–10889, 2009.

[80] G. Carmignoto, R. Canella, P. Candeo, M. C. Comelli, and L. Maffei, "Effects of nerve growth factor on neuronal plasticity of the kitten visual cortex," *The Journal of Physiology*, vol. 464, pp. 343–360, 1993.

[81] S. T. Lockhart, J. N. Mead, J. M. Pisano, J. D. Slonimsky, and S. J. Birren, "Nerve growth factor collaborates with myocyte-derived factors to promote development of presynaptic sites in cultured sympathetic neurons," *Journal of Neurobiology*, vol. 42, no. 4, pp. 460–476, 2000.

[82] Y. Liu, K. F. Meiri, M. S. Cynader, and Q. Gu, "Nerve growth factor induced modification of presynaptic elements in adult visual cortex in vivo," *Brain Research*, vol. 732, no. 1-2, pp. 36–42, 1996.

[83] K. K. McDonald, K. Abramson, M. A. Beltran et al., "Myocilin and optineurin coding variants in Hispanics of Mexican descent with POAG," *Journal of Human Genetics*, vol. 55, no. 10, pp. 697–700, 2010.

[84] T. Rezaie, A. Child, R. Hitchings et al., "Adult-onset primary open-angle glaucoma caused by mutations in optineurin," *Science*, vol. 295, no. 5557, pp. 1077–1079, 2002.

[85] B.-C. Park, M. Tibudan, M. Samaraweera, X. Shen, and B. Y. J. T. Yue, "Interaction between two glaucoma genes, optineurin and myocilin," *Genes to Cells*, vol. 12, no. 8, pp. 969–979, 2007.

[86] T. Koga, X. Shen, J.-S. Park et al., "Differential effects of myocilin and optineurin, two glaucoma genes, on neurite outgrowth," *The American Journal of Pathology*, vol. 176, no. 1, pp. 343–352, 2010.

[87] W. C. Skarnes, B. Rosen, A. P. West et al., "A conditional knockout resource for the genome-wide study of mouse gene function," *Nature*, vol. 474, no. 7351, pp. 337–342, 2011.

[88] B. S. Kim, O. V. Savinova, M. V. Reedy et al., "Targeted disruption of the myocilin gene (*Myoc*) suggests that human

glaucoma-causing mutations are gain of function," *Molecular and Cellular Biology*, vol. 21, no. 22, pp. 7707–7713, 2001.

[89] J. Kohn, R. S. Aloyz, J. G. Toma, M. Haak-Frendscho, and F. D. Miller, "Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation," *Journal of Neuroscience*, vol. 19, no. 13, pp. 5393–5408, 1999.

[90] S. O. Yoon, P. Casaccia-Bonnefil, B. Carter, and M. V. Chao, "Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival," *The Journal of Neuroscience*, vol. 18, no. 9, pp. 3273–3281, 1998.

[91] S. X. Bamji, M. Majdan, C. D. Pozniak et al., "The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death," *The Journal of Cell Biology*, vol. 140, no. 4, pp. 911–923, 1998.

[92] Z. Shi, E. Birman, and H. U. Saragovi, "Neurotrophic rationale in glaucoma: a TrkA agonist, but not NGF or a p75 antagonist, protects retinal ganglion cells in vivo," *Developmental Neurobiology*, vol. 67, no. 7, pp. 884–894, 2007.

[93] F. Lebrun-Julien, B. Morquette, A. Douillette, H. U. Saragovi, and A. Di Polo, "Inhibition of p75NTR in glia potentiates TrkA-mediated survival of injured retinal ganglion cells," *Molecular and Cellular Neuroscience*, vol. 40, no. 4, pp. 410–420, 2009.

[94] B. D. Carter and G. R. Lewint, "Neurotrophins live or let die: does p75(NTR) decide?" *Neuron*, vol. 18, no. 2, pp. 187–190, 1997.

[95] J. M. Cosgaya, J. R. Chan, and E. M. Shooter, "The neurotrophin receptor p75NTR as a positive modulator of myelination," *Science*, vol. 298, no. 5596, pp. 1245–1248, 2002.

[96] S. Capsoni, G. Ugolini, A. Comparini, F. Ruberti, N. Berardi, and A. Cattaneo, "Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 12, pp. 6826–6831, 2000.

[97] L. Olson, "NGF and the treatment of Alzheimer's disease," *Experimental Neurology*, vol. 124, no. 1, pp. 5–15, 1993.

[98] V. Colafrancesco, V. Parisi, V. Sposato et al., "Ocular application of nerve growth factor protects degenerating retinal ganglion cells in a rat model of glaucoma," *Journal of Glaucoma*, vol. 20, no. 2, pp. 100–108, 2011.

[99] A. Lambiase, L. Aloe, M. Centofanti et al., "Experimental and clinical evidence of neuroprotection by nerve growth factor eye drops: implications for glaucoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 32, pp. 13469–13474, 2009.

[100] K. Akassoglou, "Nerve growth factor-independent neuronal survival: a role for NO donors," *Molecular Pharmacology*, vol. 68, no. 4, pp. 952–955, 2005.

[101] M. H. Tuszynski, L. Thal, M. Pay et al., "A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease," *Nature Medicine*, vol. 11, no. 5, pp. 551–555, 2005.

[102] S. M. Massa, Y. Xie, and F. M. Longo, "Alzheimer's therapeutics: neurotrophin domain small molecule mimetics," *Journal of Molecular Neuroscience*, vol. 20, no. 3, pp. 323–326, 2003.

[103] K. Lee, E. A. Silva, and D. J. Mooney, "Growth factor delivery-based tissue engineering: general approaches and a review of recent developments," *Journal of the Royal Society Interface*, vol. 8, no. 55, pp. 153–170, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 547187, 13 pages
http://dx.doi.org/10.1155/2014/547187

*Research Article*

# NGF Accelerates Cutaneous Wound Healing by Promoting the Migration of Dermal Fibroblasts via the PI3K/Akt-Rac1-JNK and ERK Pathways

**Ji-Cai Chen,[1] Bei-Bei Lin,[2] Hou-Wen Hu,[2] Cai Lin,[3] Wen-Yang Jin,[4] Fa-Biao Zhang,[4] Yan-An Zhu,[4] Cai-Jiao Lu,[3] Xiao-Jie Wei,[5] and Rui-Jie Chen[6]**

[1] *Department of Gastrointestinal Surgery, The First Affiliate Hospital, Wenzhou Medical University, Wenzhou 325035, China*
[2] *School of Pharmacy, Wenzhou Medical University, Wenzhou 325035, China*
[3] *Wound Treatment Center, The First Affiliate Hospital, Wenzhou Medical University, Wenzhou 325000, China*
[4] *Emergency Department, Taizhou Hospital, Wenzhou Medical University, Taizhou 318000, China*
[5] *Translation Medicine Research Center, Cixi People's Hospital, Wenzhou Medical University, Ningbo 315300, China*
[6] *Departmant of Pharmacy, The Second Affiliate Hospital, Wenzhou Medical University, Wenzhou 325000, China*

Correspondence should be addressed to Xiao-Jie Wei; cxweixiaojie@126.com and Rui-Jie Chen; wzhoucrj@126.com

Received 6 March 2014; Accepted 12 April 2014; Published 21 May 2014

Academic Editor: Jian Xiao



As a well-known neurotrophic factor, nerve growth factor (NGF) has also been extensively recognized for its acceleration of healing in cutaneous wounds in both animal models and randomized clinical trials. However, the underlying mechanisms accounting for the therapeutic effect of NGF on skin wounds are not fully understood. NGF treatment significantly accelerated the rate of wound healing by promoting wound reepithelialization, the formation of granulation tissue, and collagen production. To explore the possible mechanisms of this process, the expression levels of CD68, VEGF, PCNA, and TGF-$\beta$1 in wounds were detected by immunohistochemical staining. The levels of these proteins were all significantly raised in NGF-treated wounds compared to untreated controls. NGF also significantly promoted the migration, but not the proliferation, of dermal fibroblasts. NGF induced a remarkable increase in the activity of PI3K/Akt, JNK, ERK, and Rac1, and blockade with their specific inhibitors significantly impaired the NGF-induced migration. In conclusion, NGF significantly accelerated the healing of skin excisional wounds in rats and the fibroblast migration induced by NGF may contribute to this healing process. The activation of PI3K/Akt, Rac1, JNK, and ERK were all involved in the regulation of NGF-induced fibroblast migration.

## 1. Introduction

The skin is the largest organ in the body, covering the entire external surface, and it has many different functions. The skin primarily acts as a protective physical barrier between the host and the external environment against numerous insults, and it serves to prevent excessive loss of body water while also possessing other critical functions including immune surveillance, sensory detection, and self-healing. The breakdown of skin integrity because of injury or illness such as diabetes, pressure, or venous stasis results in substantial physiologic imbalance and renders a patient vulnerable to a number of pathologic conditions, such as infection, fluid loss, and electrolyte imbalance [1]. Therefore, the efficient and complete healing of cutaneous wounds is undoubtedly critical.

The healing of skin wounds is a complex process involving a series of sequential and overlapping phases such as clotting, inflammatory infiltration, reepithelialization, and the formation of granulation tissue, followed by tissue remodeling and wound contraction [2]. This process requires the collaboration of a variety of tissue and cell types, including

inflammatory cells, fibroblasts, keratinocytes, endothelial cells, and macrophages. These cells are tightly regulated by cytokines, growth factors, and extracellular matrix (ECM) molecules [3]. Inflammatory cells, keratinocytes, and fibroblasts in the wound area and border produce and release a variety of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), and nerve growth factor (NGF) [4]. Local or topical application of exogenous growth factors and cytokines has also been reported to accelerate the repair of acute and chronic wounds [5].

NGF is the first isolated and best-characterized member of a neurotrophin family. NGF exerts a crucial role in survival, differentiation, and function of peripheral sensory and sympathetic nerves and brain neurons of mammals [6]. Additionally, accumulating evidence has shown that NGF plays a prominent role in promoting healing processes. NGF levels are significantly higher at wounded sites after skin punching than at uninjured control skin sites [7]. The removal of the submaxillary glands, tissues storing large amounts of NGF, substantially affects recovery of wound healing in mouse skin, and when purified NGF is topically applied to mice without submandibular glands, the rate of wound contraction is markedly accelerated [8]. The application of NGF to cutaneous wounds accelerated the rate of wound healing in both normal and healing-impaired diabetic mice [7]. Topical application of NGF also promotes skin ulcer healing in patients with pressure ulcers [9]. However, the regulatory mechanism underlying NGF-accelerated cutaneous wound healing is not yet completely understood. Thus, the underlying mechanism deserves further investigations.

Dermal fibroblasts play critical roles in all three phases of wound healing. After wounding, fibroblasts are attracted from the edge of the wound or from the bone marrow [10]. At the inflammation stage, fibroblasts produce a variety of chemokines [11]. At the stage of new tissue formation, fibroblasts are stimulated by macrophages and some differentiate into myofibroblasts. Fibroblasts interact with myofibroblasts to produce extracellular matrix, mainly in the form of collagen [12]. At the stage of tissue remodeling, most of the myofibroblasts, macrophages, and endothelial cells undergo apoptosis or exit from the wound, leaving a mass that contains few cells and consists mostly of collagen and other extracellular matrix proteins [1]. However, the effect of NGF on dermal fibroblasts is largely unknown.

To address these questions, the present study first examined the effect of topical NGF on wound healing in a rat skin excisional wound model and then further explored the possible mechanisms of wound-healing promoted by NGF. In light of the crucial role that dermal fibroblasts play in wound healing, we mainly focused on investigating the effect of NGF on dermal fibroblasts and underlying signal pathways.

## 2. Results

*2.1. NGF Accelerated the Rate of Cutaneous Wound Healing.* The healing of skin excisional wounds in rats was determined by the percentage of the wound surface covered by regenerating epidermis. NGF-treated wounds exhibited accelerated skin wound closure in Sprague-Dawley rats compared with saline-treated wounds (Figure 1(a)). The enhancement of wound healing appeared as early as at 2 days following treatment with the three different doses of NGF (Figure 1(b)). Although 10 $\mu$g or 20 $\mu$g of NGF treatment demonstrated an accelerated speed in wound closure, the group treated with 40 $\mu$g NGF did not show a significant difference from the saline-treated control between 6 days of treatment and the end of the experiment. After treatment with 10 $\mu$g or 20 $\mu$g of NGF for 10 days, the cutaneous wound was nearly closed, while the saline-treated group still showed a much larger wound area. It seems that topical NGF treatment reaches its maximal effect in wound healing at 20 $\mu$g topically per wound. Therefore, the 20 $\mu$g NGF treatment was chosen for our later experiment.

*2.2. NGF Promoted Reepithelialization, Granulation Tissue Formation, and Collagen Production.* Excisional skin wounds are healed through lateral migration of keratinocytes, called reepithelialization, followed by inward migration of dermal cells. The rate of wound closure was well matched to the histological observations in wounds by hematoxylin and eosin staining (Figure 2(a)). The NGF-treated wounds exhibited substantially more reepithelialization than the saline-treated wounds. Longer reepithelialized tongues (ReT) could be clearly visualized after 14 days of 20 $\mu$g NGF treatment compared to saline-treated controls (Figure 2(a)). In addition, the granulation tissue formed in NGF-treated wounds appeared to be thicker and larger than saline-treated control. The sections stained with Masson's trichrome were shown in Figure 2(b). There was a mild increase in collagen production in NGF-treated wounds compared with the saline-treated controls. Although collagen staining was light and unevenly distributed, the collagen content in NGF-treated wounds was higher than in saline-treated controls at 3 days after wounding. The collagen in NGF-treated wounds at 21 days after wounding was more mature than in saline-treated controls.

*2.3. NGF Enhanced the Expression of TGF-$\beta$1 and PCNA in Wound.* It has been extensively reported that collagen synthesis is modulated by the profibrotic cytokine transforming growth factor $\beta$ (TGF-$\beta$). We therefore investigated the expression of TGF-$\beta$1 in NGF-treated and saline-treated wounds. As shown in Figures 3(a) and 3(c), the expression of TGF-$\beta$1 in NGF-treated wounds was significantly increased at 3 days and 7 days after wounding compared to saline-treated controls. However, the expression of TGF-$\beta$1 in the NGF-treated wound was significantly decreased at 14 days after wounding. To explore the effect of NGF treatment on cell proliferation, the expression of PCNA, a marker of cell proliferation, in the wound tissues was detected by immunohistochemical staining using an anti-PCNA antibody. As shown in Figures 3(b) and 3(d), the expression of PCNA was significantly induced in NGF-treated wounds compared with the saline-treated control at the early time points, 3 days

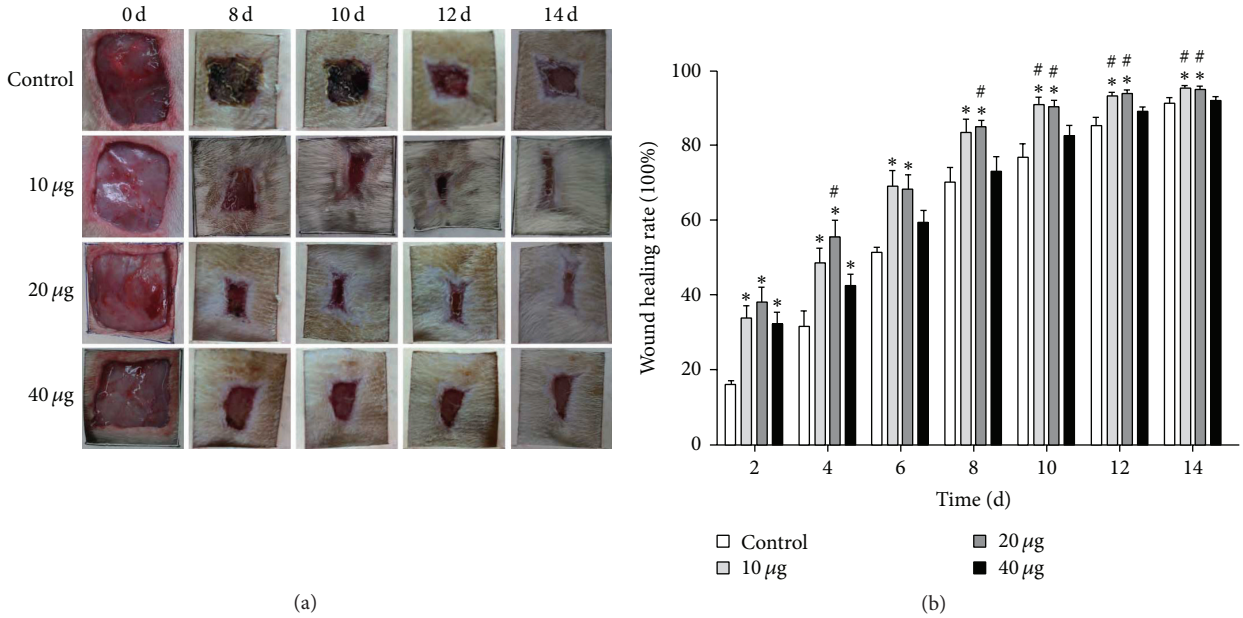



Figure 1: Wound closure after NGF treatment in rat. (a) Representative photographs of skin full-thickness excisional wounds in rat treated topically every other day with saline control or 10, 20, or 40 $\mu$g/mL of NGF for 8, 10, 12, and 14 days after wounding. (b) The rate of wound healing after different concentrations of NGF treatment. $^{*}P < 0.05$, compared to saline-treated control; $^{\#}P < 0.05$, compared to 40 $\mu$g/mL NGF group; $n = 7$.

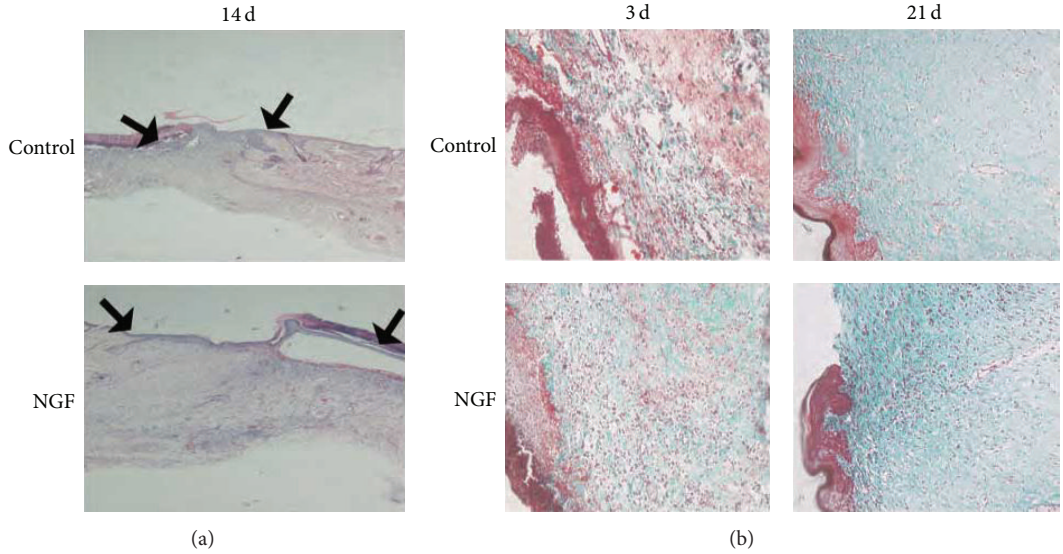



Figure 2: Histological structure of wounded skin sections. (a) Hematoxylin and eosin stainingof wound healing at 14 days after wounding (×40). As compared with saline-treated control, epithelial crawling (the distance between two black arrows as indicated) in NGF-treated wounds advanced more rapidly, and more granulation tissue was found in the NGF-treated wound. (b) Masson's trichrome staining of the wounded skin sections with saline or NGF treatment for 3 and 21 days after wounding (×200). There is a mild increase of collagen content (green) in NGF-treated wound tissue compared with saline-treated controls at different times after wounding.

and 7 days after wounding. However, there was no significant difference in PCNA expression between NGF-treated and saline-treated wounds at 14 days after wounding. These data suggest that NGF can promote cell proliferation and collagen production.

*2.4. NGF Upregulated the Levels of CD68 and VEGF in Wounds.* The inflammatory response is instrumental for supplying the growth factor and cytokine signals that orchestrate the cell and tissue movements necessary for repair during wound healing. The expression of CD68, a marker

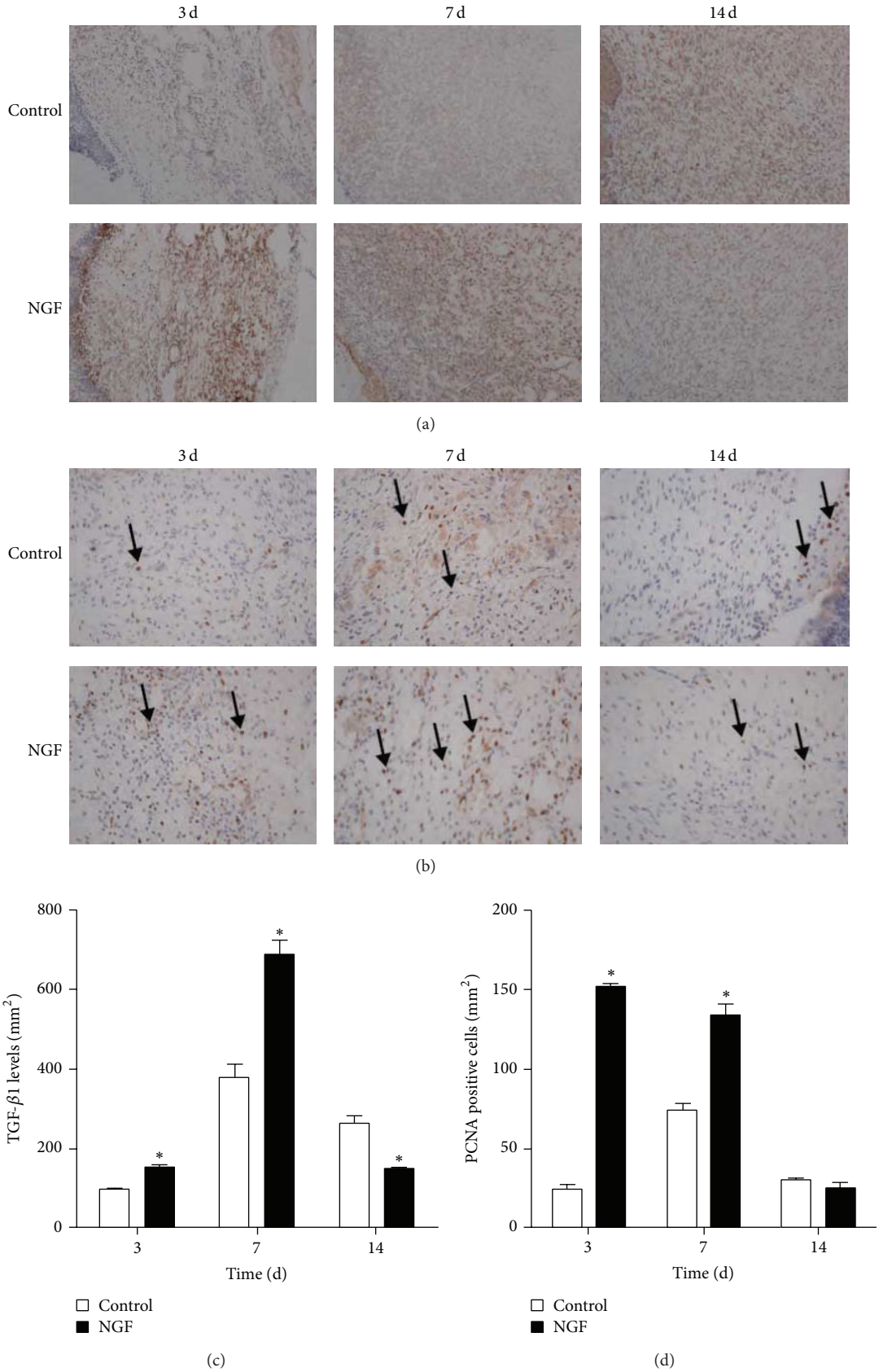



Figure 3: NGF administration increases the level of TGF-$\beta$1- and PCNA-positive cells in skin wounds. Immunohistochemical staining for (a) TGF-$\beta$1 and (b) PCNA was performed at the indicated day after wounding (×200). (c) The optical density of TGF-$\beta$1 analyzed with Image J software. (d) The number of cells positive for PCNA staining. $^{*}P < 0.05$, compared to control group.

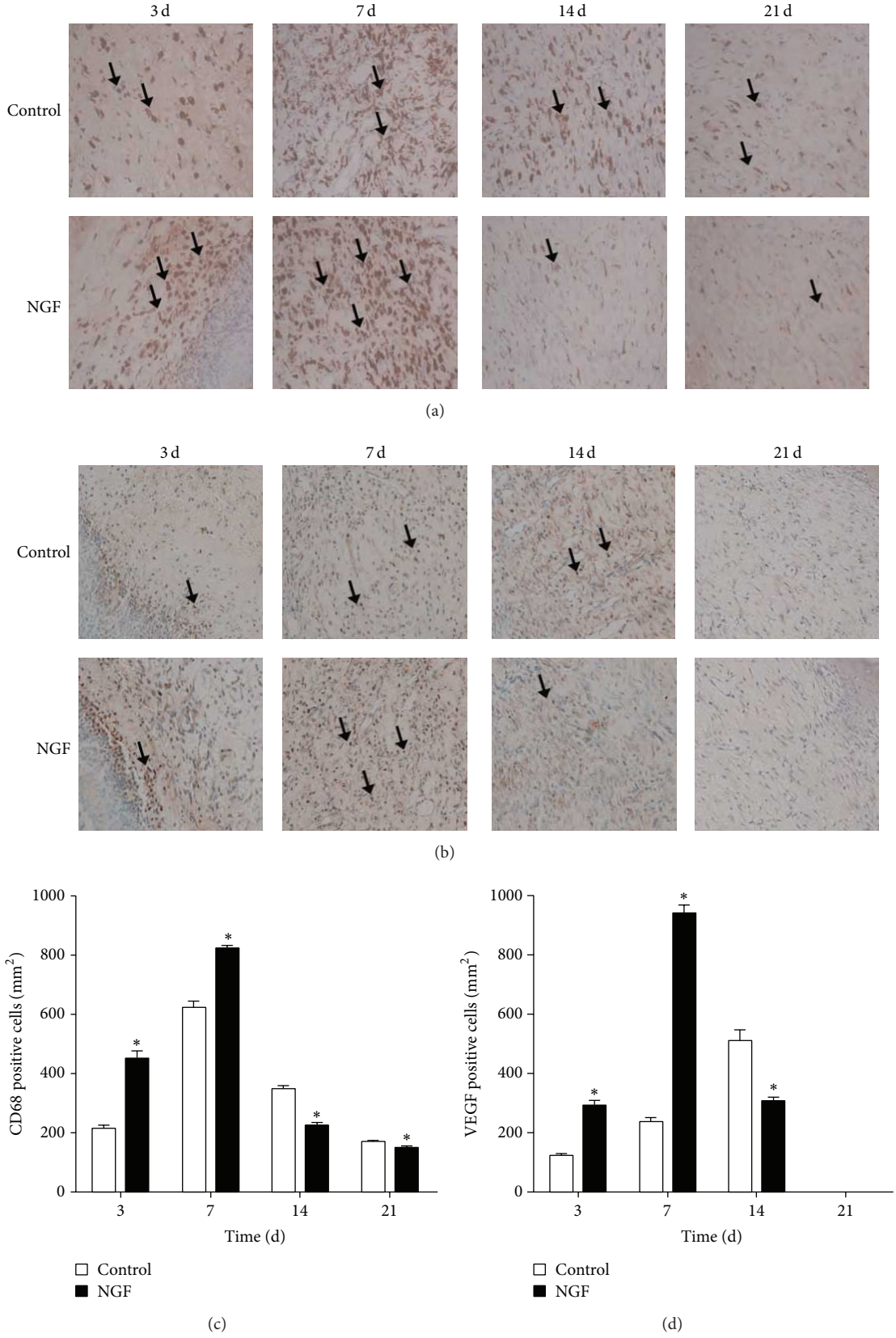



Figure 4: The expression of CD68 and VEGF was increased following NGF treatment. Immunohistochemical staining for CD68 (a) and VEGF (b) was performed at the indicated day after wounding (×200). ((c) and (d)) The number of cells positive for CD68 or VEGF in wounds. $^*P < 0.05$, compared to control group.

for macrophages, was detected by immunohistochemical staining. As shown in Figures 4(a) and 4(c), there were more cells positive for CD68 in NGF-treated wounds than in saline-treated controls at 3 days and 7 days after wounding. However, the level of CD68 expression in NGF-treated wounds was lower than in saline-treated controls at 14 days and 21 days after wounding. These results suggested that NGF was involved in the early stage of inflammation by recruiting inflammatory cells such as macrophages. The expression of VEGF, a proangiogenic growth factor, in NGF-treated wounds was investigated by immunohistochemical staining. As shown in Figures 4(b) and 4(d), there were more cells positively stained for VEGF in NGF-treated wounds than in saline-treated controls at 3 days and 7 days after wounding. However, the cells positive for VEGF were significantly downregulated at 14 days after wounding, while VEGF was undetectable in both NGF-treated and saline-treated wounds at 21 days after wounding. Thus, NGF might promote angiogenesis in cutaneous wounds by upregulating the expression of VEGF during the early stage of wound healing.

*2.5. NGF Induced the Migration, but Not Proliferation, of Cultured Dermal Fibroblasts.* In light of the critical role fibroblasts play in cutaneous wound healing, we focused on the effect of NGF on cultured human dermal fibroblast. The effect of NGF on fibroblast proliferation was investigated with MTT assays. As shown in Figure 5(a), incubating the fibroblasts with different doses of NGF for 24 h did not promote fibroblast proliferation. In contrast, basic fibroblast growth factor (bFGF) could significantly induce fibroblast proliferation when incubated for 24 h (data not shown). However, fibroblast migration was remarkably induced by treatment with 100 ng/mL of NGF as compared to saline-treated control (Figures 5(b) and 5(c)). Thus, NGF plays a crucial role in fibroblast migration. The fibroblast migration enhanced by NGF may contribute to NGF-accelerated wound healing in excisional wounds in rat.

*2.6. PI3K/Akt, Rac1, JNK, and ERK Were Involved in NGF-Promoted Fibroblast Migration.* To clarify which signaling pathways are involved in the regulation of human fibroblast migration promoted by NGF, we further investigated the respective roles of PI3K/Akt, Rac1, JNK, and ERK in NGF-accelerated fibroblast migration.

As shown in Figure 6(A1), 100 ng/mL NGF sharply raised the activity of Akt at 5, 15, and 30 min after treatment, though its peak effect was reached at 5 min. The NGF-induced increase in Akt phosphorylation in cultured human dermal fibroblasts was completely inhibited by LY294002, a specific inhibitor for Akt (Figure 6(A2)). When cells were pretreated with LY294002, NGF-induced fibroblast migration in the wound-healing assay was obviously impaired (Figures 6(A3) and 6(A4)).

The level of the phosphorylated JNK was significantly increased after stimulation with NGF (Figure 6(B1)). As shown in Figure 6(B2), when incubated with SP600125, a specific inhibitor for JNK, the NGF-induced phosphorylation of JNK in cultured fibroblasts, was completely blocked; thus the NGF-enhanced fibroblast migration was also significantly impaired in a wound-healing assay (Figure 6(B3)).

Similarly, ERK activity was significantly enhanced after treatment with 100 ng/mL of NGF (Figure 6(C1)). While the specific inhibitor for ERK, PD98059, markedly blocked the NGF-induced phosphorylation of ERK in cultured fibroblasts (Figure 6(C2)). When cells were pretreated with PD98059, NGF-induced fibroblast migration in the wound-healing assay was clearly reduced (Figure 6(C3)).

As shown in Figures 6(D1) and 6(D2), although total Rac1 content seemed unchanged after treatment with 100 ng/mL NGF, the level of active Rac1 was significantly increased over untreated controls as early as 5 min after NGF stimulation, and it remained high until 15 min. Thus, NGF can increase Rac1 activity, implying that Rac1 is probably involved in NGF-induced fibroblast migration. Taken together, these results suggest that PI3K/Akt, JNK, ERK, and Rac1 are all involved in the regulation of NGF-induced dermal fibroblast migration.

## 3. Discussion

Wound healing is an orderly and coordinated tissue repair process mainly consisting of three overlapping phases: inflammation, granulation tissue formation, and tissue remodeling. The healing process is highly regulated by cytokines, growth factors, and inflammatory mediators released from residential cells and infiltrating inflammatory cells in cutaneous wound tissue [1]. In intact skin, NGF is constitutively expressed by a variety of cell types, including fibroblasts and keratinocytes [13–15]. After skin wounding, the level of NGF is markedly upregulated, especially in the neonate [16]. The two specific receptors for NGF, p75NTR and TrkA, are expressed on the surfaces of various types of cells in the skin, including keratinocytes, melanocytes, fibroblasts, and mast cells [17–20]. The removal of the submaxillary glands, which store large amounts of NGF, substantially affects recovery after wound healing in mouse skin [8]. All of these findings suggest that NGF may play an important role in cutaneous biology and wound healing.

Indeed, it has been reported that topical application of NGF to cutaneous wounds hastened wound healing in normal and healing-impaired diabetic mice [7, 21]. A randomized clinical trial has also indicated that topical application of NGF could accelerate the wound-healing process in 18 selected patients with pressure ulcers of the foot [22]. We made a full-thickness excisional skin wound model in rats and topically applied different doses of NGF every other day to the wound. Our results showed that topical application of NGF with doses of 10 $\mu$g or 20 $\mu$g per wound also significantly accelerated the rate of wound healing in rats and enhanced wound reepithelialization and the formation of granulation tissue. Further increasing the dose to 40 $\mu$g showed less of an effect on wound healing.

By immunohistochemical staining for PCNA, NGF obviously increased the cell proliferation in wound area. NGF significantly stimulates the proliferation of normal human keratinocytes in culture in a dose-dependent manner [13]. NGF has been shown to elicit broad biologic functions in

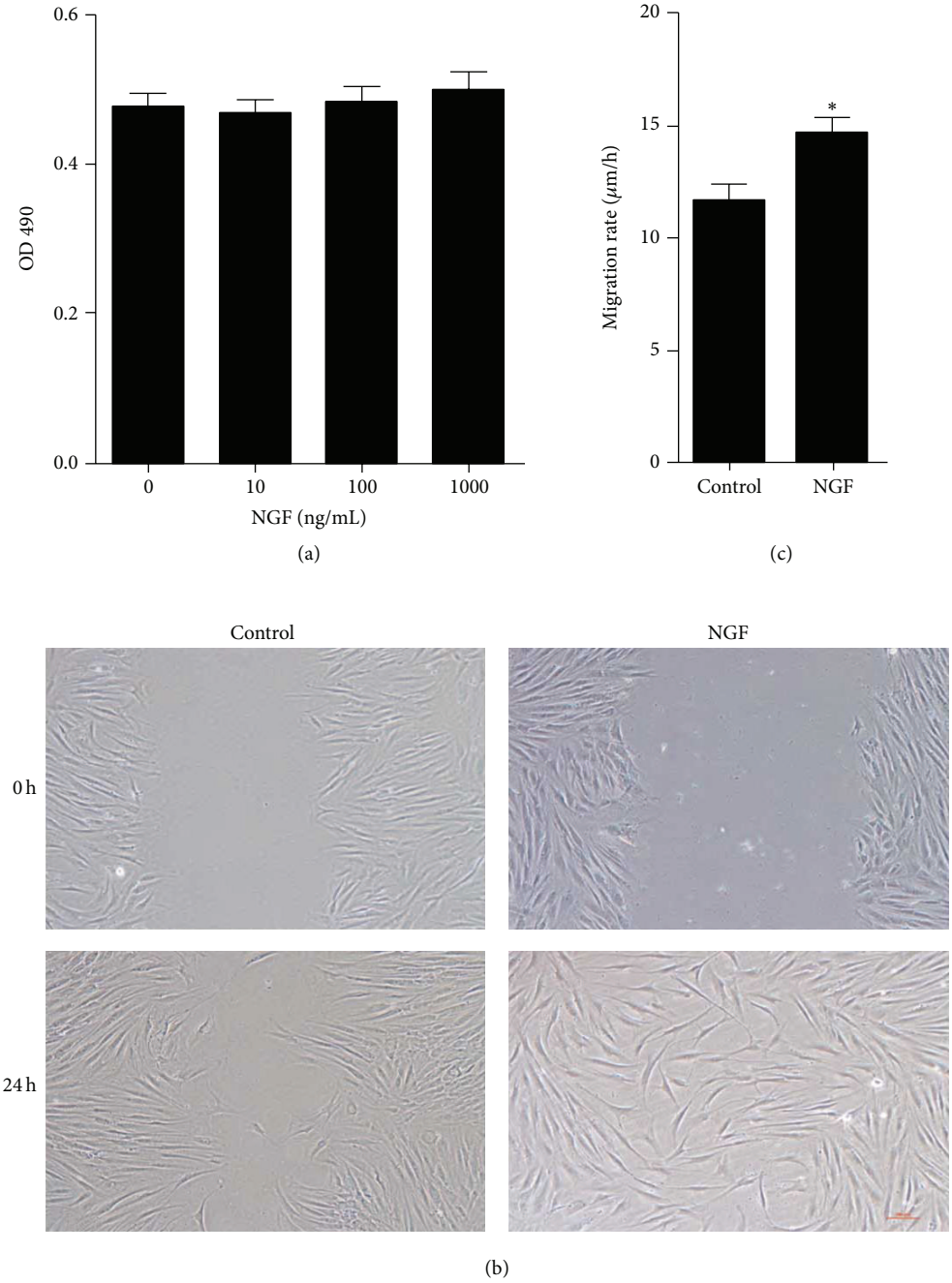



Figure 5: The effect of NGF on the proliferation and migration of human skin fibroblasts. (a) Cultured human dermal fibroblasts were incubated with different concentrations of NGF protein (0, 10, 100, and 1000 ng/mL) for 24 h, and cell proliferation was assessed by MTT assay. (b) Wound-healing assay of cultured human skin fibroblasts treated with saline or 100 ng/mL NGF for 24 h. (c) The migration rate of cultured fibroblasts after wounding is expressed as migration distance/time ($\mu$m/h). $^{*}P < 0.05$, compared to control group.

inflammatory cells such as murine neutrophils and macrophages in the process of inflammation [23]. In agreement with this, we also found a significant increase in staining for CD68, a marker for macrophages, at the first week after NGF treatment, implying that NGF could recruit inflammatory cells including macrophages into the wound area at the inflammation stage of wound healing. There was also a mild increase in collagen content in healing wounds in the NGF-treated group compared to untreated controls, as demonstrated by Masson's trichrome staining. It has been reported that NGF does not influence the production of collagen in cultured fibroblasts, which is different from our finding [24]. This difference is probably attributable to the more sophisticated healing microenvironment *in vivo* involving the interaction of NGF with other growth factors and extracellular matrix molecules.

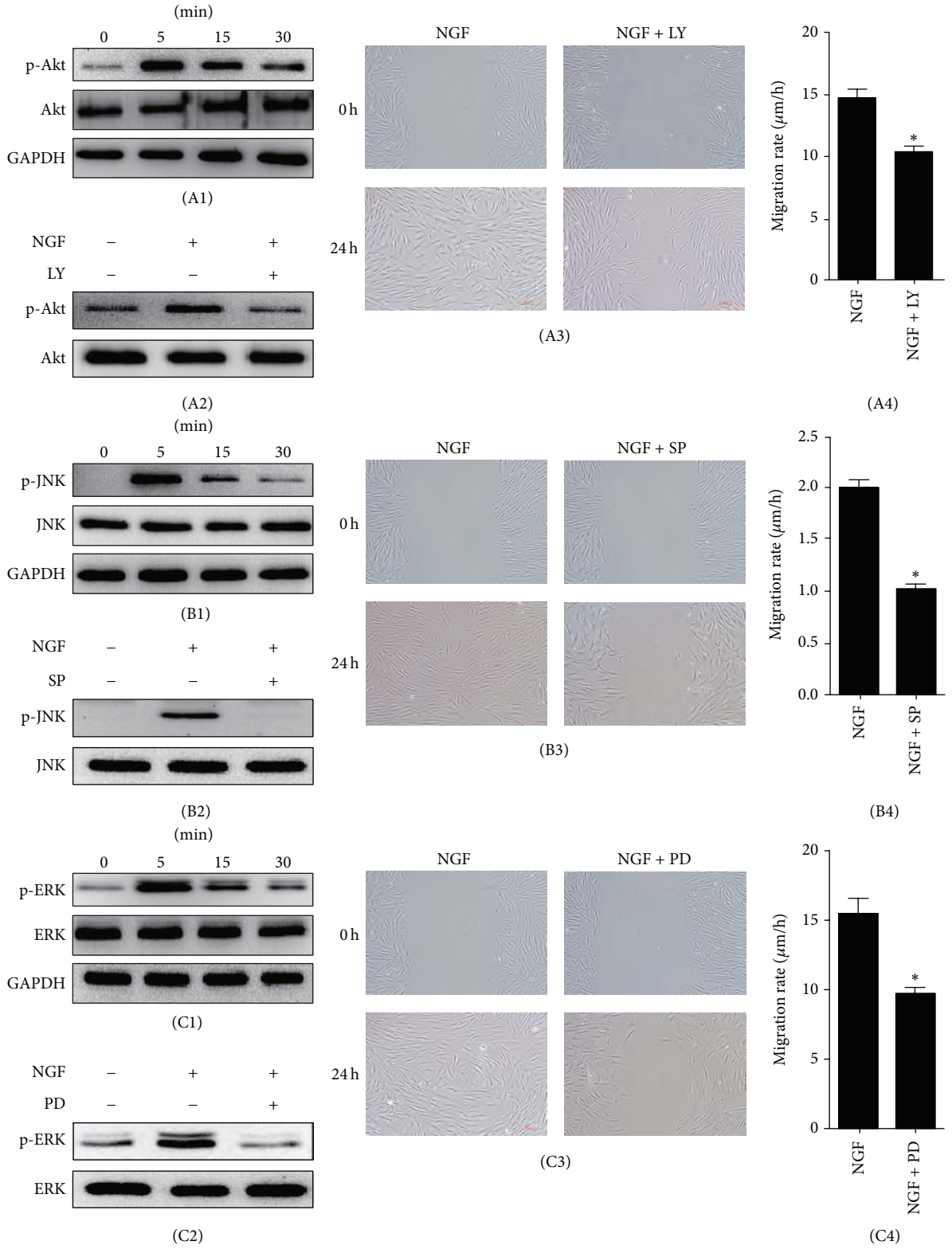



Figure 6: Continued.

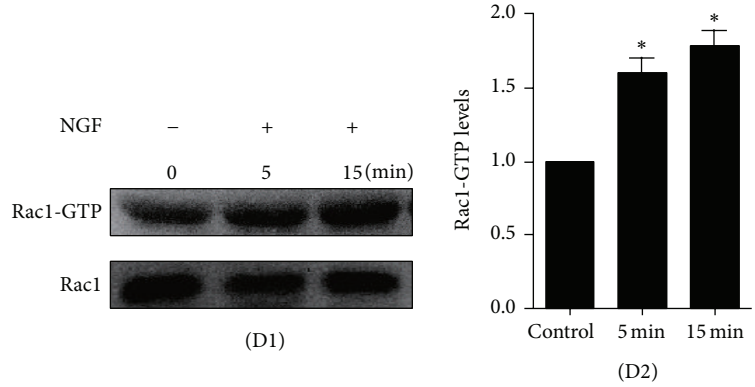



Figure 6: The effect on human skin fibroblast migration of blocking JNK, ERK, or PI3K/Akt pathways with specific inhibitors. ((A1), (B1), and (C1)) The levels of Akt, JNK, and ERK activity were all enhanced after treatment with 100 ng/mL of NGF for 5, 15, or 30 min. ((A2), (B2), and (C2)) The activity of Akt, JNK, or ERK following 5 min of NGF treatment was abolished by the corresponding specific inhibitor: LY294002 (10 $\mu$M, LY is the abbreviation for LY294002), SP600125 (10 $\mu$M, SP is the abbreviation for SP600125), or PD98059 (10 $\mu$M, PD is the abbreviation for PD98059). ((A3), (A4), (B3), (B4), (C3), and (C4)) The NGF-induced migration of human skin fibroblasts induced by NG was significantly impaired after incubation with LY294002 (10 $\mu$M), SP600125 (10 $\mu$M), or PD98059 (10 $\mu$M). $^*P < 0.05$, compared to the control group. ((D1) and (D2)) Active Rac1 was pulled down and then detected by Western blotting. $^*P < 0.05$, compared to untreated control group.

TGF-$\beta$1 has been extensively reported to elicit collagen synthesis in fibroblasts [25]; we measured the expression of TGF-$\beta$1 in wounds and found that the level of TGF-$\beta$1 was significantly increased in NGF-treated wounds compared with untreated controls. This implies that NGF may promote collagen production in wounds by upregulating the expression of TGF-beta. It has been suggested that supplementation of NGF promotes angiogenesis in mice with limb ischemia through a VEGF-dependent mechanism [26]. Consistent with this, we detected a significant increase in VEGF levels in wounds during the first week following NGF treatment. However, at 14 days after NGF treatment, the expression of VEGF in wounds was much lower than in untreated controls because the NGF-treated wounds were nearly closed. NGF did not promote the proliferation of cultured dermal fibroblasts derived from human foreskins, which is consistent with the findings of Micera et al. [24]. However, the migration ability of dermal fibroblasts was significantly enhanced following NGF treatment, suggesting that NGF-induced fibroblast migration may also contribute to NGF-accelerated skin wound healing *in vivo*. Our results, combined with others' reports, demonstrate that NGF influences wound-healing, probably by regulating inflammation and promoting angiogenesis and the proliferation of keratinocytes.

To clarify which signaling pathway is involved in the regulation of human fibroblast migration promoted by NGF, we first investigated the specific role of PI3K/Akt in NGF-accelerated fibroblast migration. The activation of the PI3K/Akt pathway plays a central role in establishing cell polarity and migration speed and is therefore required for the migration of various cell types, including fibroblasts [27–29]. NGF has also been shown to induce cell migration through the activation of PI3K/Akt pathway in rat peritoneal mast cells and aorta endothelial cells [30]. In our study, the cultured human skin fibroblasts showed an obvious increase in Akt activity after treatment with NGF. The inhibition of Akt phosphorylation by LY294002 significantly inhibited the migration of skin fibroblasts accelerated by NGF. This implies that PI3K/Akt signal transduction is involved in the regulation of NGF-promoted fibroblast migration. Accumulating evidence also suggests that the JNK pathway is important in regulating cell migration [31, 32]; by the same methods, the result is implying that the JNK signaling pathway also plays a crucial role in NGF-boosted fibroblast migration.

Rac1, a member of the Rho family of proteins, regulates actin organization and cell-cell adhesion and migration. Dominant-negative Rac1 inhibits lamellipodium extension, membrane ruffling, and migration in multiple cell types, including macrophages, T cells, epithelial cells, and fibroblasts [33]. Mutationally activated Rac1 potently and selectively activates JNK without affecting MAPK and dominant negative mutants of Rac1 block the JNK activation induced by cytokines and growth factors in COS-7 cells, suggesting that Rac1 plays a critical role in controlling the JNK signaling pathway [34]. PI3-kinase, Rac1, and JNK are all involved in bFGF-induced fibroblast migration and PI3-kinase is upstream of Rac1 and JNK is downstream of Rac1 [35]. Based on these findings, we next examined the effect of NGF on activation of Rac1. The activity of Rac1 was significantly increased after five minutes of NGF treatment. Taken together, our results suggested that PI3K/Akt, Rac1, and JNK are all involved in the regulation of NGF-induced dermal fibroblast migration and that it is mediated by a PI3K/Akt-Rac1-JNK signaling pathway.

Extracellular signal-regulated protein kinase (ERK) plays a crucial role in the regulation of cell survival, proliferation, and differentiation. ERK has also been reported to be involved in regulating cell migration, and the duration and magnitude of ERK activation associate with cell motility [36]. The inhibition of ERK activation results in markedly

reduced movement of epithelial and endothelial cells in wound-healing experiments [37, 38]. Moreover, some reports have suggested that the ERK pathway is required for fibroblast migration and is potentially involved in determining movement direction [29, 39–41]. Previous studies in rat peritoneal mast cells and aorta endothelial cells also showed that NGF was able to induce directional cell migration through activation of the ERK pathway [30, 42]. Our study indicated that ERK is significantly activated following NGF treatment. The NGF-enhanced migration ability of fibroblasts was notably impaired when ERK activity was inhibited by PD98059, suggesting that ERK was also involved in the NGF-enhanced fibroblast migration. However, the detailed signal transduction cascade underlying NGF-induced fibroblast migration remains to be elucidated.

In conclusion, in this study, we provided experimental evidences that NGF administration may be used for the treatment of wound healing. NGF could speed up the reepithelialization, the formation of granulation tissue, and collagen production in cutaneous wound via regulating the expression of CD68, VEGF, PCNA, and TGF-$\beta$1. We also found that NGF could significantly promote the migration, but not proliferation, of dermal fibroblasts, which may contribute to the healing-promoting effect of NGF in skin wound. The activation of PI3K/Akt, Rac1, JNK, and ERK was all involved in the regulation of NGF-induced fibroblast migration. However, to apply NGF for the treatment of cutaneous wound in a clinical setting, a lot of work needs to be done. For example, the best administration dose, the interval of dosing, the therapeutic window of NGF, and its synergistic effect with other growth factors in wound healing remain to be further investigated in the future. Given the confirmative effect of NGF to promote cutaneous wound healing, further study to improve the pharmacodynamic action of NGF and to explore its underlying complicated mechanisms in wound healing is definitely warranted.

## 4. Materials and Methods

*4.1. Ethics Statement.* All experimental animals were provided by the Laboratory Animals Center of Wenzhou Medical University. The care and use of laboratory animals were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experimental procedures were carried out with the approval of the Animal Experimentation Ethics Committee of Wenzhou Medical University. Human foreskins were obtained from Second Affiliated Hospital of Wenzhou Medical University. Written consent was obtained from all participants involved in this study. The study protocol was approved by Institutional Ethics Committee of Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) and informed consent was obtained from patients.

*4.2. Culture of Human Skin Fibroblasts.* Human foreskins were obtained from Second Affiliated Hospital of Wenzhou Medical University, and the primary culture of human dermal fibroblasts (HDFs) was established as described [43]. Briefly, the foreskins were washed three times in phosphate buffered saline (PBS) solution containing 1% penicillin/streptomycin sulfate. Subsequently, the tissues were digested with 0.5% dispase II overnight. The epidermis and subcutaneous tissue were excised from the tissues, which were then cut into pieces of approximately $1 \times 1 \times 0.5$ cm and placed as explants in T25 tissue culture flasks. Growth medium was Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM D-glucose, 20% fetal bovine serum (FBS), 1% penicillin/streptomycin sulfate, and 2 mM L-glutamine. The primary fibroblasts were grown at 37°C in an atmosphere of 5% $CO_2$ and were passaged every 2 days by trypsinization. Cells in passages three to six were used for all experiments in this study.

*4.3. Preparation of Rat Cutaneous Wounds and Experimental Design.* Male Sprague-Dawley rats, weighing 220–250 g, were chosen for this experiment. A wound healing model in rats was prepared as previously described [44]. Animals were anaesthetized by an intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg) and positioned on a cork platform, and then hairs on the rat dorsum were clipped and then two $2 \times 2\,cm^2$ full-thickness skin flaps were cut per rat. The two wounds were separated by at least 1.5 cm of unwounded skin. The right-side (right) wounds were treated with different doses of NGF (Sigma-Aldrich, dissolved in 0.9% saline (w/v)), while the left-side wounds received equal volumes of 0.9% saline (w/v) as untreated saline controls. Wounded rats were randomly divided into three groups (fifteen rats per group): (1) rats treated with 10 $\mu$g of NGF in right wounds; (2) rats treated with 20 $\mu$g of NGF in right wounds; (3) rats treated with 40 $\mu$g of NGF in right wounds. NGF treatments were repeated under anesthesia every other day for 14 days. Postoperative analgesics and antibiotics were not administered because these drugs may influence the healing process and thereby confound the interpretation of experimental data.

*4.4. MTT Assay.* Cultured dermal fibroblasts were seeded on 96-well plates ($1 \times 10^4$ cells/well) and treated with different concentrations of NGF (0, 10, 100, and 1000 ng/mL) for 24 h. Then, 20 $\mu$L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL in PBS) was added to each well for 4 h. Cells were washed with PBS (pH 7.4), and 150 $\mu$L of DMSO was added to each well to solubilize the formed formazan crystals. Fluorescence intensity was measured at 570 nm.

*4.5. Quantitative Assessment of Wound Healing.* The size of the wounded area was determined at 2, 4, 6, 8, 10, 12, and 14 days after wounding. Transparent paper was placed over each wound, and the shape of the wound was drawn on the paper. The transparent paper was then superimposed on $1\,mm^2$ graph paper for calculating the surface area of the wound. The percentage of wound closure was calculated using the following formula: wound closure rate = (area of original wound − area of actual wound)/area of original wound × 100% [45].

*4.6. Wound-Healing Assay.* Confluent primary fibroblasts were cultured in DMEM containing 0.5% FBS for 24 h and were then wounded with a linear scratch by a sterile pipette tip. Images of the wounded cell monolayers were taken using a microscope immediately and 24 h after wounding. To observe the effect of NGF on fibroblast migration, the cells were treated with saline or 100 ng/mL NGF just before wounding. To investigate the role of PI3-kinase, JNK, or ERK in fibroblast migration, the cells were incubated with 10 $\mu$M LY294002, 10 $\mu$M SP600125, or 10 $\mu$M PD98059, respectively, for 60 min before the wounding. Twenty cells at the wounded area per each experiment were randomly selected 24 h after wounding, and the distance between the selected cells and wound edge was measured with Image J software. Migration rate was expressed as migration distance/time (mm/h).

*4.7. Histological Examination.* Wounded skin was excised, fixed in cold 4% paraformaldehyde overnight, and embedded in paraffin. Tissue sections (5 $\mu$m thick) were stained with hematoxylin and eosin (H&E) for morphological assessment and with Masson's Trichrome staining for collagen analysis. The stained section slides were imaged at a magnification of 40 or 200x using a Nikon Eclipse E800 microscope.

*4.8. Immunohistochemical Staining.* Immunohistochemical staining for PCNA, CD68, TGF-$\beta$1, and VEGF was performed with specific antibodies for each protein (Santa Cruz Biotech, Santa Cruz, CA). Sections were dewaxed and hydrated; endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min; nonspecific binding was blocked with 1% BSA for 30 min. Sections were incubated with primary antibodies (at a dilution of 1 : 100) overnight at 4°C. Biotinylated secondary antibodies were then applied at 1 : 200 for 30 min, followed by incubation with horseradish peroxidase- (HRP-) streptavidin at 1 : 400 for 30 min. Color development was performed with DAB for 3 to 5 min for all samples, followed by hematoxylin counterstaining, dehydration, and coverslipping. The immunopositive cells in five fields per section were counted using Image-Pro Plus software (Nikon, Tokyo, Japan).

*4.9. Rac1 Pull-Down Assay.* Rac1-GTP assays were performed with the Active Rac1 Pull-Down and Detection Kit using the manufacturer's recommendations (Thermo Scientific). Briefly, the cells were scraped into 1X lysis buffer containing 25 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 1% NP-40, 5% glycerol, 150 mM NaCl, and 1% protease inhibitor cocktail and centrifuged for 15 min at 16,000 ×g. The total of 700 $\mu$L of cleared cell lysates was split into 20 $\mu$g aliquots. As negative and positive controls for the pull-down, two of the aliquots were added to 5 $\mu$L of 100 mM GDP or 10 mM GTP$\gamma$S, respectively, and incubated for 1 h at 4°C with gentle rocking. The beads were washed three times with lysis buffer and heated for 5 min at 100°C in reducing SDS-PAGE sample buffer and then analyzed for activated Rac1 by Western blotting with a monoclonal antibody for Rac1. Total Rac1 was determined to compare the level of the activated Rac1 across experimental conditions.

*4.10. Western Blot.* Western blotting was performed as follows. The cells were lysed, and the collected protein samples were denatured and separated on 10% polyacrylamide gels before being transferred to polyvinylidene difluoride membranes. The membranes were incubated in TBS containing 5% nonfat milk and 0.05% Tween-20 for 1 h at room temperature and blotted with the specified primary antibodies overnight at 4°C. The primary antibodies used were anti-phospho-Akt (1 : 300, Santa Cruz Biotech, Santa Cruz, CA), anti-Akt (1 : 300, Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-JNK (1 : 1000, Cell Signaling Technology), anti-JNK (1 : 1000, Cell Signaling Technology), anti-phospho-ERK (1 : 300, Santa Cruz Biotech, Santa Cruz, CA), anti-ERK (1 : 300, Santa Cruz Biotech, Santa Cruz, CA), and anti-GAPDH (1 : 300, Santa Cruz Biotech, Santa Cruz, CA). The membranes were washed with TBST for 15 min the next day. Next, the membranes were incubated for 1 h with an anti-mouse or anti-rabbit HRP-linked secondary antibody (1 : 3000 dilution) and washed with TBST for 15 min. The signals were then detected using Western blotting detection reagent. The Western blot results were further analyzed using Image J software.

*4.11. Statistical Analysis.* Data are expressed as the mean ± SEM. Statistical significance was determined with Student's $t$-test when there were two experimental groups. For more than two groups, statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. For all tests, $P < 0.05$ was considered significant.

## Conflict of Interests

The authors have declared that no conflict of interests exists.

## Acknowledgments

This work was supported by National Natural Science Funding of China (no. 81372112), Ningbo City Natural Science Foundation (no. 2012A610254), and Zhejiang Provincial Project of Key Group (no. 2010R50042). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

## References

[1] G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, "Wound repair and regeneration," *Nature*, vol. 453, no. 7193, pp. 314–321, 2008.

[2] A. J. Singer and R. A. F. Clark, "Cutaneous wound healing," *The New England Journal of Medicine*, vol. 341, no. 10, pp. 738–746, 1999.

[3] G. S. Schultz, J. M. Davidson, R. S. Kirsner, P. Bornstein, and I. M. Herman, "Dynamic reciprocity in the wound microenvironment," *Wound Repair and Regeneration*, vol. 19, no. 2, pp. 134–148, 2011.

[4] J. Li, J. Chen, and R. Kirsner, "Pathophysiology of acute wound healing," *Clinics in Dermatology*, vol. 25, no. 1, pp. 9–18, 2007.

[5] R. Braund, S. Hook, and N. J. Medlicott, "The role of topical growth factors in chronic wounds," *Current Drug Delivery*, vol. 4, no. 3, pp. 195–204, 2007.

[6] M. V. Sofroniew, C. L. Howe, and W. C. Mobley, "Nerve growth factor signaling, neuroprotection, and neural repair," *Annual Review of Neuroscience*, vol. 24, pp. 1217–1281, 2001.

[7] H. Matsuda, H. Koyama, H. Sato et al., "Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice," *Journal of Experimental Medicine*, vol. 187, no. 3, pp. 297–306, 1998.

[8] A. K. Li, M. J. Koroly, M. E. Schattenkerk, R. A. Malt, and M. Young, "Nerve growth factor: acceleration of the rate of wound healing in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 7, pp. 4379–4381, 1980.

[9] R. Bernabei, F. Landi, S. Bonini et al., "Effect of topical application of nerve-growth factor on pressure ulcers," *The Lancet*, vol. 354, no. 9175, p. 307, 1999.

[10] S. R. Opalenik and J. M. Davidson, "Fibroblast differentiation of bone marrow-derived cells during wound repair," *The FASEB Journal*, vol. 19, no. 11, pp. 1561–1563, 2005.

[11] C. S. Nirodi, R. Devalaraja, L. B. Nanney et al., "Chemokine and chemokine receptor expression in keloid and normal fibroblasts," *Wound Repair and Regeneration*, vol. 8, no. 5, pp. 371–382, 2000.

[12] S. Werner, T. Krieg, and H. Smola, "Keratinocyte-fibroblast interactions in wound healing," *Journal of Investigative Dermatology*, vol. 127, no. 5, pp. 998–1008, 2007.

[13] C. Pincelli, C. Sevignani, R. Manfredini et al., "Expression and function of nerve growth factor and nerve growth factor receptor on cultured keratinocytes," *Journal of Investigative Dermatology*, vol. 103, no. 1, pp. 13–18, 1994.

[14] K. Murase, Y. Murakami, K. Takayanagi, Y. Furukawa, and K. Hayashi, "Human fibroblast cells synthesize and secrete nerve growth factor in culture," *Biochemical and Biophysical Research Communications*, vol. 184, no. 1, pp. 373–379, 1992.

[15] V. A. Tron, M. D. Coughlin, D. E. Jang, J. Stanisz, and D. N. Sauder, "Expression and modulation of nerve growth factor in murine keratinocytes (PAM 212)," *Journal of Clinical Investigation*, vol. 85, no. 4, pp. 1085–1089, 1990.

[16] J. Oger, B. G. W. Arnason, and N. Pantazis, "Synthesis of nerve growth factor by L and 3T3 cells in culture," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 4, pp. 1554–1558, 1974.

[17] H. A. Bull, T. A. Leslie, S. Chopra, and P. M. Dowd, "Expression of nerve growth factor receptors in cutaneous inflammation," *British Journal of Dermatology*, vol. 139, no. 5, pp. 776–783, 1998.

[18] E. Shibayama and H. Koizumi, "Cellular localization of the Trk neurotrophin receptor family in human non-neuronal tissues," *The American Journal of Pathology*, vol. 148, no. 6, pp. 1807–1818, 1996.

[19] G. Nilsson, K. Forsberg-Nilsson, Z. Xiang et al., "Human mast cells express functional TrkA and are a source of nerve growth factor," *European Journal of Immunology*, vol. 27, no. 9, pp. 2295–2301, 1997.

[20] C. Pincelli, A. R. Haake, L. Benassi et al., "Autocrine nerve growth factor protects human keratinocytes from apoptosis through its high affinity receptor (TRK): a role for BCL-2," *Journal of Investigative Dermatology*, vol. 109, pp. 757–764, 1997.

[21] P. Muangman, L. A. Muffley, J. P. Anthony et al., "Nerve growth factor accelerates wound healing in diabetic mice," *Wound Repair and Regeneration*, vol. 12, no. 1, pp. 44–52, 2004.

[22] F. Landi, L. Aloe, A. Russo et al., "Topical treatment of pressure ulcers with nerve growth factor: a randomized clinical trial," *Annals of Internal Medicine*, vol. 139, no. 8, pp. 635–641, 2003.

[23] Y. Susaki, S. Shimizu, K. Katakura et al., "Functional properties of murine macrophages promoted by nerve growth factor," *Blood*, vol. 88, pp. 4630–4637, 1996.

[24] A. Micera, E. Vigneti, D. Pickholtz et al., "Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 11, pp. 6162–6167, 2001.

[25] D. A. Bettinger, D. R. Yager, R. F. Diegelmann, and I. K. Cohen, "The effect of TGF-beta on keloid fibroblast proliferation and collagen synthesis," *Plastic and Reconstructive Surgery*, vol. 98, pp. 827–833, 1996.

[26] C. Emanueli, M. B. Salis, A. Pinna, G. Graiani, L. Manni, and P. Madeddu, "Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs," *Circulation*, vol. 106, no. 17, pp. 2257–2262, 2002.

[27] J. M. Haugh, F. Codazzi, M. Teruel, and T. Meyer, "Spatial sensing in fibroblasts mediated by 3' phosphoinositides," *Journal of Cell Biology*, vol. 151, no. 6, pp. 1269–1280, 2000.

[28] A. T. Sasaki, C. Chun, K. Takeda, and R. A. Firtel, "Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement," *Journal of Cell Biology*, vol. 167, no. 3, pp. 505–518, 2004.

[29] L. Sepe, M. C. Ferrari, C. Cantarella, F. Fioretti, and G. Paolella, "Ras activated ERK and PI3K pathways differentially affect directional movement of cultured fibroblasts," *Cellular Physiology and Biochemistry*, vol. 31, pp. 123–142, 2013.

[30] J. Sawada, A. Itakura, A. Tanaka, T. Furusaka, and H. Matsuda, "Nerve growth factor functions as a chemoattractant for mast cells through both mitogen-activated protein kinase and phosphatidylinositol 3- kinase signaling pathways," *Blood*, vol. 95, no. 6, pp. 2052–2058, 2000.

[31] R. K. Barr and M. A. Bogoyevitch, "The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs)," *International Journal of Biochemistry and Cell Biology*, vol. 33, no. 11, pp. 1047–1063, 2001.

[32] C. Huang, K. Jacobson, and M. D. Schaller, "MAP kinases and cell migration," *Journal of Cell Science*, vol. 117, no. 20, pp. 4619–4628, 2004.

[33] A. J. Ridley, "Rho GTPases and cell migration," *Journal of Cell Science*, vol. 114, no. 15, pp. 2713–2722, 2001.

[34] O. A. Coso, M. Chiarielio, J.-C. Yu et al., "The small GTP-binding proteins rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway," *Cell*, vol. 81, no. 7, pp. 1137–1146, 1995.

[35] S. Kanazawa, T. Fujiwara, S. Matsuzaki et al., "bFGF regulates PI3-Kinase-Rac1-JNK pathway and promotes fibroblast migration in wound healing," *PLoS ONE*, vol. 5, no. 8, Article ID e12228, 2010.

[36] G. L. Johnson and R. Lapadat, "Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases," *Science*, vol. 298, no. 5600, pp. 1911–1912, 2002.

[37] Y. Matsubayashi, M. Ebisuya, S. Honjoh, and E. Nishida, "ERK activation propagates in epithelial cell sheets and regulates their migration during wound healing," *Current Biology*, vol. 14, no. 8, pp. 731–735, 2004.

[38] W.-L. Chen, C.-T. Lin, J.-W. Li, F.-R. Hu, and C.-C. Chen, "ERK1/2 activation regulates the wound healing process of

rabbit corneal endothelial cells," *Current Eye Research*, vol. 34, no. 2, pp. 103–111, 2009.

[39] E. Ranzato, M. Patrone, M. Pedrazzi, and B. Burlando, "Hmgb1 promotes wound healing of 3T3 mouse fibroblasts via RAGE-dependent ERK1/2 activation," *Cell Biochemistry and Biophysics*, vol. 57, no. 1, pp. 9–17, 2010.

[40] R. Buonomo, F. Giacco, A. Vasaturo et al., "PED/PEA-15 controls fibroblast motility and wound closure by ERK1/2-dependent mechanisms," *Journal of Cellular Physiology*, vol. 227, no. 5, pp. 2106–2116, 2012.

[41] T. Makino, M. Jinnin, F. C. Muchemwa et al., "Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways," *British Journal of Dermatology*, vol. 162, no. 4, pp. 717–723, 2010.

[42] U. L. Rahbek, S. Dissing, C. Thomassen, A. J. Hansen, and K. Tritsaris, "Nerve growth factor activates aorta endothelial cells causing PI3K/Akt- and ERK-dependent migration," *Pflugers Archiv*, vol. 450, no. 5, pp. 355–361, 2005.

[43] A. S. Colwell, T.-T. Phan, W. Kong, M. T. Longaker, and P. H. Lorenz, "Hypertrophic scar fibroblasts have increased connective tissue growth factor expression after transforming growth factor-$\beta$ stimulation," *Plastic and Reconstructive Surgery*, vol. 116, no. 5, pp. 1387–1391, 2005.

[44] H. X. Shi, C. Lin, B. B. Lin et al., "The anti-scar effects of basic fibroblast growth factor on the wound repair in vitro and in vivo," *PLoS ONE*, vol. 8, Article ID e59966, 2013.

[45] K. Meena, A. V. Mohan, B. Sharath, S. N. Somayaji, and K. L. Bairy, "Effect of topical phenytoin on burn wound healing in rats," *Indian Journal of Experimental Biology*, vol. 49, no. 1, pp. 56–59, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 697935, 12 pages
http://dx.doi.org/10.1155/2014/697935

*Review Article*

# Neuropathologic Implication of Peripheral Neuregulin-1 and EGF Signals in Dopaminergic Dysfunction and Behavioral Deficits Relevant to Schizophrenia: Their Target Cells and Time Window

**Hiroyuki Nawa, Hidekazu Sotoyama, Yuriko Iwakura, Nobuyuki Takei, and Hisaaki Namba**

*Department of Molecular Neurobiology, Brain Research Institute, Niigata University, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8585, Japan*

Correspondence should be addressed to Hiroyuki Nawa; hnawa@bri.niigata-u.ac.jp

Received 1 February 2014; Accepted 10 April 2014; Published 13 May 2014

Academic Editor: Saverio Bellusci

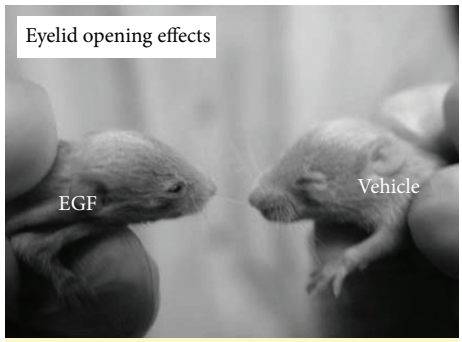


Neuregulin-1 and epidermal growth factor (EGF) are implicated in the pathogenesis of schizophrenia. To test the developmental hypothesis for schizophrenia, we administered these factors to rodent pups, juveniles, and adults and characterized neurobiological and behavioral consequences. These factors were also provided from their transgenes or infused into the adult brain. Here we summarize previous results from these experiments and discuss those from neuropathological aspects. In the neonatal stage but not the juvenile and adult stages, subcutaneously injected factors penetrated the blood-brain barrier and acted on brain neurons, which later resulted in persistent behavioral and dopaminergic impairments associated with schizophrenia. Neonatally EGF-treated animals exhibited persistent hyperdopaminergic abnormalities in the nigro-pallido-striatal system while neuregulin-1 treatment resulted in dopaminergic deficits in the corticolimbic dopamine system. Effects on GABAergic and glutamatergic systems were transient or limited. Even in the adult stage, intracerebral administration and transgenic expression of these factors produced similar but not identical behavioral impairments, although the effects of intracerebral administration were reversible. These findings suggest that dopaminergic development is highly vulnerable to circulating ErbB ligands in the pre- and perinatal stages. Once maldevelopment of the dopaminergic system is established during early development, dopamine-associating behavioral deficits become irreversible and manifest at postpubertal stages.

## 1. EGF-Like Ligands and Their ErbB Receptors in the Brain

Epidermal growth factor (EGF) was first purified from mouse salivary gland, together with nerve growth factor (NGF), and was found to induce eyelid opening activity [1] (Figure 1). Molecular cloning verified the presence of many EGF-related peptides such as heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor alpha (TGF$\alpha$), amphiregulin, and neuregulins (NRG) [2, 3]. All members of the EGF family have the capability to promote eyelid opening and are contained by various body fluids such as saliva, urine, serum, and amniotic fluids [1, 4, 5] (Figure 1). In addition to these endogenous ligands, several viruses encode EGF-like peptides in their genome and potentate host cell proliferation [6, 7]. These EGF-like peptides in blood can penetrate into the immature brain and influence neural stem cell proliferation and neuronal/glial differentiation and maturation [8–10].

All members in the EGF family interact with ErbB receptor tyrosine kinases ErbB1, ErbB2, ErbB3, and ErbB4 (note: ErbB3 lacks the kinase activity). These ligands promote ErbB receptor dimer formation and transphosphorylate the partner ErbB molecule [11] (Figure 2). Each ligand has a specific binding preference with an ErbB subtype; for instance,




| Conc (nM) | Saliva | Serum | Urine | Amniotic |
|---|---|---|---|---|
| EGF | 1-3 | 0.02-0.31 | 4-20 | 0.1-0.5 |
| Neuregulin-1 | ND | 0.01-0.48 | ND | ND |

Figure 1: EGF and neuregulin-1 (NGR1) in body fluids mediate eyelid opening. The picture depicts the acceleration of eyelid opening following subcutaneous injections of EGF into pups. EGF (1.0 mg/kg) was subcutaneously injected to rat neonates daily from postnatal day 2 to postnatal day 10. The table shows that both EGF and NGR1 are detected in human body fluids including saliva, serum, urine, and amniotic fluids. ND: not determined.

EGF has high affinity to ErbB1, HB-EGF interacts with ErbB1 and ErbB4, and NRG1 binds to ErbB3 and ErbB4. Regardless of the ErbB subtype bound by the ligand, their signals will be transmitted from both the partner ErbB and receptor ErbB [2, 11]. Interestingly, the activated ErbB tyrosine kinase also can form a dimer with other receptor tyrosine kinases such as MET [12]. In this context, different ligands in the EGF family often evoke distinct tyrosine kinase signaling in various types of cells.

*In situ* hybridization techniques have revealed widespread expression of ErbB1-4 mRNAs in various types of neurons and glial cells [13–16]. EGF receptors (EGF-R or ErbB1) are highly expressed in neural stem cells. In later stages, however, lower levels of ErbB1 are also detected in GABAergic and dopaminergic neurons, which often coexpress the ErbB4 subunit as well [14, 17] (Figure 3). In contrast, the expression of ErbB3 is relatively restricted to oligodendrocytes and Schwann cells [15, 18]. The localizations of ErbB1-4 are consistent with the reports of EGF and NRG1 actions. EGF and NRG1 exert various neurotrophic activities on midbrain dopaminergic neurons [19–21] although their actions on GABAergic neurons are inconsistent among the ErbB ligands [17, 22–24].

EGF and NRG1 have been researched extensively in relation to schizophrenia. In 2002, DeCode Genetics Inc. reported a genetic association of the *NRG1* gene with schizophrenia [25] and our group found abnormal expression of EGF and ErbB1 in the postmortem brains of patients with schizophrenia [4]. Subsequently, a Finland group reported a genetic association between the *EGF* gene and schizophrenia [26–28], although this has not been replicated in all ethnic populations examined [29]. Indeed, these human studies were the impetus for our research on animal modeling of schizophrenia using EGF and NRG1.

## 2. Neurobehavioral Impact of Peripheral EGF and NRG1 Administration during Development

To test the contribution of these neurotrophic factors to the neurodevelopmental abnormality of schizophrenia, we subcutaneously administered the EGF protein into rats and mice at various developmental stages, neonate, juveniles, and young adults [30, 31] (Figure 4). We then monitored their behavioral traits such as prepulse inhibition scores at the adult stage. We found that neonatal exposure to EGF resulted in various behavioral deficits, most of which are implicated in schizophrenia behavioral endophenotypes. These deficits include lower prepulse inhibition, impaired latent inhibition of fear learning, reduced social behaviors, and higher sensitivity to methamphetamine and a D2 receptor agonist [30–33] (Table 1). These behavioral deficits are persistent as we have detected the prepulse inhibition deficits at postnatal month six. Although we tested more than 10 cytokines and growth factors with the above experimental procedure, EGF and NRG1 appeared to exhibit the most remarkable and persistent abnormality in behaviors [30–38].

In contrast to the neonatal injection model, the administration of EGF into the skin of juvenile or young adult rats (at the same dose) failed to induce the above behavioral abnormalities (unpublished data). Why EGF administered at the different stages has no obvious effects remains to be explored; however, it is likely to involve the differences in (1) the supply of EGF to the target (i.e., the brain permeability of EGF), (2) the sensitivity of a target to EGF (i.e., EGF receptor expression), and (3) the phenotypic nature of the reaction of the target.

Thus we first monitored the permeability of EGF through the blood-brain barrier. We found that subcutaneous injection of EGF and neuregulin-1 to rat and mouse neonates resulted in the activation (phosphorylation) of ErbB receptors in the brain and led to behavioral deficits [38] (Table 1). The receptor activation in the brain was the most remarkable at the perinatal stages and gradually diminished during the postnatal stage. After postnatal day 10, subcutaneously-injected EGF and neuregulin-1 failed to trigger marked ErbB phosphorylation in the brain [38]. When we explored the permeability of the blood-brain barrier with interleukin-1, immunohistochemistry verified the efficient diffusion of interleukin-1 from the blood vessels into the neural spaces at the rat neonatal stage but not at rat postnatal day 14 [36]. These results are in agreement with the previous microscopic observations in rats where morphological maturation of endotheliocytes and pericytes occurs on postnatal day 7 and a decrease in permeability of the hematoencephalic barrier takes place on postnatal day 10 [39]. These results suggest that the establishment of the blood-brain barrier is attained around postnatal day 10 and may be one of the key factors for determining the time dependency for the induction of behavioral impairments [36]. Even at the adult stage, however, EGF and neuregulins-1 are reported to penetrate the established blood-brain barrier in a limited degree [21, 40, 41].

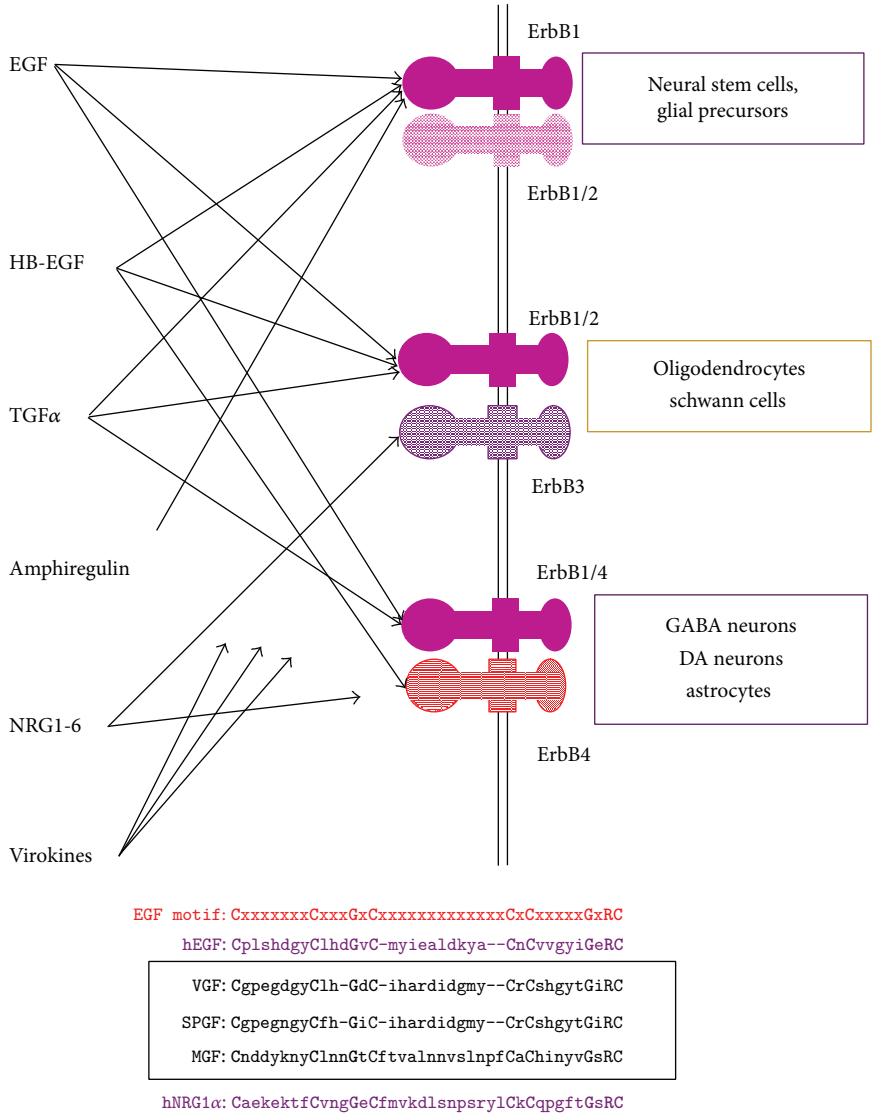



Figure 2: Ligands in the EGF family interact with heteromeric or homomeric ErbB receptors in GABAergic, dopaminergic (DA), and glial cells. ErbB ligands include EGF, HB-EGF (heparin-binding EGF-like growth factor), TGF$\alpha$ (transforming growth factor alpha), amphiregulin, NRG1-6, and virokines (VGF, SPGF, MGF, etc.), which associate with these ErbB receptor complexes to evoke both EGF-like and NRG-like signals. ErbB1-4 selectivity of the virokines remains to be characterized. These virokines carry the EGF-like amino acid motif common to human EGF and NRG1alpha. VGF: vaccinia virus growth factor; SPGF: smallpox virus growth factor; MGF: myxoma virus growth factor.

The sensitivity of brain cells to EGF might also associate with the observed EGF efficacy. It is reported that ErbB1 expression gradually diminishes in the nigrostriatal system as well as in the cortical structures during postnatal development [15, 42]. This is another explanation that illustrates the developmental difference of EGF effects on animal behaviors.

We tested the first hypothesis that the penetration of the blood-brain barrier would be critical for the effectiveness of EGF injection. If it is the case, the direct EGF supply to the brain of adult rats should mimic neonatal EGF injection [43]. Indeed when EGF was subchronically infused into the striatum of adult rats from an osmotic minipump, EGF induced the deficits of prepulse inhibition and impaired latent inhibition of fear learning as was observed in the neonatal injection model. EGF infusion simultaneously elevated dopamine content and turnover as well as the enzyme activity of tyrosine hydroxylase and protein levels of dopamine transporter in the striatum, supporting its neurotrophic actions on dopamine neurons [43]. The apparent difference between the neonatal EGF model and the adult infusion model is the persistency or reversibility of the deficits. In the adult EGF-brain infusion model, the behavioral deficits ceased soon after EGF was depleted from the pump [39], whereas in the neonatal model, the deficits persisted more than five months after EGF administration was completed at the neonatal stage [31].

Table 1: Immediate and delayed effects of neonatal NRG1 and EGF administration.

| | | NRG1 Injection | EGF Injection |
|---|---|---|---|
| Behaviors | Sound startle | No change | No change |
| | Fear-learning | No change | No change |
| | Locomotor | No change | No change |
| | PPI | DECREASE | DECREASE |
| | Social behaviors | DECREASE | DECREASE |
| GABA | GAD 65/67 | No change | (DECREASE)* |
| | PV | No change | (DECREASE)* |
| Glutamate | NR1 (GluN1) | No change | (DECREASE)* |
| | NR2 (GluN2) | No change | no change |
| | GluR1 | (INCREASE) | (DECREASE)* |
| Dopamine | TH | INCREASE (cortex) | INCREASE (pallidus) |
| | DA | No change | INCREASE (pallidus) |
| | DOPAC | INCREASE (cortex) | INCREASE (pallidus) |

*represent transient changes during neonatal administration. GAD: glutamate decarboxylase; PV: parvalbumin; TH: tyrosine hydroxylase; DA: dopamine; DOPAC: 3,4-dihydroxy-phenylacetic acid. Statistical significance represents $P < 0.05$.

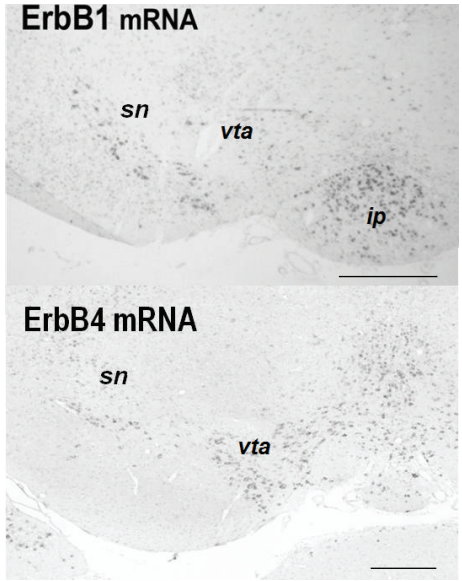



Figure 3: Distributions of ErbB1 mRNA and ErbB4 mRNA in rodent midbrain. *In situ* hybridization reveals enrichment of ErbB1 mRNA in the substantia nigra (sn) of rat pups (postnatal day 2). ErbB4 mRNA is expressed in both the sn and ventral tegmental area (vta) of mouse pups (postnatal day 2). Ip: interpeduncular nucleus. Scale bars = 250 $\mu$m.

A similar behavioral abnormality was also detected in the EGF-injection model of a nonhuman primate [44]. Apparent behavioral deficits such as stereotypic movement, vocalization, alert motion, and self-injury only emerged 5-6 years (i.e., monkey puberty) after EGF administration to a cynomolgus monkey neonate and were ameliorated by chronic treatment of the antipsychotic drug risperidone [44]. Therefore, the EGF-injection model may be established in a wide variety of animal species.

## 3. Life-Long Overexpression of EGF or NRG1 from Their Transgenes

In addition to the above injection models, we also analyzed the two types of transgenic (Tg) mice lines: type 1 neuregulin-1 overexpressing Tg mice and EGF-overexpressing Tg mice [45, 46]. In these transgenic lines, both the transgenes of EGF and NRG1 were expressed in the whole body, although their relative expression levels were higher in the central nervous system compared with those in the peripheral tissues.

EGF-Tg mice exhibited normal locomotion in the exploratory condition, and moderate deficits in context learning (Table 2). Sound startle responses of EGF-Tg mice were normal but their prepulse inhibition of startle responses was markedly lower than that of wild-type littermates [46]. In addition, EGF-Tg mice exhibited higher sensitivity to repeated cocaine administration in a locomotor test. Our preliminary studies indicate that social behavior scores of EGF-Tg mice appeared to diminish as well. Overall, EGF-Tg mice showed gross behavioral similarities to the EGF-injection model. In agreement to these behavioral traits, there was an enhancement in dopamine metabolism in the basal ganglia regions [46]. The results from EGF-Tg mice rule out the possibilities of experimental artifacts in the EGF-injection model; the behavioral deficits would be ascribed to any impurities in recombinant EGF samples or the production of anti-EGF antibody following EGF injections.

In our previous study, NRG1-Tg lines showed increased locomotor activity, a nonsignificant trend toward decreasing prepulse inhibition, and decreased context-dependent fear learning, but they exhibited normal levels of tone-dependent learning [45] (Table 2). In addition, our preliminary study indicates that social scores of both Tg lines were reduced. In contrast to the results from the NRG1-injection model, NRG1-Tg mice exhibited downregulation of dopamine metabolism in the corticolimbic regions. This reduction of dopaminergic phenotypes in NRG1-Tg mice had not been expected. The

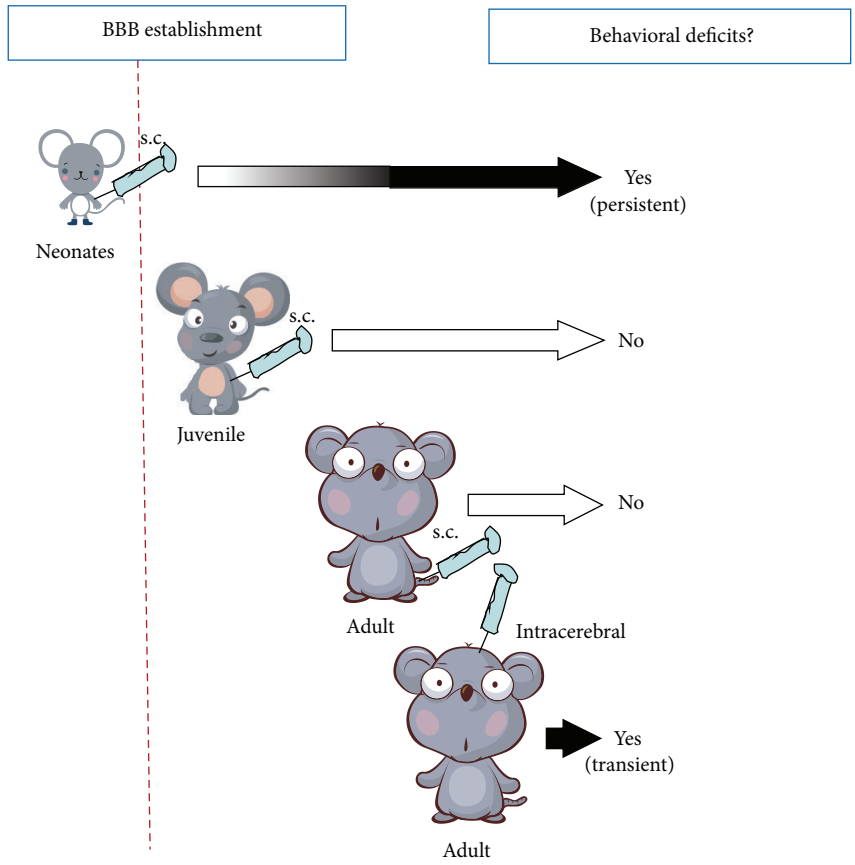



Figure 4: Neurobehavioral consequences following subcutaneous/intracerebral administration of EGF to neonatal, juvenile, and adult rats. Intracerebral administration to adult rats was achieved with cannula implantation to the stratum; EGF was subchronically supplied from an osmotic pump at the rate of 75 ng/h. There is a critical time window for the induction of behavioral deficits following peripheral EGF administration.

Table 2: Behavioral and neurochemical effects of persistent NRG1 and EGF overexpression from their transgenes.

| | | NRG-1 TG | EGF-TG |
|---|---|---|---|
| Behaviors | Sound startle | No change | No change |
| | Fear-learning | INCREASE | Modest decrease |
| | Locomotor | INCREASE | No change |
| | PPI | Modest decrease | DECREASE |
| | Social behaviors | DECREASE | DECREASE |
| GABA | GAD 65/67 | No change | No change |
| | PV | INCREASE (cortex) | No change |
| Glutamate | NR1 (GluN1) | No change | No change* |
| | NR2 (GluN2) | No change | No change* |
| | GluR1 (GluA1) | No change | No change* |
| Dopamine | TH | DECREASE (cortex) | DECREASE (striatum) |
| | DAT | No change | No change |
| | DA | DECREASE (hippocampus) | INCREASE (accumbens) |
| | DOPAC | DECREASE (hippocampus) | INCREASE (accumbens) |

*represent effects on basal ganglia regions GAD: glutamate decarboxylase; PV: parvalbumin; TH: tyrosine hydroxylase; DA: dopamine; DAT: dopamine transporter; DOPAC: 3,4-dihydroxy-phenylacetic acid. Statistical significance represents $P < 0.05$ and "modest" indicates marginal changes with $0.05 < P < 0.10$.

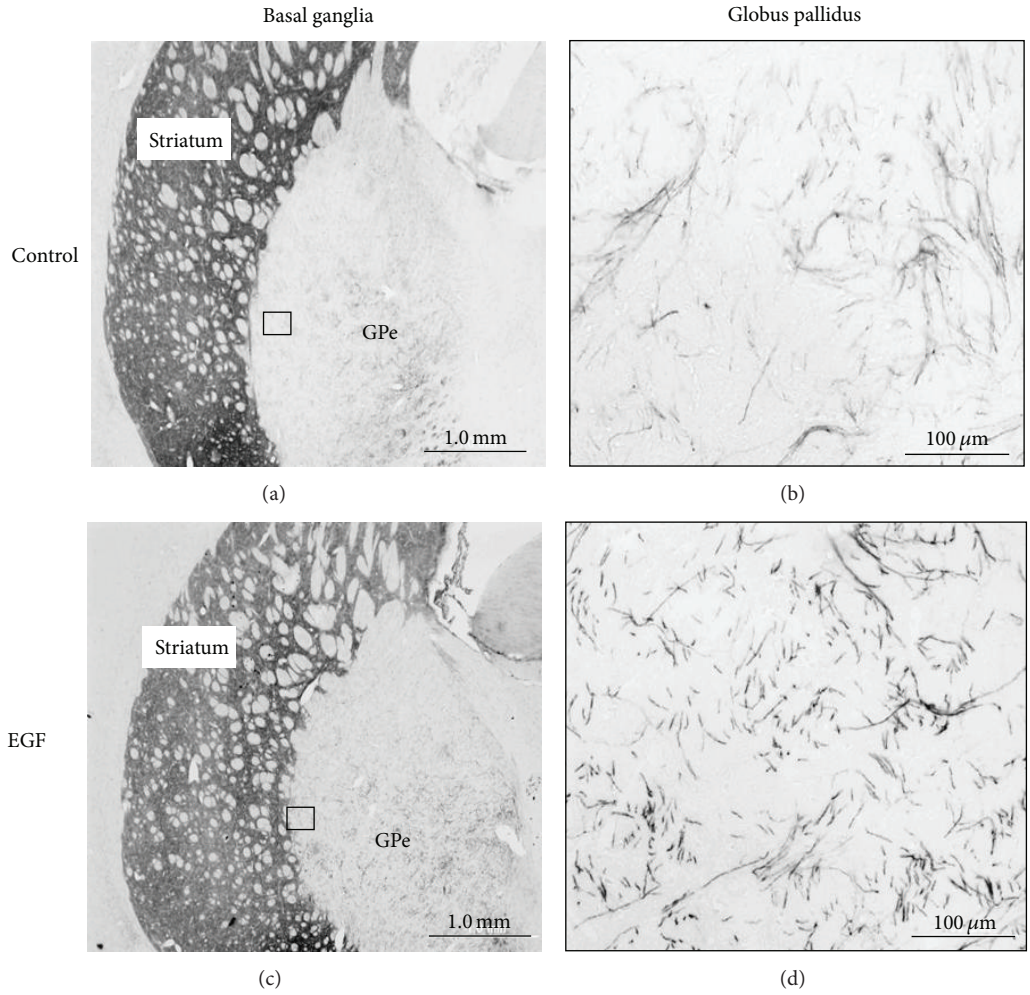



Figure 5: Neonatal EGF treatment enhances and maintains collateral sprouting of dopaminergic fibers in the globus pallidus (GPe). Neonatal rats were subcutaneously challenged with EGF (1 mg/kg body/day) for 9 days and grown until adulthood. EGF-treated and vehicle-treated rats were subjected to immunohistochemistry at the adult stage. In comparison with the tyrosine hydroxylase staining in the striatum (left), immunoreactivity in the lateral area of the GPe is elevated in the EGF-treated rats. Scale bars = 1000 and 100 $\mu$m.

controversial results from the NRG1-Tg mice should be explored with respect to the neurotrophic activity of NRG1 on dopaminergic neurons [21, 38].

Some of the behavioral traits of the NRG1-Tg mice significantly resemble those of NRG1 knockout mice [see reviews; [47–53]]. Gene targeting of another ErbB1 ligand HB-EGF also generates the animal model for schizophrenia [50]. Although here we avoid to repeat the details of their behavioral phenotypes, it is noteworthy that both hypomorphic and hypermorphic expressions of the NRG1 or EGF-related gene produce several common behavioral phenotypes in mice. This commonality is quite surprising and raises a challenging question about the molecular and cellular mechanisms underlying the behavioral deficits induced by the opposite signals.

There is another fundamental question whether schizophrenia is associated with the upregulation or downregulation of the NRG1-ErbB4 signaling [5, 47]. A postmortem study indicates the upregulation of NRG1 expression [47], while an analysis of patients' blood suggests the downregulation of NRG1 expression that associates with schizophrenia and the risk SNP [5]. The clarification of this controversy should be the first step before arguing the pathologic implication of NRG1.

## 4. Neurobiological Underpinnings of the Behavioral Deficits Triggered by EGF and Neuregulin-1 Hypersignals

There is a large time gap between factor treatment (via injection) and the emergence of behavioral deficits in neonatal EGF- and NRG1-injection models [54–57]. Thus, we aimed to determine what kinds of signals from EGF or NGR1 contribute to the delayed emergence of the behavioral deficits at the postpubertal stages. In light of reported neurobiological activities of the ErbB ligands EGF and NRG1, we initially focused on the three neurotransmitter systems: GABAergic,

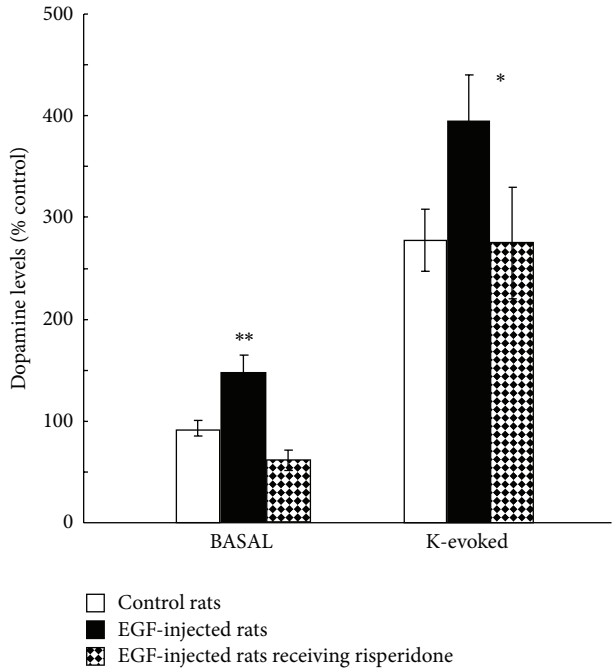



Figure 6: Local dopamine release from the globus pallidus was monitored by microdialysis. Dialysis probes were implanted in the globus pallidus of EGF-treated, vehicle-treated, and EGF-treated plus risperidone-medicated rats. Dopamine release of EGF-treated rats was elevated in both basal and high potassium (K) evoked conditions. These increases were normalized by subchronic treatment with the antipsychotic risperidone (1 mg/kg/day, 14 days total). $^{*}P < 0.05$ and $^{**}P < 0.01$.

glutamatergic (AMPA and NMDA receptors), and dopaminergic systems. The individual neuronal systems are composed of particular neurons expressing ErbB1 and/or ErbB4 and thus are reactive to EGF and NRG1.

When EGF was given to pups, GABAergic, glutamatergic, and dopaminergic markers were markedly affected in the acute phase. Specifically, GAD67, parvalbumin, GluA1 (AMPA-R), and GluN1 (NMDA-R) levels were influenced in the frontal cortex and/or midbrain of EGF-treated animals [17, 55, 56]. Dopamine synthesis, metabolism, and axon terminal arborization were shown to be markedly upregulated in most of the basal ganglia regions [20, 57]. In the postpubertal stages, however, most of these phenotypic influences became modest or undetectable, except for the presence of a hyperdopaminergic state in the globus pallidus [58]; tyrosine hydoxylase-positive fibers and varicosities were denser in the lateral regions of the globus pallidus of EGF-injected rats, compared with vehicle-injected rats.

The immediate influences of NRG1 injection in neonates were limited to dopaminergic and glutamatergic systems [24, 38]. Increases in dopamine synthesis, metabolism, and terminal arborization were found [38] as well as those in AMPA-type glutamate receptor levels [24] (Table 1). Although the acute effects on dopamine markers were similar to those seen in the EGF-injection model, those on AMPA receptors (GluA1) were opposite to that seen in EGF-injection models [17]. Again, the synaptic increase in AMPA receptor expression and function gradually diminished following the cessation of NRG1 injection in the postnatal stage [24].

As mentioned above, the NRG1-injection model also transiently exhibited gross abnormalities in the dopamine system. For instance, during postnatal NRG1-treatment, dopamine synthesis, metabolism, and axon terminal arborization were elevated in various brain regions including the basal ganglia and corticolimbic system [38]. The dopaminergic abnormalities in most of the brain regions were transient but those in the corticolimbic system continued until the postpubertal stages when the behavioral deficits emerged (Table 1). Therefore, we conclude that EGF-injection and NRG1-injection models share a persistent hyperdopaminergic abnormality; however, the target regions appear to differ between these models. The differential distributions of their receptors (ErbB1 and ErbB4) in the midbrain may illustrate the distinct influences of EGF and NRG1 on the dopamine system [15, 59]. Our quantitative study of *in situ* hybridization suggests that the EGF receptor (ErbB1) has a limited distribution in the nigrostriatal system and is less enriched in the ventral tegmental area (VTA)-corticolimbic system [20].

When we compared the behavioral and neurochemical phenotypes between the injection and TG models, we found significant differences (Tables 1 and 2): the magnitude of prepulse inhibition deficit was less pronounced or modest in NRG1-TG mice than in NRG1-injected mice. Moreover, context-dependent fear learning and exploratory motor activity deficits were prominent only in NRG1-TG mice. In this context, we suggest that spatial and temporal differences of NRG1 supply might alter target cell populations and their responses as various endophenotypes were seen among distinct NRG1-TG lines [45, 60–62].

## 5. Neurobehavioral Implication of a Pallidal Hyperdopaminergic State in the EGF-Injection Model

We further analyzed the EGF-injection model, focusing on the neuropathological abnormality of the dopaminergic system that continued over the postpubertal stage (Figure 5). The globus pallidus is implicated in the motor and cognitive regulation of the indirect pathway and is one of the major targets of the antipsychotics exhibiting D2 antagonism [63–65]. We found that more dense collaterals of nigrostriatal dopamine fibers innervate the globus pallidus in EGF-treated rats. Thus, we attempted to correlate any of the behavioral deficits of EGF-treated rats with pallidal dopamine dysfunction [58, 66].

In the globus pallidus of the EGF-injection model, there were persistent increases in tyrosine hydroxylase levels and dopamine content in the globus pallidus. Furthermore, pallidal dopamine release was also elevated in EGF-injected rats; however, the increased dopamine release was normalized by subchronic treatment with the antipsychotic drug risperidone (Figure 6). The amounts of pallidal dopamine release in individual animals were correlated with the magnitude of their prepulse inhibition levels. Single-unit recordings

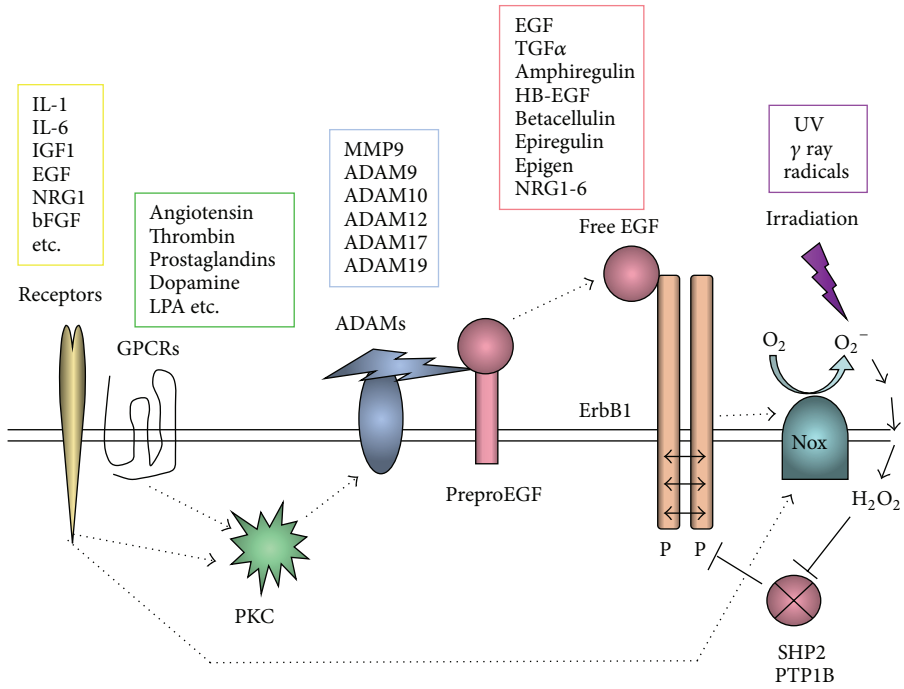



Figure 7: Ectodomain shedding of EGF-like precursors and transactivation of ErbB receptors. Cytokines (yellow box) and GPCR ligands (green box) activate metalloproteases in a disintegrin and metalloprotease (ADAM) family (blue box) via the activation of protein kinase C (PKC). These metalloproteases cleave the precursor proteins for EGF-like factors in the cell membrane and liberates the core EGF domain (red box). The soluble EGF-like factor diffuses into blood stream and acts on ErbB receptors. Alternatively, cytokine-triggered NADPH oxidase (NOX) activation results in the production of reactive oxygen species (ROS) and hydrogen peroxide, which inhibits the protein phosphatases (i.e., SHP2 or PTP1B) for ErbB kinases. UV or gamma ray irradiation (purple box) directly produces ROS and hydrogen peroxide. The attenuation of the phosphatases SHP2 (Src homology 2-containing protein tyrosine phosphatase) or PTP1B (protein tyrosine phosphatase 1B) markedly elevates basal phosphorylation (P) levels of ErbB receptors.

verified that the pallidal hyperdopaminergic state resulted in pallidal dysfunction with hyperactivation [66]. Corroborating these observations, the administration of dopamine D2-like receptor antagonists indeed ameliorated prepulse inhibition levels of EGF-treated rats as well as pallidal hyperactivity [58, 66]. Similarly, administration of ErbB inhibitors also normalized these behavioral impairments with their antidopaminergic actions in the globus pallidus [67].

Conversely, dopamine D2-like receptor agonist (quinpirole) administration to the pallidus of control rats induced prepulse inhibition deficits and pallidal frequent firing, confirming the pathophysiologic role of the pallidal hyperdopaminergic state [58, 66]. Impaired eye saccade, one of the common endophenotypes of schizophrenia patients, might reflect such a dopaminergic dysfunction of the indirect pathway in patients [68, 69].

## 6. Interactions of EGF-NRG1 Signals with Other Cytokines and Neurotransmitters

It is noteworthy that EGF-NRG1 signaling is secondarily evoked by other cytokines and neurotransmitters. This concept, namely, "ErbB transactivation," has been well established in cancer biology and cell biology [70–73]. Inflammatory cytokines (IL-1, IL-6, TNF$\alpha$) and their mediators of prostaglandins (PGEs) are potent transactivators of ErbB1 (EGF receptor) and trigger cell proliferation, leading to inflammation, wound healing or cancer priming. This process initiates with the protein kinase C activation, followed by ADAM (a disintegrin and metalloproteinase) activation (Figure 7). The activated ADAMs on cell surface shed (cleave) the membrane-linked precursor proteins of EGF, HB-EGF, and NRG1 (or their homologues) and liberate soluble EGF or NRG1, allowing them to bind to neighboring ErbB receptors or to diffuse into blood stream. Such transactivators for ErbB receptors now include GPCR agonists (angiotensin1, glutamate, dopamine, prostaglandins, thrombin, etc.), physicochemicals (UV light and ROS), growth factors, and cytokines (IGF1, EGF, NRG1, bFGF, IL-1, IL-6, etc.) [70–73]. Thus, dopamine itself is a potent activator for ErbBs and may provide positive feedback signals through the precursor shedding [74–76]. Accordingly, several other cytokines such as IL-6, which are implicated in maternal immune models for schizophrenia, potentially involve EGF-NRG1 signaling as well. IL-1, IL-6, and other inflammatory cytokines in the periphery can secondarily liberate EGF-like factors into the blood stream and may evoke ErbB signaling in various tissues including the brain.

## 7. Conclusion

The production of inflammatory cytokines and neurotrophic factors, such as EGF and NRG1, is regulated dynamically in the central nervous system as well as in the peripheral organs

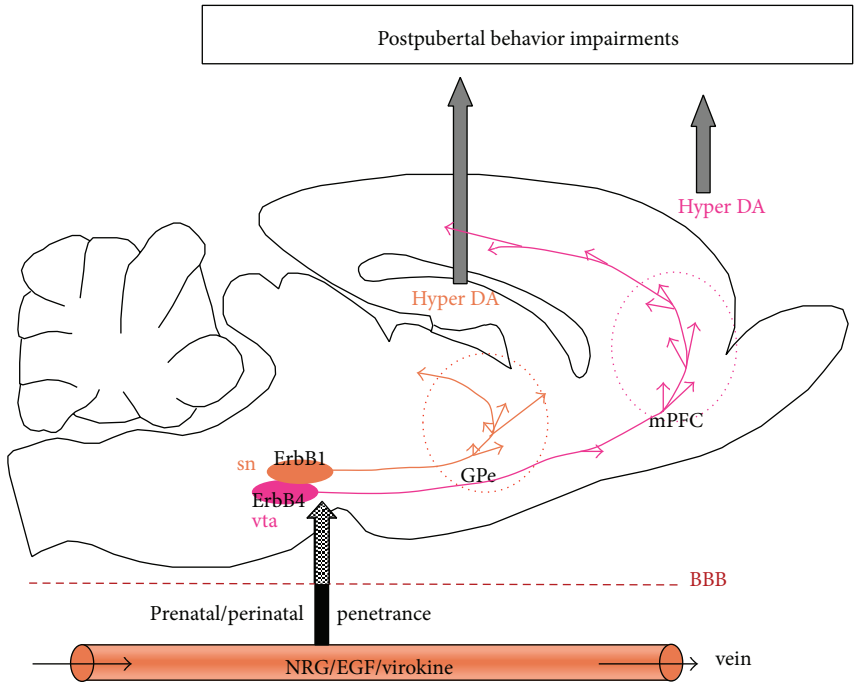



Figure 8: Ligands in the EGF family in circulation can penetrate the blood-brain barrier (BBB) during early development and act on the soma and terminals of dopamine neurons carrying ErbB1 and/or ErbB4. Exogenous EGF and NRG1 in the brain accelerate dopaminergic development and trigger ectopic hyperinnervation. The hyperdopaminergic innervation specifically persists in the globus pallidus and/or prefrontal cortex (mPFC). When the dopaminergic neurons are highly activated during and after adolescence, the excess amount of dopamine is released at the inappropriate sites, producing abnormal behavior and cognition.

[10, 77, 78]. When the blood-brain barrier is not established, or when the blood-brain barrier is disrupted, these factors can efficiently reach brain neurons (Figure 8). In particular, EGF-like ErbB ligands are overproduced in the periphery or provided to blood stream following ischemic injury, inflammation, viral infection, and obstetric complications, which recruit inflammatory cytokines and trigger shedding and release of EGF-like precursors. For instance, peripherally produced EGF can penetrate the blood-brain barrier and act on immature nigral dopamine neurons, perturb their phenotypic development, and circuit connectivity in the basal ganglia, which presumably leads to life-long dysfunction [58, 64]. Alternatively, pox virus infection in fetuses or neonates may result in the production of NRG-like factors, which hamper the target connectivity of VTA dopaminegic neurons in the front-limbic regions [11].

In the postpubertal stage, when dopamine neurons are most highly activated at the basal state, the neurobehavioral consequences from the hyperdopaminergic dysfunction or ectopic innervation of dopaminergic terminals manifest (Figure 8). This cytokine-driven dopaminergic dysfunction might illustrate some of the psychopathological features of schizophrenia, although it is possible that the responsible factor(s) might be other cytokines other than EGF, NRG1, or virokine. In this context, the cytokine hypothesis for schizophrenia might occlude the other hypotheses such as the immunoinflammatory hypothesis, developmental hypothesis, and dopamine hypothesis.

## Conflict of Interests

Recombinant EGF was provided from Higeta-Shoyu Co. Ltd. The authors declare that there is no other conflict of interests regarding the publication of this paper.

## Acknowledgments

The results obtained in the authors' laboratory were partly supported by MEXT KAKENHI (no. 24116010) and JSPS KAKENHI (no. 22300107) as well as by a special grant from the Takeda Science Foundation and the Naito Foundation.

## References

[1] S. Cohen, "The epidermal growth factor (EGF)," *Cancer*, vol. 51, no. 10, pp. 1787–1791, 1983.

[2] S. Higashiyama and D. Nanba, "ADAM-mediated ectodomain shedding of HB-EGF in receptor cross-talk," *Biochimica et Biophysica Acta*, vol. 1751, no. 1, pp. 110–117, 2005.

[3] L. Mei and W.-C. Xiong, "Neuregulin 1 in neural development, synaptic plasticity and schizophrenia," *Nature Reviews Neuroscience*, vol. 9, no. 6, pp. 437–452, 2008.

[4] T. Futamura, K. Toyooka, S. Iritani et al., "Abnormal expression of epidermal growth factor and its receptor in the forebrain and serum of schizophrenic patients," *Molecular Psychiatry*, vol. 7, no. 7, pp. 673–682, 2002.

[5] M. Shibuya, E. Komi, R. Wang et al., "Measurement and comparison of serum neuregulin 1 immunoreactivity in control subjects and patients with schizophrenia: an influence of its genetic polymorphism," *Journal of Neural Transmission*, vol. 117, no. 7, pp. 887–895, 2010.

[6] D. Jeng, Z. Ma, J. W. Barrett, G. McFadden, J. A. Loeb, and K. Essani, "The tanapoxvirus 15L protein is a virus-encoded neuregulin that promotes viral replication in human endothelial cells," *Journal of Virology*, vol. 87, no. 6, pp. 3018–3026, 2013.

[7] M. Kim, H. Yang, S. K. Kim et al., "Biochemical and functional analysis of smallpox growth factor (SPGF) and anti-SPGF monoclonal antibodies," *Journal of Biological Chemistry*, vol. 279, no. 24, pp. 25838–25848, 2004.

[8] A. Y. Galvez-Contreras, A. Quiñones-Hinojosa, and O. Gonzalez-Perez, "The role of EGFR and ErbB family related proteins in the oligodendrocyte specification in germinal niches of the adult mammalian brain," *Frontiers in Cellular Neuroscince*, vol. 7, article 258, 2013.

[9] C. J. Xian and X. F. Zhou, "EGF family of growth factors: essential roles and functional redundancy in the nerve system," *Frontiers in Bioscience*, vol. 9, pp. 85–92, 2004.

[10] H. Nawa and N. Takei, "Recent progress in animal modeling of immune inflammatory processes in schizophrenia: implication of specific cytokines," *Neuroscience Research*, vol. 56, no. 1, pp. 2–13, 2006.

[11] Y. Iwakura and H. Nawa, "ErbB1-4-dependent EGF/neuregulin signals and their cross talk in the central nervous system: pathological implications in schizophrenia and Parkinson's disease," *Frontiers in Cellular Neuroscince*, vol. 7, article 4, 2013.

[12] M. Jo, D. B. Stolz, J. E. Esplen, K. Dorko, G. K. Michalopoulos, and S. C. Strom, "Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells," *Journal of Biological Chemistry*, vol. 275, no. 12, pp. 8806–8811, 2000.

[13] H. I. Kornblum, D. S. Yanni, M. C. Easterday, and K. B. Seroogy, "Expression of the EGF receptor family members ErbB2, ErbB3, and ErbB4 in germinal zones of the developing brain and in neurosphere cultures containing CNS stem cells," *Developmental Neuroscience*, vol. 22, no. 1-2, pp. 16–24, 2000.

[14] I. J. Fox and H. I. Kornblum, "Developmental profile of ErbB receptors in murine central nervous system: implications for functional interactions," *Journal of Neuroscience Research*, vol. 79, no. 5, pp. 584–597, 2005.

[15] Y. Abe, H. Namba, Y. Zheng, and H. Nawa, "In situ hybridization reveals developmental regulation of ErbB1-4 mRNA expression in mouse midbrain: implication of ErbB receptors for dopaminergic neurons," *Neuroscience*, vol. 161, no. 1, pp. 95–110, 2009.

[16] J. Neddens, K. N. Fish, L. Tricoire et al., "Conserved interneuron-specific ErbB4 expression in frontal cortex of rodents, monkeys, and humans: implications for schizophrenia," *Biological Psychiatry*, vol. 70, no. 7, pp. 636–645, 2011.

[17] T. Nagano, H. Namba, Y. Abe, H. Aoki, N. Takei, and H. Nawa, "In vivo administration of epidermal growth factor and its homologue attenuates developmental maturation of functional excitatory synapses in cortical GABAergic neurons," *European Journal of Neuroscience*, vol. 25, no. 2, pp. 380–390, 2007.

[18] C. Birchmeier and K. A. Nave, "Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation," *Glia*, vol. 56, no. 14, pp. 1491–1497, 2008.

[19] L. Zhang, A. Fletcher-Turner, M. A. Marchionni et al., "Neurotrophic and neuroprotective effects of the neuregulin glial growth factor-2 on dopaminergic neurons in rat primary midbrain cultures," *Journal of Neurochemistry*, vol. 91, no. 6, pp. 1358–1368, 2004.

[20] Y. Iwakura, R. Wang, Y. Abe et al., "Dopamine-dependent ectodomain shedding and release of epidermal growth factor in developing striatum: target-derived neurotrophic signaling (Part 2)," *Journal of Neurochemistry*, vol. 118, no. 1, pp. 57–68, 2011.

[21] T. Carlsson, F. R. Schindler, M. Höllerhage et al., "Systemic administration of neuregulin-1$\beta$1 protects dopaminergic neurons in a mouse model of Parkinson's disease," *Journal of Neurochemistry*, vol. 117, no. 6, pp. 1066–1074, 2011.

[22] P. Fazzari, A. V. Paternain, M. Valiente et al., "Control of cortical GABA circuitry development by Nrg1 and ErbB4 signalling," *Nature*, vol. 464, no. 7293, pp. 1376–1380, 2010.

[23] A. K. Ting, Y. Chen, L. Wen et al., "Neuregulin 1 promotes excitatory synapse development and function in GABAergic interneurons," *Journal of Neuroscience*, vol. 31, no. 1, pp. 15–25, 2011.

[24] Y. Abe, H. Namba, T. Kato, Y. Iwakura, and H. Nawa, "Neuregulin-1 signals from the periphery regulate AMPA receptor sensitivity and expression in GABAergic interneurons in developing neocortex," *Journal of Neuroscience*, vol. 31, no. 15, pp. 5699–5709, 2011.

[25] H. Stefansson, E. Sigurdsson, V. Steinthorsdottir et al., "Neuregulin 1 and Susceptibility to Schizophrenia," *The American Journal of Human Genetics*, vol. 71, no. 4, pp. 877–892, 2002.

[26] S. Anttila, A. Illi, O. Kampman, K. M. Mattila, T. Lehtimäki, and E. Leinonen, "Association of EGF polymorphism with schizophrenia in Finnish men," *NeuroReport*, vol. 15, no. 7, pp. 1215–1218, 2004.

[27] K. Y. Lee, Y. M. Ahn, E. J. Joo et al., "Partial evidence of an association between epidermal growth factor A61G polymorphism and age at onset in male schizophrenia," *Neuroscience Research*, vol. 56, no. 4, pp. 356–362, 2006.

[28] K. Hänninen, H. Katila, S. Anttila et al., "Epidermal growth factor a61g polymorphism is associated with the age of onset of schizophrenia in male patients," *Journal of Psychiatric Research*, vol. 41, no. 1-2, pp. 8–14, 2007.

[29] Y. Watanabe, N. Fukui, T. Muratake, N. Kaneko, and T. Someya, "No association of EGF polymorphism with schizophrenia in a Japanese population," *NeuroReport*, vol. 16, no. 4, pp. 403–405, 2005.

[30] M. Tohmi, N. Tsuda, M. Mizuno, N. Takei, P. W. Frankland, and H. Nawa, "Distinct influences of neonatal epidermal growth factor challenge on adult neurobehavioral traits in four mouse strains," *Behavior Genetics*, vol. 35, no. 5, pp. 615–629, 2005.

[31] T. Futamura, A. Kakita, M. Tohmi, H. Sotoyama, H. Takahashi, and H. Nawa, "Neonatal perturbation of neurotrophic signaling results in abnormal sensorimotor gating and social interaction in adults: implication for epidermal growth factor in cognitive development," *Molecular Psychiatry*, vol. 8, pp. 19–29, 2003.

[32] M. Mizuno, R. S. Malta Jr., T. Nagano, and H. Nawa, "Conditioned place preference and locomotor sensitization after repeated administration of cocaine or methamphetamine in rats treated with epidermal growth factor during the neonatal period," *Annals of the New York Academy of Sciences*, vol. 1025, pp. 612–618, 2004.

[33] H. Sotoyama, H. Namba, N. Takei, and H. Nawa, "Neonatal exposure to epidermal growth factor induces dopamine D 2-like receptor supersensitivity in adult sensorimotor gating," *Psychopharmacology*, vol. 191, no. 3, pp. 783–792, 2007.

[34] Y. Watanabe, S. Hashimoto, A. Kakita et al., "Neonatal impact of leukemia inhibitory factor on neurobehavioral development in rats," *Neuroscience Research*, vol. 48, no. 3, pp. 345–353, 2004.

[35] M. Tohmi, N. Tsuda, Y. Watanabe, A. Kakita, and H. Nawa, "Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin," *Neuroscience Research*, vol. 50, no. 1, pp. 67–75, 2004.

[36] M. Tohmi, N. Tsuda, Y. Zheng et al., "The cellular and behavioral consequences of interleukin-1 alpha penetration through the blood-brain barrier of neonatal rats: a critical period for efficacy," *Neuroscience*, vol. 150, no. 1, pp. 234–250, 2007.

[37] N. Tsuda, M. Mizuno, T. Yamanaka, T. Komurasaki, M. Yoshimoto, and H. Nawa, "Common behavioral influences of the ErbB1 ligands transforming growth factor alpha and epiregulin administered to mouse neonates," *Brain and Development*, vol. 30, no. 8, pp. 533–543, 2008.

[38] T. Kato, Y. Abe, H. Sotoyama et al., "Transient exposure of neonatal mice to neuregulin-1 results in hyperdopaminergic states in adulthood: implication in neurodevelopmental hypothesis for schizophrenia," *Molecular Psychiatry*, vol. 16, no. 3, pp. 307–320, 2011.

[39] R. P. Kleshcheva, "The development of components of the blood-brain barrier in the neocortex of the white rat," *Arkhiv Anatomii, Gistologii i Embriologii*, vol. 95, no. 11, pp. 22–26, 1988.

[40] A. J. Kastin, V. Akerstrom, and W. Pan, "Neuregulin-1-$\beta$1 enters brain and spinal cord by receptor-mediated transport," *Journal of Neurochemistry*, vol. 88, no. 4, pp. 965–970, 2004.

[41] T. W. Rösler, C. Depboylu, O. Arias-Carrión et al., "Biodistribution and brain permeability of the extracellular domain of neuregulin-1-$\beta$1," *Neuropharmacology*, vol. 61, no. 8, pp. 1413–1418, 2011.

[42] K. B. Seroogy, S. Numan, C. M. Gall, D. C. Lee, and H. I. Kornblum, "Expression of EGF receptor mRNA in rat nigrostriatal system," *NeuroReport*, vol. 6, no. 1, pp. 105–108, 1994.

[43] M. Mizuno, H. Sotoyama, E. Narita et al., "A cyclooxygenase-2 inhibitor ameliorates behavioral impairments induced by striatal administration of epidermal growth factor," *Journal of Neuroscience*, vol. 27, no. 38, pp. 10116–10127, 2007.

[44] M. Sakai, M. Kashiwahara, A. Kakita, and H. Nawa, "An attempt of non-human primate modeling of schizophrenia with neonatal challenges of epidermal growth factor," *Behavioral Pharmacology in Addiction Research & Therapy*, vol. 5, article 170, 2014.

[45] T. Kato, A. Kasai, M. Mizuno et al., "Phenotypic characterization of transgenic mice overexpressing neuregulin-1," *PLoS ONE*, vol. 5, no. 12, Article ID e14185, 2010.

[46] T. Eda, M. Mizuno, K. Araki et al., "Neurobehavioral deficits of epidermal growth factor-overexpressing transgenic mice: impact on dopamine metabolism," *Neuroscience Letters*, vol. 547, pp. 21–25, 2013.

[47] C. M. O'Tuathaigh, D. Babovic, G. O'Meara, J. J. Clifford, D. T. Croke, and J. L. Waddington, "Susceptibility genes for schizophrenia: characterisation of mutant mouse models at the level of phenotypic behaviour," *Neuroscience and Biobehavioral Reviews*, vol. 31, no. 1, pp. 60–78, 2007.

[48] A. Mouri, T. Nagai, D. Ibi, and K. Yamada, "Animal models of schizophrenia for molecular and pharmacological intervention and potential candidate molecules," *Neurobiology of Disease*, vol. 53, pp. 61–74, 2013.

[49] T. Karl, "Neuregulin 1: a prime candidate for research into gene-environment interactions in schizophrenia? Insights from genetic rodent models," *Frontiers in Behavioral Neuroscience*, vol. 7, article 106, 2013.

[50] A. Oyagi, Y. Oida, K. Kakefuda et al., "Generation and characterization of conditional heparin-binding EGF-like growth factor knockout mice," *PLoS ONE*, vol. 4, no. 10, Article ID e7461, 2009.

[51] C. G. Hahn, H. Y. Wang, D. S. Cho et al., "Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia," *Nature Medicine*, vol. 12, no. 7, pp. 824–828, 2006.

[52] T. Karl, L. Duffy, A. Scimone, R. P. Harvey, and P. R. Schofield, "Altered motor activity, exploration and anxiety in heterozygous neuregulin 1 mutant mice: Implications for understanding schizophrenia," *Genes, Brain and Behavior*, vol. 6, no. 7, pp. 677–687, 2007.

[53] I. del Pino, C. García-Frigola, N. Dehorter et al., "ErbB4 deletion from fast-spiking interneurons causes schizophrenia-like phenotypes," *Neuron*, vol. 79, no. 6, pp. 1152–1168, 2013.

[54] Y. Watanabe, T. Someya, and H. Nawa, "Cytokine hypothesis of schizophrenia pathogenesis: evidence from human studies and animal models," *Psychiatry and Clinical Neurosciences*, vol. 64, no. 3, pp. 217–230, 2010.

[55] H. Namba, T. Nagano, Y. Iwakura et al., "Transforming growth factor alpha attenuates the functional expression of AMPA receptors in cortical GABAergic neurons," *Molecular and Cellular Neuroscience*, vol. 31, no. 4, pp. 628–641, 2006.

[56] H. Namba, Y. Zheng, Y. Abe, and H. Nawa, "Epidermal growth factor administered in the periphery influences excitatory synaptic inputs onto midbrain dopaminergic neurons in postnatal mice," *Neuroscience*, vol. 158, no. 4, pp. 1731–1741, 2009.

[57] D. Yokomaku, H. Jourdi, A. Kakita et al., "ErbB1 receptor ligands attenuate the expression of synaptic scaffolding proteins, GRIP1 and SAP97, in developing neocortex," *Neuroscience*, vol. 136, no. 4, pp. 1037–1047, 2005.

[58] H. Sotoyama, Y. Zheng, Y. Iwakura et al., "Pallidal hyperdopaminergic innervation underlying d2 receptor-dependent behavioral deficits in the schizophrenia animal model established by EGF," *PLoS ONE*, vol. 6, no. 10, Article ID e25831, 2011.

[59] Y. Zheng, A. Watakabe, M. Takada et al., "Expression of ErbB4 in substantia nigra dopamine neurons of monkeys and humans," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 33, no. 4, pp. 701–706, 2009.

[60] I. H. Deakin, W. Nissen, A. J. Law et al., "Transgenic overexpression of the type I isoform of neuregulin 1 affects working memory and hippocampal oscillations but not long-term potentiation," *Cerebral Cortex*, vol. 22, no. 7, pp. 1520–1529, 2012.

[61] D. M. Yin, Y. J. Chen, Y. S. Lu et al., "Reversal of behavioral deficits and synaptic dysfunction in mice overexpressing neuregulin 1," *Neuron*, vol. 78, no. 4, pp. 644–657, 2013.

[62] X. Luo, W. He, X. Hu, and R. Yan, "Reversible overexpression of Bace1-cleaved neuregulin-1 N-terminal fragment induces schizophrenia-like phenotypes in mice," *Biological Psychiatry*, 2013.

[63] M. L. Perreault, A. Hasbi, M. Alijaniaram et al., "The dopamine D1-D2 receptor heteromer localizes in dynorphin/enkephalin neurons: Increased high affinity state following amphetamine-andin schizophrenia," *Journal of Biological Chemistry*, vol. 285, no. 47, pp. 36625–36634, 2010.

[64] C. R. Gerfen and D. J. Surmeier, "Modulation of striatal projection systems by dopamine," *Annual Review of Neuroscience*, vol. 34, pp. 441–466, 2011.

[65] M. Cazorla, F. D. de Carvalho, M. O. Chohan et al., "Dopamine d2 receptors regulate the anatomical and functional balance of Basal Ganglia circuitry," *Neuron*, vol. 81, no. 1, pp. 153–164, 2014.

[66] H. Sotoyama, H. Namba, S. Chiken, A. Nambu, and H. Nawa, "Exposure to the cytokine EGF leads to abnormal hyperactivity of pallidal GABA neurons: implications for schizophrenia and its modeling," *Journal of Neurochemistry*, vol. 126, no. 4, pp. 518–528, 2013.

[67] M. Mizuno, H. Sotoyama, H. Namba et al., "ErbB inhibitors ameliorate behavioral impairments of an animal model for schizophrenia: implication of their dopamine-modulatory actions," *Translational Psychiatry*, vol. 3, article e252, 2013.

[68] D. L. Levy, A. B. Sereno, D. C. Gooding, and G. A. O'Driscoll, "Eye tracking dysfunction in schizophrenia: characterization and pathophysiology," *Current Topics in Behavioral Neurosciences*, vol. 4, pp. 311–347, 2010.

[69] M. Furman and M. Gur, "And yet it moves: perceptual illusions and neural mechanisms of pursuit compensation during smooth pursuit eye movements," *Neuroscience and Biobehavioral Reviews*, vol. 36, no. 1, pp. 143–151, 2012.

[70] S. Higashiyama, D. Nanba, H. Nakayama, H. Inoue, and S. Fukuda, "Ectodomain shedding and remnant peptide signalling of EGFRs and their ligands," *Journal of Biochemistry*, vol. 150, no. 1, pp. 15–22, 2011.

[71] M. R. Schneider and E. Wolf, "The epidermal growth factor receptor ligands at a glance," *Journal of Cellular Physiology*, vol. 218, no. 3, pp. 460–466, 2009.

[72] H. C. Crawford, P. J. Dempsey, G. Brown, L. Adam, and M. L. Moss, "ADAM10 as a therapeutic target for cancer and inflammation," *Current Pharmaceutical Design*, vol. 15, no. 20, pp. 2288–2299, 2009.

[73] L. Peng, B. Li, T. Du et al., "Astrocytic transactivation by $\alpha$2A-adrenergic and 5-HT2B serotonergic signaling," *Neurochemistry International*, vol. 57, no. 4, pp. 421–431, 2010.

[74] Y. Iwakura, Y. Zheng, M. Sibilia et al., "Qualitative and quantitative re-evaluation of epidermal growth factor-ErbB1 action on developing midbrain dopaminergic neurons in vivo and in vitro: target-derived neurotrophic signaling (Part 1)," *Journal of Neurochemistry*, vol. 118, no. 1, pp. 45–56, 2011.

[75] S. Yoon and J. H. Baik, "Dopamine D2 receptor-mediated epidermal growth factor receptor transactivation through a disintegrin and metalloprotease regulates dopaminergic neuron development via extracellular signal-related kinase activation," *The Journal of Biological Chemistry*, vol. 288, no. 40, pp. 28435–28446, 2013.

[76] G. C. O'Keeffe, P. Tyers, D. Aarsland, J. W. Dalley, R. A. Barker, and M. A. Caldwellf, "Dopamine-induced proliferation of adult neural precursor cells in the mammalian subventricular zone is mediated through EGF," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 21, pp. 8754–8759, 2009.

[77] U. Meyer, J. Feldon, and B. K. Yee, "A review of the fetal brain cytokine imbalance hypothesis of schizophrenia," *Schizophrenia Bulletin*, vol. 35, no. 5, pp. 959–972, 2009.

[78] S. Bauer, B. J. Kerr, and P. H. Patterson, "The neuropoietic cytokine family in development, plasticity, disease and injury," *Nature Reviews Neuroscience*, vol. 8, no. 3, pp. 221–232, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 807874, 9 pages
http://dx.doi.org/10.1155/2014/807874

*Research Article*

# ATF4- and CHOP-Dependent Induction of FGF21 through Endoplasmic Reticulum Stress

**Xiao-shan Wan,[1] Xiang-hong Lu,[2] Ye-cheng Xiao,[3] Yuan Lin,[4] Hong Zhu,[5] Ting Ding,[2] Ying Yang,[3] Yan Huang,[3] Yi Zhang,[5] Yan-Long Liu,[3] Zhu-mei Xu,[3] Jian Xiao,[2,3] and Xiao-kun Li[1,3]**

[1] *College of Basic Medical Sciences, Jilin University, Changchun 130021, China*
[2] *Translation Medicine Research Center, Lishui People's Hospital, Wenzhou Medical University, Lishui, Zhejiang 323000, China*
[3] *Molecular Pharmacology Research Center, Key Laboratory of Biotechnology and Pharmaceutical Engineering, School of Pharmaceutical Science, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China*
[4] *Department of Ophthalmology, Xiamen Hospital of Traditional Chinese Medicine, Xiamen 361000, China*
[5] *Department of Endocrinology, The First Affiliated Hospital, Wenzhou Medical University, Wenzhou 323000, China*

Correspondence should be addressed to Jian Xiao; xfxj2000@126.com and Xiao-kun Li; xiaokunli@163.net

Received 16 January 2014; Accepted 22 April 2014; Published 11 May 2014

Academic Editor: Yadong Wang



Fibroblast growth factor 21 (FGF21) is an important endogenous regulator involved in the regulation of glucose and lipid metabolism. FGF21 expression is strongly induced in animal and human subjects with metabolic diseases, but little is known about the molecular mechanism. Endoplasmic reticulum (ER) stress plays an essential role in metabolic homeostasis and is observed in numerous pathological processes, including type 2 diabetes, overweight, nonalcoholic fatty liver disease (NAFLD). In this study, we investigate the correlation between the expression of FGF21 and ER stress. We demonstrated that TG-induced ER stress directly regulated the expression and secretion of FGF21 in a dose- and time-dependent manner. FGF21 is the target gene for activating transcription factor 4 (ATF4) and CCAAT enhancer binding protein homologous protein (CHOP). Suppression of CHOP impaired the transcriptional activation of FGF21 by TG-induced ER stress in CHOP−/− mouse primary hepatocytes (MPH), and overexpression of ATF4 and CHOP resulted in FGF21 promoter activation to initiate the transcriptional programme. In mRNA stability assay, we indicated that ER stress increased the half-life of mRNA of FGF21 significantly. In conclusion, FGF21 expression is regulated by ER stress via ATF- and CHOP-dependent transcriptional mechanism and posttranscriptional mechanism, respectively.

## 1. Introduction

The fibroblast growth factor family contains 22 members with a wide range of biological functions relevant to regulating cell growth, differentiation, wound healing, development, and angiogenesis [1–3]. Fibroblast growth factor 21 (FGF21) is a unique member of the FGF family and has broad metabolic functions, including stimulating glucose uptake insulin-independently and improving hyperglycemia and dyslipidemia [4–7]. FGF21 has a protective effect on the preservation of pancreatic $\beta$-cell function and promotes hepatic and peripheral insulin sensitivity via the prevention of lipolysis, which improves insulin resistance [8–10]. In addition, FGF21 can resist the diet-induced obesity and induce fatty acid oxidation [8, 11, 12]. At present, FGF21 is considered as a novel metabolism regulator and has become a focus of metabolic disease research.

FGF21 is expressed predominantly in liver and, to a lower extent, in white adipose tissue, thymus, skeletal muscle, and pancreatic $\beta$-cells [4, 9, 13]. Substantial clinical research has focused on detecting FGF21 expression levels in various pathological states. It has been reported that serum FGF21 and hepatic mRNA expression levels in patients with NAFLD are significantly higher than levels in control subjects, which correlates with a substantial increase in liver triglyceride levels [14–16]. Plasma FGF21 was also found to elevate

in type 2 diabetic or impaired glucose tolerance patients [17–19]. Circulating FGF21 levels were significantly higher in overweight subjects than those in lean individuals [20, 21]. Animal studies have reported similar results, showing increased FGF21 mRNA levels and serum FGF21 concentrations in the hepatic and adipose tissue of high fat diet-induced and genetically obese mice compared with wild-type mice [6, 8, 22]. An increase in FGF21 mRNA levels is similarly induced by fasting [23–25]. It seems likely that FGF21 levels are unchanged in different physiological states but increased with stress in individuals who are either overweight or have type 2 diabetes, or NAFLD. Based on these findings, we propose that the mechanism of increased FGF21 levels in metabolism disease may be due to feedback regulation, but the mechanism responsible for the effect is still unclear.

Numerous studies indicated that ER stress was closely related to metabolic diseases and it contributed to triggering insulin resistance, obesity, and type 2 diabetes [26–29]. ER is the site of synthesis, folding, and routing of proteins and it plays a prominent role in maintaining $Ca^{2+}$ homeostasis in the cytosol. ER stress is a compensatory process that aims to preserve cellular functions and survival and induce by hypoxia, toxicity, infection, unfold protein accumulation, and perturbation of $Ca^{2+}$ homeostasis [30]. ER stress transducers, including PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1), can be activated [31]. Phosphorylation of eukaryotic initiation factor $\alpha$ (eIF2$\alpha$), via activation by PERK, leads to translational induction of ATF4. BiP-free pATF6(p) is transported to the Golgi apparatus where it is processed to a transcriptionally active nuclear form pATF6(N). Activated IRE1 site-specifically cleaves x-box-binding protein 1 (XBP1) mRNA precursor to create the mature XBP1 mRNA (XBP1-sp). ATF4, pATF6(N), and XBP1-sp then activate transcription of CCAAT enhancer binding protein homologous protein (CHOP) by binding to the appropriate promoter region, and CHOP plays a crucial role in ER stress-mediated apoptosis and in diseases including diabetes, brain ischemia, and neurodegenerative disease [32].

Several studies have shown that upregulation of FGF21 is mediated by ATF4 under conditions causing cellular stress, such as amino acid deprivation, autophagy, and mitochondrial dysfunction [33–36]. ATF4 directly increases FGF21 expression in cells with ER stress by binding to both amino acid-responsive element 1 (AARE1) and amino acid-responsive element 2 (AARE2) sequence on FGF21 [35, 37]. ATF4 activates the CHOP gene downstream, but not much is known on the relationship between CHOP and FGF21. To investigate whether FGF21 is regulated by ER stress via effects on AFT4 and CHOP, we establish an ER stress cell model using TG (thapsigargin) in which we detect FGF21 and ER stress-specific gene expression levels. We then demonstrated that TG-induced ER stress upregulates the expression and secretion of FGF21 by influencing ATF4 and CHOP, providing insights on the mechanisms that link FGF21 and metabolic diseases.

## 2. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin (p-s), newborn calf serum (NCS), and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). High-Capacity cDNA Reverse Transcription Kits were obtained from Applied Biosystems (Foster City, CA, USA). QIAprep spin miniprep kits were obtained from Qiagen. Restriction endonucleases *Hind* III and *Xho* I were purchased from NEB (Ipswich, MA, USA). Vector pGL4.17-Luc, Fugene HD reagents, and Luciferase Assay System were obtained from Promega (Sunnyvale, CA, USA). Mouse FGF21 ELISA Kits were obtained from R&D Systems (Minneapolis, MN, USA). Isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX), Insulin, Thapsigargin (TG), Actinomycin D, and all other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO).

## 3. Methods

*3.1. Cell Culture and Differentiation.* 3T3-L1 murine preadipocytes were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM containing 10% NCS and 1% p-s; cells were induced to differentiate with DMEM plus 10% FBS, 1% p-s, 0.5 mM IBMX, 1 $\mu$M of DEX, and 1.7 $\mu$M Insulin for two days. Then the induction medium was replaced by DMEM with 10% FBS, 1% p-s, and 1.7 $\mu$M Insulin for another two days, followed by 10% FBS/DMEM medium, which was changed every two days. After 5-6 additional days, more than 85% cells differentiated to mature adipocytes, which can be used for the experiments.

*3.2. Isolation and Culture of Mouse Primary Hepatocytes.* Primary hepatocytes were isolated from C57BL/6J wild type (WT) and CHOP knockout (CHOP−/−) mice (male, 8 weeks) and cultured as described previously [38]. Cells were maintained in serum-free William'E medium containing 0.1 $\mu$M Dex, 1% penicillin, and 1 $\mu$M thyroxine. Before treatment, cells were incubated at 37°C, in 5% $CO_2$ for approximately 16 h or until they had attached.

*3.3. RNA Isolation and Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR).* Total RNA was extracted from 3T3-L1 adipocytes using the TRIzol reagent according to the manufacturer's instructions. Total RNA (2 $\mu$g) was used as a template for first-strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit. The mRNA levels of ATF4, splicing of XBP1 (XBP1-sp), CHOP, and FGF21 were quantified using the following primers. ATF4 forward primer 5′-CCT AGG TCT CTT AGA TGA CTA TCT GGA GG-3′, ATF4 reverse primer 5′-CCA GGT CAT CCA TTC GAA ACA GAG CAT CG-3′; XBP1-sp forward primer 5′-TGA GTC CGC AGC AGG TG-3′, XBP1-sp reverse primer 5′-GAC AGG GTC CAA CTT GT-3′; CHOP forward primer 5′-GCT CCT GCC TTT CAC CTT GG-3′, CHOP reverse primer 5′-GGT TTT TGA

TTC TTC CTC TTC-3′; FGF21 forward primer 5′-GCA GTC CAG AAA GTC TCC-3′, FGF21 reverse primer 5′-TGT AAC CGT CCT CCA GCA G-3′; iQ SYBR Green Supermix was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA levels of each target gene were normalized to an endogenous control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH forward primer 5′-GTC GTG GAT CTG ACG TGC C-3′, GAPDH reverse primer 5′-GAT GCC TGC TTC ACC ACC TT-3′. The ratio of normalized mean value for each treatment group to vehicle control group (DMSO) was calculated.

*3.4. Enzyme-Linked Immunosorbent Assay (ELISA) of FGF21.* 3T3-L1 adipocytes were treated with TG (0, 12.5, 25, 50, and 100 nM) for 24 h, or TG (100 nM) for 0, 2, 4, 8, 16, and 24 h. The accumulated FGF21 in the culture medium was determined using ELISA Kit according to the manufacturer's instructions. The total protein concentrations of viable cells were determined using the Bio-Rad Protein Assay reagent. The total amounts of the FGF21 in medium were normalized to the total protein amounts and reported as pg/mg protein.

*3.5. Plasmids Construction and Luciferase Assay.* The mouse FGF21 promoter constructs −1497/+5 were generously provided by Dr. Wenke Feng (The University of Louisville, Louisville, USA) and subcloned into pGL4.17-Luc luciferase report vector using *Hind* III and *Xho* I sites. The expression vector containing the coding sequence of ATF4 or CHOP was preserved in our laboratory. All plasmids were propagated in *Escherichia coli* DH5$\alpha$ and isolated using QIAprep spin miniprep kit (Qiagen). 293T cells were plated in 6-well plates 24 h before transfection. Cells were transfected with 2 $\mu$g of pGL4.17 promoter FGF21 (−1497/+5), 2 $\mu$g of ATF4, or CHOP expression vector using Fugene HD (Promega). 48 h after transfection, the cells were harvested and lysed, and the luciferase activity was measured using the Luciferase Assay System (Promega). The transfection efficiency was normalized to cotransfection of 1 $\mu$g of GFP vector.

*3.6. Assessment of FGF21 mRNA Stability.* 3T3-L1 mature adipocytes were treated with TG (100 nM)/DTT or vehicle control for 4 h; then Actinomycin D (5.0 $\mu$g/mL) was added to the medium (time 0 h). The mRNA of the cells was isolated after added Actinomycin D for 0.5, 1, 2, 4, and 6 h. FGF21, ATF4, XBP1-sp, and CHOP mRNA levels were detected using real-time RT-PCR as described in the previous section; the results are expressed as the fold of the mRNA value at the time of Actinomycin D addition.

*3.7. Statistical Analysis.* All of the experiments were repeated at least three times; results were stated as the mean ± standard error. One-way ANOVA was employed to analyze the differences between sets of data. Statistics were performed using GraphPad Pro. A value of $P < 0.05$ was considered significant.

## 4. Results

*4.1. ER Stress Increases FGF21 Expression.* To investigate the effect of ER stress on FGF21 mRNA levels, we treated 3T3-L1 adipocytes with TG, a potent ER stress activator, by disturbing ER calcium homeostasis. The mRNA levels of ER stress-specific genes (ATF4, XBP1-sp, and CHOP) and FGF21 were detected using real-time RT-PCR. We observed that TG increased FGF21mRNA expression in a time-dependent manner (Figure 1(a)). However, the expression levels at 24 h were lower than those at 16 h, perhaps due to cell toxicity. As shown in Figure 1(b), after the 3T3-L1 adipocytes were incubated with 12.5, 25, and 100 nM TG for 16 h, the levels of FGF21 mRNA were significantly increased in a concentration-dependent manner compared with the vehicle control group.

*4.2. ER Stress Induces FGF21 Secretion.* Based on the above findings, a model of TG-induced stress in 3T3-L1 adipocytes was established, and we used this model to examine whether ER stress increases FGF21 secretion. Differentiated 3T3-L1 cells were treated with TG; the FGF21 protein levels in the medium were measured using ELISA. As shown in Figures 2(a) and 2(b), TG-induced ER stress led to increase in secreted FGF21 in a time- and dose-dependent manner. TG induced FGF21 protein level to a 40-fold rise at concentration of 100 nM for 24 h.

*4.3. Knockout of CHOP Decreases FGF21 Expression.* CHOP is a major transcription factor involved in ER stress. To determine whether CHOP expression contributes to ER stress-induced upregulation of FGF21, we isolated MPH from WT and CHOP−/− mice and treated the cells with TG for 24 h. In WT MPH, TG promoted the mRNA levels of CHOP and FGF21. However, in CHOP−/− MPH, TG failed to induce FGF21 expression, because ATF4 is upstream gene of CHOP and there is no effect of CHOP knockout on the activation of ATF4. Moreover, much research indicated that ATF4 can induce FGF21 expression under stress [33–37]. As depicted in Figure 3, an absence of CHOP expression significantly increased FGF21 gene expression by 30%. These results indicate that CHOP may be a key player in the mechanism by which TG-induces increased FGF21 expression in MPH.

*4.4. ATF4 and CHOP Increase FGF21 Promoter-Driven Transcription.* To address the mechanism of TG-induced stress regulating FGF21 expression, we subcloned the FGF21 promoter (−1497/+5) into the pGL4.17-Luc luciferase report vector and measured the ability of ATF4 or CHOP to regulate the activation of the FGF21 promoter using a cotransfection assay. A previous study has reported that FGF21 expression could be mimicked by overexpression of ATF4 [37]. Unlike 3T3-L1 cell line, 293T cells can be transfected with high efficiency. 293T cells were cotransfected with pGL4.17-Luc luciferase report vector, which was inserted the mouse FGF21 promoter and expression vector for ATF4 or CHOP. Luciferase activity was determined at 48 h after transfection. Figure 4(a) demonstrates that, compared with the control group, ATF4

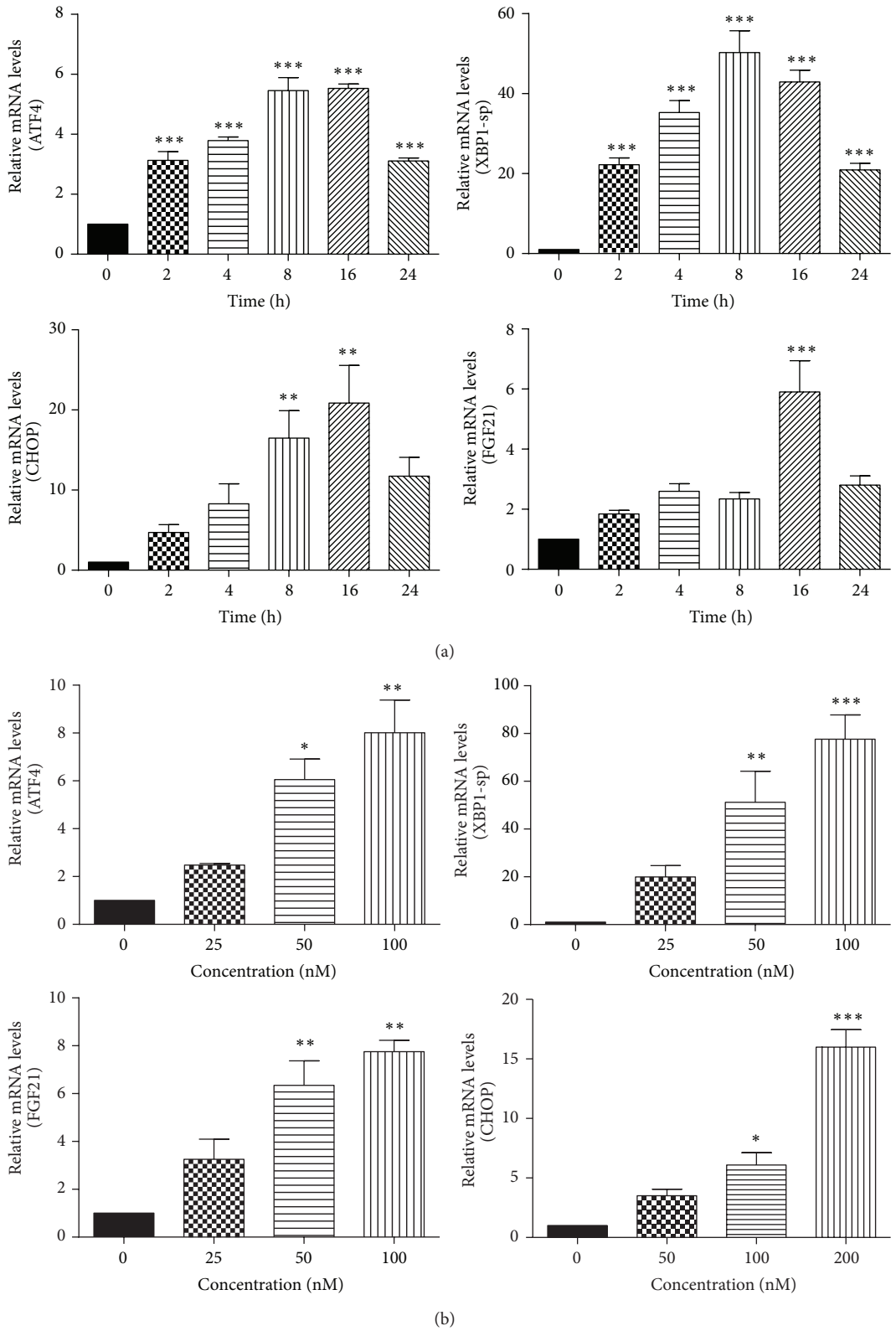



Figure 1: ER stress increases FGF21 mRNA levels. (a) 3T3-L1 adipocytes were treated with TG (100 nM) for 0, 2, 4, 8, 16, and 24 h; (b) 3T3-L1 adipocytes were treated with TG (25, 50, and 100 nM) for 16 h. Total cellular RNA was isolated. The mRNA levels of ATF4, XBP1-sp, CHOP, and FGF21 were measured by real-time RT-PCR. Values are mean ± S.E. of three independent experiments. Statistical significance relative to vehicle control: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

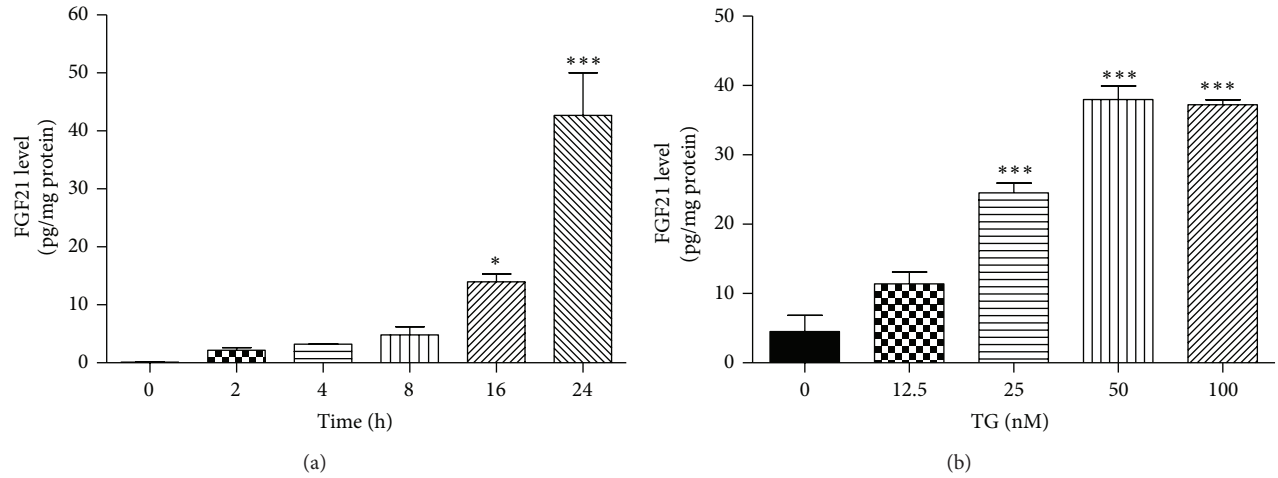



Figure 2: ER stress induces FGF21 secretion. Differentiated 3T3-L1 cells were treated with 100 nM TG for 0, 2, 4, 8, 16, and 24 h (a); or different concentrations of TG for 24 h (b), at the end of treatment, cell culture medium was collected. The protein level of FGF21 was determined by ELISA. Values are mean ± S.E. of three independent experiments. Statistical significance relative to vehicle control: $^{*}P < 0.05$; $^{***}P < 0.001$.

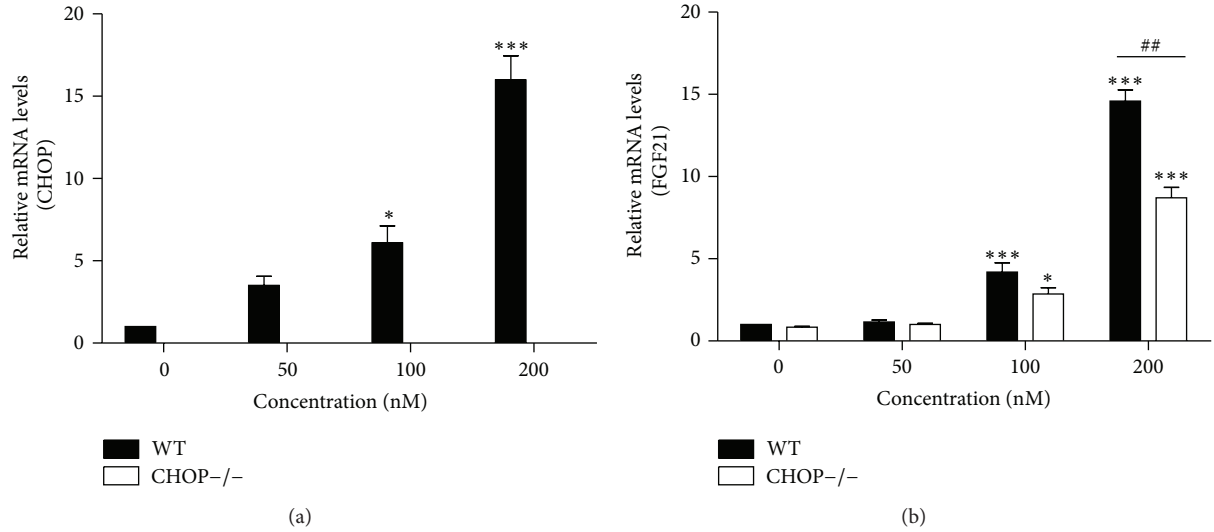



Figure 3: Knockout of CHOP decreases FGF21 expression. WT and CHOP−/− mouse primary hepatocytes were treated for 24 h with increasing concentration of TG. Total cellular RNA was isolated and the mRNA levels of CHOP and FGF21 were measured by real-time RT-PCR. Values are mean ± S.E. of three independent experiments. Statistical significance relative to WT vehicle control: $^{*}P < 0.05$; $^{***}P < 0.001$; statistical significance relative of the same TG concentration between WT group and CHOP−/− group: $^{\#\#}P < 0.01$.

overexpression enhanced FGF21 promoter activity more than 3-fold. This is consistent with the result of a previous study [37] that reported that there are two conserved ATF4-binding sites in the promoter region of the FGF21 gene and that FGF21 expression can be mimicked by overexpression of ATF4. CHOP is one of the genes that is downstream of ATF4. We hypothesized that CHOP would induce FGF21 expression similar to ATF4. We transfected HEK293 cells with the FGF21 reporter construct and CHOP. As shown in Figure 4(b), similar to ATF4, CHOP overexpression significantly increased the transcription of an FGF21 promoter-driven reporter. These findings indicate that ATF4 and CHOP upregulate FGF21 expression by activating the promoter in an environment of TG-induced ER stress.

*4.5. ER Stress Increases FGF21 mRNA Stability.* Posttranscriptional regulation is a major mechanism for the expression of cytokines. To determine whether TG- or DTT-(dithiothreitol-) induced ER stress increases FGF21 expression by regulating mRNA stability, we examined the effects of TG/DTT on the mRNA stability of FGF21 in 3T3-L1 adipocytes. The results indicated that TG and DTT increased the half-life of mRNA of FGF21 significantly but had no effect

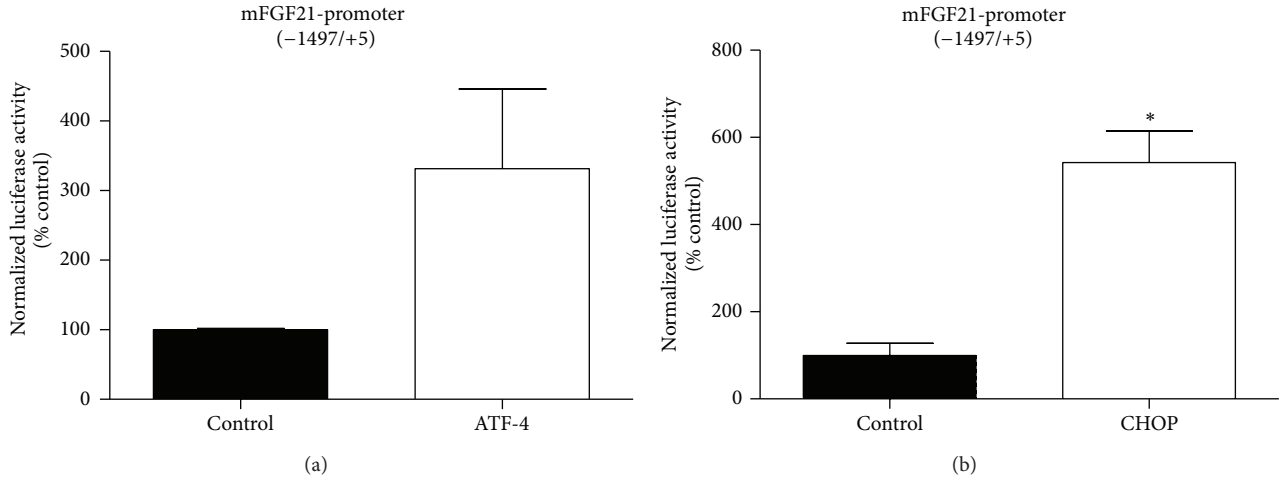



Figure 4: ATF4 and CHOP increase FGF21 promoter-driven transcription. 293T cells were transfected with FGF21 promoter reporter construct along with the expression plasmid ATF4 or CHOP. Values are mean ± S.E. of three independent experiments. Statistical significance relative to control vector: $^{*}P < 0.05$.

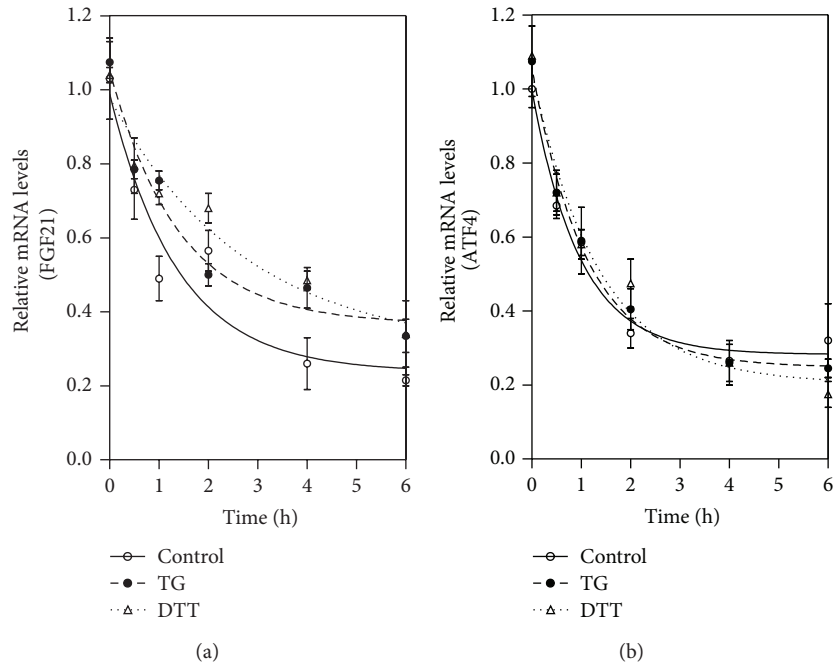



Figure 5: ER stress increases FGF21 mRNA stability. 3T3-L1 adipocytes were pretreated with 100 nM TG/DTT or vehicle control (DMSO) for 4 h and then treated with 5.0 $\mu$g/mL actinomycin D (time 0). Total cellular RNA was extracted at 0, 0.5, 1, 2, 4, and 6 h after actinomycin D addition. FGF21 mRNA levels were determined by real-time RT-PCR. Values are mean ± S.E. of three independent experiments.

on ER stress-specific genes (Figure 5). This result suggested TG- and DTT-induced ER stress activate FGF21 expression by increasing mRNA stability specifically.

## 5. Discussion

FGF21 acts as a hormone-like cytokine on multiple tissues to coordinate carbohydrate and lipid metabolism [4]. Clinical research has shown that serum FGF21 levels are higher in subjects who are overweight, have NAFLD, or are type 2 diabetic [14–18, 20]. Similarly, circulating FGF21 concentrations in *db/db* mice were much higher than normal, as were the FGF21 mRNA levels in both the liver and white adipose tissue [6, 8, 22]. Previous studies have reported that FGF21 expression is mediated by several transcriptional activators and their DNA response elements. Gene expression of FGF21 is induced directly by PPAR$\alpha$ in response to starvation and ketotic states and PPAR$\alpha$ agonists in liver [23, 25] as well as in cultured adipocytes and adipose tissue by PPAR$\gamma$ [39–41].

Activation of the farnesoid X receptor (FXR) increased FGF21 gene expression and secretion was mediated by FXR/retinoid X receptor binding site in 5′-flanking region of the FGF21 gene [42]. A study demonstrated that glucose activation of carbohydrate response element binding protein (ChREBP) is involved in the upregulation of FGF21 mRNA expression in liver [43]. Retinoic acid receptor-related receptor $\alpha$ (ROR$\alpha$) also induces expression and secretion of FGF21, and there is a canonical ROR response element in the proximal promoter of FGF21 gene that exhibits functional activity [44]. PGC-1$\alpha$-mediated reduction of FGF21 expression is dependent on the expression of its ligand, ALAS-1, and Rev-Erb$\alpha$ [45].

In addition, studies by Schaap et al. suggest that FGF21 expression is regulated by ER stress [37]. The authors reported that FGF21 mRNA is increased by TG-induced ER stress in rat H4IIE cells and rat primary hepatocytes. Moreover, intraperitoneal injection of the ER stressor tunicamycin induced hepatic FGF21 expression in mice and resulted in marked elevation of serum FGF21 levels [37]. Consistent with these new findings, we observed that TG-induced ER stress elevated FGF21 expression and secretion in murine 3T3-L1 adipocytes along with increasing ATF4 expression.

PERK (PKR-like ER kinase) is one of the major ER stress pathways. PERK can induce CHOP via activating ATF4. However, there was no information regarding the regulation of FGF21 by CHOP. We show for the first time that CHOP can increase FGF21 expression by activating transcription via promoter elements and enhancing mRNA stability in ER stress. We analyzed mouse FGF21 (−1497/+5) promoter and confirmed the absence of the conserved CHOP binding site 5′-(A/G) (A/G) TGCAAT (A/C) CCC-3′. Thus, FGF21 was not directly responsive to CHOP directly. To the contrary, our data demonstrates that CHOP can induce the transcription of a FGF21 promoter-driven reporter (Figure 4(b)). CHOP may also regulate the expression of FGF21 indirectly by activating other cytokines and intracellular stress signaling pathways, though this remains to be determined conclusively.

Gene expression can be regulated by posttranscriptional control of mRNA stability [46]. The presence of AU-rich elements (AREs) in the 3′-untranslated region (3′-UTR) is essential for stabilization or degradation of mRNA of inflammatory factor [47]. The RNA-binding proteins (RBPs), such as HuR, AUF1, and CUG-BP1, positively regulate stability of many target mRNA via binding AREs present in the 3′-UTR [48, 49]. In this study, we identified for the first time that increased FGF21 mRNA stability, through the binding of RBPs to its target mRNAs, is responsible for elevated FGF21 levels by TG- or DTT-induced ER stress in differentiated 3T3-L1 cells.

In conclusion, these findings suggest that FGF21 is the target gene for ATF4 and CHOP, and transcription and mRNA stabilization are responsible for ATF4 and CHOP mediated induction of FGF21 expression in ER stress. Thus, we indicate ER stress is the key mechanism for regulating FGF21 in several metabolic diseases. Moreover, our studies provide important information about the FGF21 signaling pathway and the clinical significance of FGF21 in the development of metabolic diseases. Compared with WT MPH, FGF21 mRNA levels are reduced in CHOP−/− MPH treated with TG; however, the effects of CHOP overexpression on FGF21 levels are not understood. And it remains to be detected that the synergistic effect of ATF4 and CHOP on FGF21 expression. Moreover, further prospective studies are needed to determine the specific RBPs and their binding sites in FGF21 3′-UTR as well as the signaling pathway of CHOP-dependent activation of FGF21 in ER stress.

## Conflict of Interests

The authors confirm that the content of this paper has no conflict of interests.

## Acknowledgments

This work was supported by National Natural Science Funding of China (81170813), Zhejiang Natural Science Foundation (Y2100048, Y2110328, and Y12H030018), Zhejiang Provincial Project of Key Group (2010R50042), and Fujian Provincial for Medical Innovative Health Project (2011-CXB-44).

## References

[1] A. Beenken and M. Mohammadi, "The FGF family: biology, pathophysiology and therapy," *Nature Reviews Drug Discovery*, vol. 8, no. 3, pp. 235–253, 2009.

[2] S. K. Olsen, M. Garbi, N. Zampieri et al., "Fibroblast growth factor (FGF) homologous factors share structural but not functional homology with FGFs," *The Journal of Biological Chemistry*, vol. 278, no. 36, pp. 34226–34236, 2003.

[3] P. M. Smallwood, I. Munoz-Sanjuan, P. Tong et al., "Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 18, pp. 9850–9857, 1996.

[4] A. Kharitonenkov, T. L. Shiyanova, A. Koester et al., "FGF-21 as a novel metabolic regulator," *Journal of Clinical Investigation*, vol. 115, no. 6, pp. 1627–1635, 2005.

[5] A. Kharitonenkov, V. J. Wroblewski, A. Koester et al., "The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21," *Endocrinology*, vol. 148, no. 2, pp. 774–781, 2007.

[6] T. Coskun, H. A. Bina, M. A. Schneider et al., "Fibroblast growth factor 21 corrects obesity in mice," *Endocrinology*, vol. 149, no. 12, pp. 6018–6027, 2008.

[7] J. A. Seo and N. H. Kim, "Fibroblast growth factor 21: a novel metabolic regulator," *Diabetes & Metabolism Journal*, vol. 36, no. 1, pp. 26–28, 2012.

[8] J. Xu, D. J. Lloyd, C. Hale et al., "Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice," *Diabetes*, vol. 58, no. 1, pp. 250–259, 2009.

[9] W. Wente, A. M. Efanov, M. Brenner et al., "Fibroblast growth factor-21 improves pancreatic $\beta$-cell function and survival by activation of extracellular signal-regulated kinase 1/2 and Akt signaling pathways," *Diabetes*, vol. 55, no. 9, pp. 2470–2478, 2006.

[10] P. Arner, A. Pettersson, P. J. Mitchell, J. D. Dunbar, A. Kharitonenkov, and M. Rydén, "FGF21 attenuates lipolysis in human

adipocytes—a possible link to improved insulin sensitivity," *FEBS Letters*, vol. 582, no. 12, pp. 1725–1730, 2008.

[11] K. Mai, J. Andres, K. Biedasek et al., "Free fatty acids link metabolism and regulation of the insulin-sensitizing fibroblast growth factor-21," *Diabetes*, vol. 58, no. 7, pp. 1532–1538, 2009.

[12] Y. Hotta, H. Nakamura, M. Konishi et al., "Fibroblast growth factor 21 regulates lipolysis in white adipose tissue but is not required for ketogenesis and triglyceride clearance in liver," *Endocrinology*, vol. 150, no. 10, pp. 4625–4633, 2009.

[13] F. L. Mashili, R. L. Austin, A. S. Deshmukh et al., "Direct effects of FGF21 on glucose uptake in human skeletal muscle: implications for type 2 diabetes and obesity," *Diabetes/Metabolism Research and Reviews*, vol. 27, no. 3, pp. 286–297, 2011.

[14] H. Li, Q. Fang, F. Gao et al., "Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride," *Journal of Hepatology*, vol. 53, no. 5, pp. 934–940, 2010.

[15] J. Dushay, P. C. Chui, G. S. Gopalakrishnan et al., "Increased fibroblast growth factor 21 in obesity and nonalcoholic fatty liver disease," *Gastroenterology*, vol. 139, no. 2, pp. 456–463, 2010.

[16] Y. Yilmaz, F. Eren, O. Yonal et al., "Increased serum FGF21 levels in patients with nonalcoholic fatty liver disease," *European Journal of Clinical Investigation*, vol. 40, no. 10, pp. 887–892, 2010.

[17] W.-W. Chen, L. Li, G.-Y. Yang et al., "Circulating FGF-21 levels in normal subjects and in newly diagnose patients with type 2 diabetes mellitus," *Experimental and Clinical Endocrinology and Diabetes*, vol. 116, no. 1, pp. 65–68, 2008.

[18] L. Li, G. Yang, H. Ning, M. Yang, H. Liu, and W. Chen, "Plasma FGF-21 levels in type 2 diabetic patients with ketosis," *Diabetes Research and Clinical Practice*, vol. 82, no. 2, pp. 209–213, 2008.

[19] A. O. Chavez, M. Molina-Carrion, M. A. Abdul-Ghani, F. Folli, R. A. DeFronzo, and D. Tripathy, "Circulating fibroblast growth factor-21 is elevated in impaired glucose tolerance and type 2 diabetes and correlates with muscle and hepatic insulin resistance," *Diabetes Care*, vol. 32, no. 8, pp. 1542–1546, 2009.

[20] X. Zhang, D. C. Y. Yeung, M. Karpisek et al., "Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans," *Diabetes*, vol. 57, no. 5, pp. 1246–1253, 2008.

[21] M. Mraz, M. Bartlova, Z. Lacinova et al., "Serum concentrations and tissue expression of a novel endocrine regulator fibroblast growth factor-21 in patients with type 2 diabetes and obesity," *Clinical Endocrinology*, vol. 71, no. 3, pp. 369–375, 2009.

[22] F. M. Fisher, J. L. Estall, A. C. Adams et al., "Integrated regulation of hepatic metabolism by fibroblast growth factor 21 (FGF21) in vivo," *Endocrinology*, vol. 152, no. 8, pp. 2996–3004, 2011.

[23] M. K. Badman, P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier, and E. Maratos-Flier, "Hepatic fibroblast growth factor 21 is regulated by PPAR$\alpha$ and is a key mediator of hepatic lipid metabolism in ketotic states," *Cell Metabolism*, vol. 5, no. 6, pp. 426–437, 2007.

[24] T. Inagaki, P. Dutchak, G. Zhao et al., "Endocrine regulation of the fasting response by PPAR$\alpha$-mediated induction of fibroblast growth factor 21," *Cell Metabolism*, vol. 5, no. 6, pp. 415–425, 2007.

[25] T. Lundåsen, M. C. Hunt, L.-M. Nilsson et al., "PPAR$\alpha$ is a key regulator of hepatic FGF21," *Biochemical and Biophysical Research Communications*, vol. 360, no. 2, pp. 437–440, 2007.

[26] U. Özcan, Q. Cao, E. Yilmaz et al., "Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes," *Science*, vol. 306, no. 5695, pp. 457–461, 2004.

[27] G. S. Hotamisligil, "Endoplasmic reticulum stress and the inflammatory basis of metabolic disease," *Cell*, vol. 140, no. 6, pp. 900–917, 2010.

[28] L. Ozcan and I. Tabas, "Role of endoplasmic reticulum stress in metabolic disease and other disorders," *Annual Review of Medicine*, vol. 63, pp. 317–328, 2012.

[29] S. S. Cao and R. J. Kaufman, "Targeting endoplasmic reticulum stress in metabolic disease," *Expert Opinion on Therapeutic Targets*, vol. 17, no. 4, pp. 437–448, 2013.

[30] R. J. Kaufman, "Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls," *Genes and Development*, vol. 13, no. 10, pp. 1211–1233, 1999.

[31] S. Oyadomari and M. Mori, "Roles of CHOP/GADD153 in endoplasmic reticulum stress," *Cell Death and Differentiation*, vol. 11, no. 4, pp. 381–389, 2004.

[32] H. Zinszner, M. Kuroda, X. Wang et al., "CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum," *Genes and Development*, vol. 12, no. 7, pp. 982–995, 1998.

[33] A. L. De Sousa-Coelho, P. F. Marrero, and D. Haro, "Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation," *Biochemical Journal*, vol. 443, no. 1, pp. 165–171, 2012.

[34] K. H. Kim, Y. T. Jeong, H. Oh et al., "Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine," *Nature Medicine*, vol. 19, no. 1, pp. 83–92, 2013.

[35] X. Jiang, C. Zhang, Y. Xin et al., "Protective effect of FGF21 on type 1 diabetes-induced testicular apoptotic cell death probably via both mitochondrial- and endoplasmic reticulum stress-dependent pathways in the mouse model," *Toxicology Letters*, vol. 219, no. 1, pp. 65–76, 2013.

[36] Y. Luo and W. L. McKeehan, "Stressed liver and muscle call on adipocytes with FGF21," *Frontiers in Endocrinology*, vol. 4, p. 194, 2013.

[37] F. G. Schaap, A. E. Kremer, W. H. Lamers et al., "Fibroblast growth factor 21 is induced by endoplasmic reticulum stress," *Biochimie*, vol. 95, no. 4, pp. 692–699, 2013.

[38] H. Zhou, E. C. Gurley, S. Jarujaron et al., "HIV protease inhibitors activate the unfolded protein response and disrupt lipid metabolism in primary hepatocytes," *American Journal of Physiology*, vol. 291, no. 6, pp. G1071–G1080, 2006.

[39] E. S. Muise, B. Azzolina, D. W. Kuo et al., "Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor $\gamma$ and altered metabolic states," *Molecular Pharmacology*, vol. 74, no. 2, pp. 403–412, 2008.

[40] J. S. Moyers, T. L. Shiyanova, F. Mehrbod et al., "Molecular determinants of FGF-21 activity—synergy and cross-talk with PPAR$\gamma$ signaling," *Journal of Cellular Physiology*, vol. 210, no. 1, pp. 1–6, 2007.

[41] H. Wang, L. Qiang, and S. R. Farmer, "Identification of a domain within peroxisome proliferator-activated receptor $\gamma$ regulating expression of a group of genes containing fibroblast growth factor 21 that are selectively repressed by SIRT1 in adipocytes," *Molecular and Cellular Biology*, vol. 28, no. 1, pp. 188–200, 2008.

[42] H. A. Cyphert, X. Ge, A. B. Kohan et al., "Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21," *The Journal of Biological Chemistry*, vol. 287, no. 30, pp. 25123–25138, 2012.

[43] K. Iizuka, J. Takeda, and Y. Horikawa, "Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes," *FEBS Letters*, vol. 583, no. 17, pp. 2882–2886, 2009.

[44] Y. Wang, L. A. Solt, and T. P. Burris, "Regulation of FGF21 expression and secretion by retinoic acid receptor-related orphan receptor $\alpha$," *The Journal of Biological Chemistry*, vol. 285, no. 21, pp. 15668–15673, 2010.

[45] J. L. Estall, J. L. Ruas, C. S. Choi et al., "PGC-1$\alpha$ negatively regulates hepatic FGF21 expression by modulating the heme/Rev-Erb$\alpha$ axis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 52, pp. 22510–22515, 2009.

[46] B. Schwanhüusser, D. Busse, N. Li et al., "Global quantification of mammalian gene expression control," *Nature*, vol. 473, no. 7347, pp. 337–342, 2011.

[47] C. M. Brennan and J. A. Steitz, "HuR and mRNA stability," *Cellular and Molecular Life Sciences*, vol. 58, no. 2, pp. 266–277, 2001.

[48] I. Raineri, D. Wegmueller, B. Gross, U. Certa, and C. Moroni, "Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference," *Nucleic Acids Research*, vol. 32, no. 4, pp. 1279–1288, 2004.

[49] J.-Y. Lu and R. J. Schneider, "Tissue dstribution of AU-rich mRNA-binding proteins involved in regulation of mRNA decay," *The Journal of Biological Chemistry*, vol. 279, no. 13, pp. 12974–12979, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 261672, 13 pages
http://dx.doi.org/10.1155/2014/261672

*Review Article*

# Association between Risk Factors for Vascular Dementia and Adiponectin

**Juhyun Song,[1] Won Taek Lee,[1] Kyung Ah Park,[1] and Jong Eun Lee[1,2]**

[1] *Department of Anatomy, Yonsei University College of Medicine, 50 Yonsei-ro, Seoul 120-752, Republic of Korea*
[2] *BK21 Plus Project for Medical Sciences and Brain Research Institute, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Republic of Korea*

Correspondence should be addressed to Jong Eun Lee; jelee@yuhs.ac

Received 24 January 2014; Revised 27 March 2014; Accepted 30 March 2014; Published 17 April 2014

Academic Editor: Xiaokun Li



Vascular dementia is caused by various factors, including increased age, diabetes, hypertension, atherosclerosis, and stroke. Adiponectin is an adipokine secreted by adipose tissue. Adiponectin is widely known as a regulating factor related to cardiovascular disease and diabetes. Adiponectin plasma levels decrease with age. Decreased adiponectin increases the risk of cardiovascular disease and diabetes. Adiponectin improves hypertension and atherosclerosis by acting as a vasodilator and antiatherogenic factor. Moreover, adiponectin is involved in cognitive dysfunction via modulation of insulin signal transduction in the brain. Case-control studies demonstrate the association between low adiponectin and increased risk of stroke, hypertension, and diabetes. This review summarizes the recent findings on the association between risk factors for vascular dementia and adiponectin. To emphasize this relationship, we will discuss the importance of research regarding the role of adiponectin in vascular dementia.

## 1. Introduction

Vascular dementia is the second most common type of dementia, accounting for 15 to 20% of all cases of dementia [1]. It is characterized by cognitive impairment and cerebrovascular pathologies [2]. According to the World Alzheimer Report 2011, an estimated 36 million people worldwide were afflicted with dementia. This number is increasing twofold every 20 years and will likely reach 115 million people by 2050 [3, 4]. Among the subtypes of dementia, vascular dementia is important because it results from a variety of causes, including cerebrovascular dysfunction. Vascular dementia and cerebrovascular diseases have common risk factors including hypertension, insulin resistance, diabetes, obesity, hyperhomocystinemia, and hyperlipidemia [5–8]. Recent clinical-pathological studies have focused on cognitive impairment and increased risk of dementia in patients with cerebrovascular disease [2, 9, 10]. In addition, vascular dementia is the most severe form of vascular cognitive impairment (VCI) [2, 11], and it results from subclinical vascular brain injury and stroke. VCI reflects the full range of cognitive alterations due to vascular factors [12]. A previous study demonstrates that reducing vascular risk factors inhibits cognitive decline progression [12]. Type 2 diabetes mellitus (T2DM), a risk factor for vascular dementia, is a heterogeneous metabolic disease characterized by reduced insulin sensitivity and relative insulin deficiency. T2DM and dyslipidemia frequently coexist with vascular dementia [13]. Adiponectin is almost exclusively secreted by adipocytes, and it appears to act as a modulator of anti-inflammation and insulin-sensitizer [14]. Adiponectin has beneficial effects on endothelial cells and affects the progression of stroke, atherosclerosis, and hypertension [15–20]. Plasma adiponectin levels are decreased in patients with cardiovascular disease and several metabolic disorders [21]. Several studies reported an inverse relationship between plasma adiponectin and T2DM [22–26]. In this review, we examine current research regarding the relationship between risk factors for vascular dementia and adiponectin.

## 2. Risk Factors for Vascular Dementia

Vascular dementia is regarded as the most severe form of VCI characterized by the presence of clinical stroke or vascular

brain injury as well as cognitive impairment [2, 11, 27]. Several studies suggest that the risk factors for vascular dementia are almost identical as the risk factors for VCI. Common risk factors in both animal models and humans include hypertension, insulin resistance, hyperlipidemia, hyperhomocystinemia, atherosclerosis, and diabetes [28–32]. Age is also a risk factor for vascular dementia, suggesting that dementia in patients after the age of 65 increased gradually [33]. In addition, cerebrovascular dysfunction is a risk factor because the cerebrovascular function is reduced in patients with dementia [34–42]. In addition, another study suggests that metabolic syndrome, including insulin resistance, hypertension, and dyslipidemia, is associated with cognitive decline, a typical feature of vascular dementia [30]. Figure 1 shows that vascular dementia risk factors include aging, diabetes, hypertension, atherosclerosis, and stroke (Figure 1).

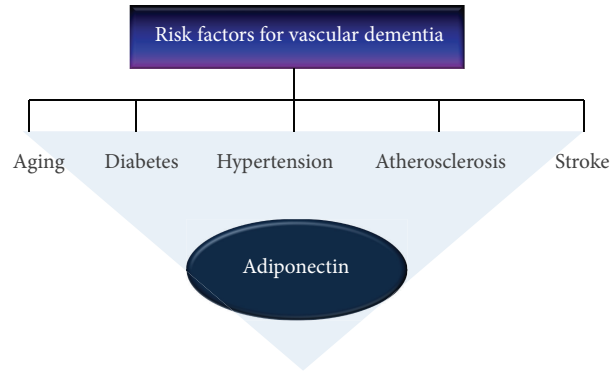



Figure 1: Risk factors for vascular dementia and adiponectin. Vascular dementia risk factors include aging, diabetes, hypertension, atherosclerosis, and stroke. Adiponectin is related to aging, diabetes, hypertension, atherosclerosis, and stroke by acting as a modulator or regulator in various mechanisms. Current researches have reported the role of adiponectin in diabetes, hypertension, atherosclerosis, and stroke.

## 3. Adiponectin

Adiponectin is one of the most abundant adipokines [43, 44]. It has significant sequence similarities with complement factor C1q, whose protein is termed Acrp30 because it is a 30 kDa adipocyte complement-related protein [45, 46]. Adiponectin is the protein produced by adipose's most abundant gene transcript 1 (*APM1*) gene, and *APM1* gene is located on chromosome 3q27, a region associated with T2DM and metabolic syndrome susceptibility [47–50]. Several human genetic association studies emphasized that hypoadiponectinemia caused by the single nucleotides polymorphisms (SNPs) in *APM1* gene is important to investigate the role of adiponectin in a variety of diseases [14, 51–54] including insulin resistance, T2DM, and metabolic syndrome, such as obesity [55]. In white French subjects, 2 SNPs in the promoter region of *APM1* gene, SNP 11377 and SNP 11391, were strongly related to hypoadiponectinemia and T2DM [50]. In white German and North American subjects, the +276 G/T SNP was associated with obesity and insulin resistance [52, 56]. In Chinese subjects, the +276 G/T SNP was significantly involved in the coronary heart disease [57]. Adiponectin acts via binding its receptors, adiponectin receptor type 1 (AdipoR1) and type 2 (AdipoR2). AdipoR1 has a higher binding affinity to the globular form of adiponectin, whereas AdipoR2 has a higher binding affinity to full-length adiponectin [58]. Adiponectin binds to the C-terminal extracellular domain of AdipoR, and the N-terminal cytoplasmic domain interacts with APPL1 [59]. Adiponectin receptors are expressed in liver, hypothalamus, and brain vascular endothelial cells [60–62]. Adiponectin is associated with insulin resistance, obesity, T2DM, dyslipidemia, and cardiovascular diseases [63–70]. It is an effective insulin sensitizer [64, 71, 72], and it promotes peripheral insulin sensitivity [14] and inhibits liver gluconeogenesis [73]. Decreases in circulating adiponectin in the prediabetic state lead to insulin resistance [74]. Adiponectin activates AMP-activated protein kinase (AMPK), which activates insulin-independent glucose uptake by muscle, downregulates gluconeogenic enzymes, and increases muscle fatty acid oxidation [73]. Unlike other adipocyte-derived hormones, adiponectin gene expression and plasma concentration are inversely associated with body mass index (BMI) [75]. Reduced plasma adiponectin levels have also been reported in patients with coronary artery disease [19] as well as those with increased carotid intima media thickness [76]. Plasma adiponectin levels are inversely related to the platelet activation status of patients with cardiovascular risk factors [20]. Adiponectin suppresses platelet aggregation in hyperlipidemic rats by reversing the increase in inducible nitric oxide synthase expression while enhancing endothelial nitric oxide synthase activation [77, 78]. Current studies have reported the association between adiponectin and various diseases because adiponectin has multiple roles in glucose and lipid metabolisms and vascular system.

## 4. Adiponectin, Aging, and Diabetes

*4.1. Aging, Insulin Signal Transduction, and Adipocytokines.* Recently, the number of elderly patients with dementia has been increasing rapidly [79]. One epidemiology study suggests an exponential increase in the incidence of dementia after the age of 65, doubling roughly every 5 years, such that greater than 50% of centenarians are expected to suffer from dementia [33]. Aging induces an oxidative redox shift by attenuating mitochondrial metabolism and changing glycolysis metabolism [80]. These alterations initiate a damaging pathway involving signaling molecules, transcription factors, and epigenetic transcriptional regulators [80, 81]. Among several important pathways for maintaining longevity, insulin sensitivity has been considered a key factor for the healthy aging phenotype in humans [82, 83] and mice [84, 85]. Several studies have reported that insulin and insulin growth factor-1 (IGF-1) receptor regulate the lifespan of mice [86, 87]. In humans, growth hormone (GH) and IGF-1 deficiencies are also associated with life expectancy [88]. Insulin sensitivity normally decreases during aging, and the prevalence of

metabolic syndrome (MetS) and insulin resistance substantially increases [89, 90]. In elderly persons, decreased insulin receptor (IR) levels and impaired insulin signaling have been observed predominantly in the hippocampus cortex and choroid plexus [81]. Impaired insulin receptor binding promotes chronic insulin resistance [91]. Muller et al. [92] reported that IGF-I signaling deteriorated in the brains of aged mice. This study demonstrated that activation of the brain IGF-1R/Akt/GSK-3$\beta$ pathway was evidently reduced although older mice have higher brain IGF-1R levels [92]. In humans, insulin sensitivity decreases with aging and the prevalence of T2DM increases with advancing age [89, 90]. Reduced mitochondrial function contributes to decline in glucose uptake with advancing age and leads to insulin resistance [93–97]. IGF-1 concentrations decline with age and are associated with age-related changes in body composition by both increasing fat mass and decreasing muscle mass [98–100]. Aging alters the function and number of adipose cells which cause alterations in the secretion and function of the adipocytokines such as leptin and adiponectin [101]. A recent study demonstrated that cellular senescence of adipose tissue causes insulin resistance [102]. Considering these evidences, aging alters the function of adipose cells, and alteration in secretion of adipocytokines attenuates insulin sensitivity.

*4.2. Adiponectin and Insulin Signal Transduction.* Insulin and IRs are ubiquitously expressed in the brain [81, 103] where insulin can reach levels 10- to 100-fold greater than in plasma, particularly in the hippocampus, cortex, hypothalamus, olfactory bulb, and pituitary [81, 104]. IRs are largely localized in neurons and are less abundant in glia [103, 105]. Insulin produced by pancreatic $\beta$-cells is transported by cerebrospinal fluid (CSF) to the brain where it crosses the blood-brain barrier (BBB) [106, 107]. Similar to IRs, IGF-1Rs are widely distributed in the brain [107, 108]. Insulin/IGF-1-mediated activation of Akt leads to GSK-3$\beta$ inactivation, which triggers multiple cascades, including synthesis of proteins involved in neuronal glucose metabolism and antiapoptotic mechanisms [104, 109]. Regarding brain glucose metabolism, recent studies suggest that changes in circulating insulin levels modulate glucose transporter (GLUT) expression [110, 111]. Cerebral IRs and IGF-1Rs are involved in cortical and hippocampal synaptic plasticity, thereby affecting memory and learning [105, 112]. In brain, insulin contributes to memory function through regulation of neurotransmitter receptors and synaptic function [113, 114]. Additionally, insulin signal transduction also promotes neurite outgrowth and axonal regeneration in the brain [105, 112, 115]. In the brain, insulin resistance results from perturbation of insulin signal transduction, causing systemic hyperglycemia. Decreased insulin and IGF-1 have been observed in Alzheimer's disease brain [116, 117]. Also, decreased insulin receptor substrate (IRS) protein levels related to insulin resistance [118] are associated with cognitive decline in dementia [119]. Impaired insulin transduction aggravates features of Alzheimer's disease including formation of neurofibrillary tangle caused by the decreasing brain glucose level and the increase of amyloid $\beta$ aggregation [104, 106, 118, 120–122]. In addition, insulin resistance is closely linked with other metabolic symptoms, including hypertension and hyperlipidemia [123]. Adiponectin directly regulates glucose metabolism and insulin sensitivity. Adiponectin, via activation of AMPK and adiponectin, stimulates GLUT4 translocation and glucose uptake [124]. Adiponectin receptors activate AMPK, PPAR-$\alpha$, and p38 MAPK to increase insulin sensitivity [58, 125]. An adaptor protein, APPL1, binds to adiponectin receptors that activate the AMPK and p38 MAPK pathways [126]. In addition, adiponectin decreases insulin resistance by decreasing triglyceride content in obese mice [127]. Increased tissue triglyceride content has been reported to interfere with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and subsequent GLUT 4 translocation and glucose uptake, thus leading to insulin resistance. Adipose tissue deficiency or lipodystrophy is associated with insulin resistance and metabolic dysregulation [128]. *Adiponectin* knockout mice show impaired insulin secretion, and intravenous adiponectin injection into C57BL/6 mice induces insulin secretion [129, 130]. *AdipoR1* and *2* double knockout mice have increased triglyceride levels in the liver and exhibit insulin resistance and glucose intolerance, suggesting that AdipoR1 and AdipoR2 regulate lipid and glucose homeostasis [14, 131]. In conclusion, adiponectin and adiponectin receptors improve insulin resistance by modulating triglyceride level and impaired insulin signal transduction. Thus, regulation of adiponectin is important impaired insulin signal transduction to improve and also adiponectin may contribute to the improvement of cognitive decline in dementia.

*4.3. Adiponectin, Diabetes, and Vascular Dementia.* Diabetes characterized by reduced insulin sensitivity is associated with thrombosis, myocardial infarction, and cerebrovascular disease, which can lead to infarctions and white matter ischemia [132]. Macrovascular disease causes approximately 80% of mortality in patients with T2DM. The risk of vascular diseases in patients with T2DM is decreased by lowering the blood pressure of patients with hypertension [133–135]. In addition, diabetes and hypoglycemia are associated with cognitive impairment [136–138]. Yaffe et al., in a 4-year prospective study, suggested that older women with impaired fasting glucose levels performed poorly on cognitive tests compared to those with normal glycemia [139]. Considering these associations, diabetes may be regarded as a risk factor of vascular dementia. Adiponectin levels are elevated in type I diabetics compared with healthy controls [140]. Several studies have consistently found that increased adiponectin levels are associated with reduced risk for T2DM [22, 25, 26]. Hypoadiponectinemia has been considered an underlying mechanism of insulin resistance in T2DM [141–145]. In cross-sectional studies, plasma adiponectin concentrations were significantly lower in patients with diabetes [146]. In a 5-year follow-up study of 1096 nondiabetics, the association between adiponectin and T2DM was attenuated after adjustment for homeostatic model assessment of insulin resistance (HOMA-IR) and was eliminated after adjustment for insulin sensitivity. These data suggest that the antidiabetic effect of

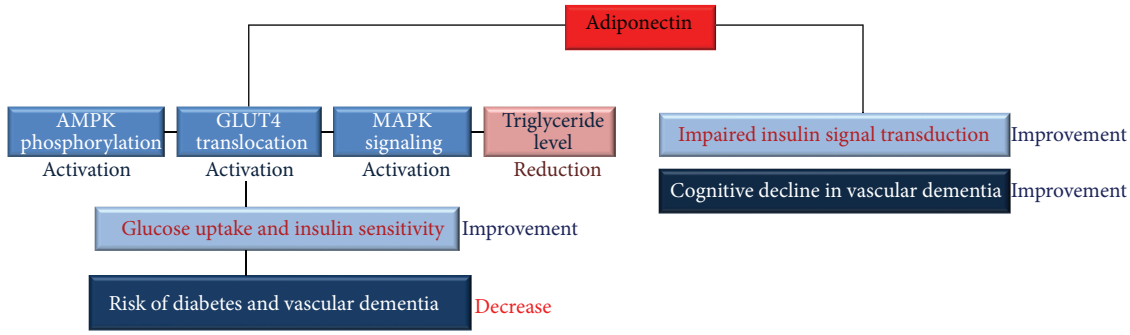



Figure 2: Adiponectin improves insulin sensitivity and reduces diabetes and vascular dementia risk. Adiponectin stimulates AMPK phosphorylation, GLUT4 translocation, and MAPK pathways. Adiponectin also reduces the levels of triglyceride. Consequentially, adiponectin increases glucose uptake and insulin sensitivity and reduces insulin resistance. In addition, adiponectin improves the cognitive decline by modulating impaired insulin signal transduction in vascular dementia brain. These mechanisms decrease the risk of diabetes and vascular dementia. Also, these mechanisms improve the memory dysfunction in dementia. AMPK: AMP-activated kinase, GLUT4: glucose transporter type 4, and MAPK: mitogen activated protein kinase.

adiponectin is due to insulin sensitization [147]. Adiponectin predicts against diabetes onset, and diabetic patients always show lower plasma adiponectin levels compared to the general population [148]. Thus, adiponectin reduces the risk of diabetes by regulating insulin signal transduction and insulin resistance. Suppression of adiponectin aggravates diabetes as a risk for vascular dementia. Figure 2 shows that adiponectin stimulates the phosphorylation of AMPK and GLUT4 translocation and attenuates levels of triglyceride. As a result, adiponectin enhances glucose uptake and insulin sensitivity. This indicates that adiponectin reduces the risk of diabetes and vascular dementia (Figure 2).

## 5. Adiponectin, Hypertension, and Stroke

*5.1. Adiponectin and Hypertension.* Hypertension has been reported as the most common risk factor for stroke worldwide and has also been gradually recognized as a risk factor for dementia [149]. Arterial hypertension contributes to the development and progression of cerebrovascular disease [150]. Hypertension exposes the cerebral microvasculature to pulsatile pressure and flow that cause vascular endothelium and smooth muscle cell tearing [151]. Many cross-sectional and longitudinal studies have demonstrated that dementia and VCI are associated with hypertension [152–156]. Therefore, previous studies suggest that hypertension is the most important risk factor for cerebral vessel dysfunction, and it contributes to cognitive decline [157, 158]. Pulse pressure (PP), a marker of arterial stiffness, has been connected with the risk of cognitive decline [159] and AD [160, 161]. Elevated pulse pressure increases the risk of cognitive decline and impaired language abilities [162]. Decreased blood pressure (BP) is a clinical manifestation of dementia in elderly subjects [163, 164]. Endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) are crucial regulators of vascular homeostasis and, in particular, endothelial function [165, 166]. Endothelium-derived NO is a beneficial factor that promotes vasodilation and inhibits platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation [167]. Adiponectin, acting via AdipoR1 and AdipoR2, promotes NO production through AMPK signaling pathway activation. AMPK activates eNOS through phosphorylation at $Ser^{1177}$ and facilitates complex formation between eNOS and heat shock protein 90 (HSP-90), which is required for eNOS activation [167]. *Adiponectin* knockout mice have reduced endothelial NO levels in vessel walls [168]. Adiponectin inhibits the inflammatory response and causes vasodilatation largely through AMPK/eNOS [169–172]. Adiponectin-induced AMPK signaling promotes phosphatidylinositol 3-kinase-Akt signaling, leading to angiogenic growth factor synthesis [170, 173]. A recent study also suggests that adiponectin inhibits vascular endothelial growth factor-(VEGF-) induced ROS generation and has an antioxidant role in the vasculature [174]. These actions of adiponectin are also mediated via inhibition of growth factor-stimulated extracellular signal regulated kinase (ERK) signaling. In addition, several studies indicate that adiponectin plays a role in the regulation of microvascular network flow and function [175, 176]. Some clinical research demonstrates that plasma adiponectin levels are positively associated with arterial vasodilation [177]. Considering the role of adiponectin in vascular function, decreased adiponectin raises the risk of hypertension. Figure 3 shows that adiponectin increases AMPK phosphorylation and NO production. Platelet aggregation is decreased and vasodilation is increased due to NO production. Finally, adiponectin decreases the risk of hypertension and improves vascular cognitive impairment (Figure 3).

*5.2. Adiponectin and Atherosclerosis.* Atherosclerosis is a degenerative vessel disease that frequently affects large- to medium-sized arteries. In the brain, vessels of the circle of Willis are often involved [178]. Atherosclerotic plaques are prone to rupture with subsequent thrombosis [179, 180]. The thrombus resulting from plaque rupture leads to vessel occlusion or embolizes a smaller artery [181]. Atherosclerosis plaque rupture is related to inflammation, including secretion of cytokines and matrix-metalloproteinases,

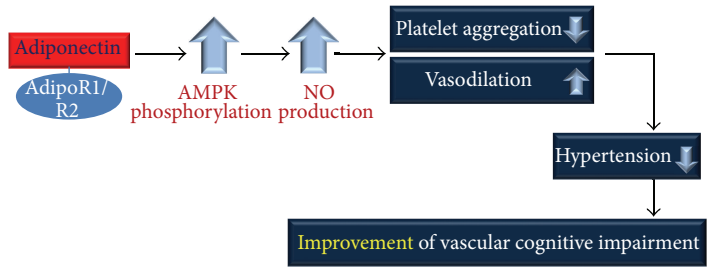



Figure 3: Adiponectin improves vascular cognitive impairment by stimulating NO production. Adiponectin, acting via AdipoR1 and AdipoR2, promotes AMPK phosphorylation and NO production. Increased NO reduces platelet aggregation and increases vasodilation. Consequentially, adiponectin decreases the risk of hypertension and improves vascular cognitive impairment. AMPK: AMP-activated kinase and NO: nitric oxide.

which are involved in vessel wall degradation [182–186]. Adiponectin plays the role of an antiatherogenic and anti-inflammatory modulator [18]. Several studies suggest that adiponectin inhibits many peptides and cytokines related to atherosclerosis progression [187]. Adiponectin inhibits monocyte adherence to TNF-$\alpha$-stimulated endothelial cells by suppressing adhesion molecule expression [188, 189]. Adiponectin directly inhibits atherogenic molecules, such as intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, and E-selectin, which are molecules associated with heightened leukocyte trafficking [189]. Adiponectin also attenuates expression of class A scavenger receptor in human macrophages and inhibits transformation of macrophages to foam cells [190]. The association between adiponectin and atherogenic factors indicates that regulation of adiponectin is important in atherosclerosis.

*5.3. Adiponectin and Stroke.* Several studies reported that stroke doubles the risk for dementia (poststroke dementia), and approximately 30% of stroke patients develop cognitive dysfunction within 3 years [191–194]. An association between stroke and dementia is also observed in patients younger than 50 years, and up to 50% of these patients exhibit cognitive deficits after a decade [195]. One of the first population-based studies to assess the relationship between stroke and dementia was conducted in Rochester, Minnesota [196]. Many stroke patients show gradual but continuous deterioration after a single-stroke lesion. This deterioration is characterized clinically by cognitive and behavioral dysfunction [197]. Several cross-sectional and retrospective case-control studies have reported an association between low adiponectin levels and increased stroke risk [16, 198–201]. In addition, adiponectin levels are associated with coronary heart disease such as coronary vascular disease [202, 203]. Several studies demonstrate that hypoadiponectinemia increases the prevalence of coronary vascular disease [189, 204]. Several studies demonstrated that adiponectin knockout (APN-KO) mice showed severe injuries during cerebral ischemia-reperfusion [205, 206], while adiponectin injected APN-KO mice were reduced pathological ischemia-induced damage [207]. Adiponectin blocks the interaction between the endothelial cells and leukocytes in ischemia-reperfusion and also inhibits the secondary inflammation in cerebral ischemia-reperfusion [208]. Adiponectin reduced the infarct size through nitric oxide synthase dependent mechanism in cerebral ischemic stroke mice model [209, 210]. In addition, adiponectin activates AMPK phosphorylation in cerebral ischemic stroke mice model [211]. Then, the activation of VEGF by the activated AMPK signaling promotes angiogenesis in cerebral ischemic brain [212, 213]. Considering the results of the above studies, adiponectin is associated with the risk of stroke and reduces cerebral ischemia induced damage. This may be due to the roles of adiponectin as an antiatherogenic modulator and a vasodilator in vascular system. Figure 4 shows that adiponectin decreases the expression of atherogenic molecules and plaque formation in blood vessels. Consequentially, adiponectin attenuates the risk of stroke and vascular dementia (Figure 4).

## 6. Conclusions

Risk factors for vascular dementia include advanced age, diabetes, hypertension, atherosclerosis, and stroke. Adiponectin, an adipokine, acts as an antidiabetic and antiatherogenic regulator. Insulin sensitivity is a key cellular mechanism related to diabetes, cerebrovascular dysfunction, and cognitive decline. Adiponectin is involved in insulin sensitivity, and increased adiponectin levels improve impaired insulin signaling. Moreover, adiponectin affects the cerebrovascular function by stimulating NO production and inhibiting transformation of macrophages to foam cells. Specifically, we summarize the findings as follows.

(1) Vascular dementia characterized by cognitive decline is associated with increased age because insulin receptors, which are related to cognitive function, decrease with age. Adiponectin is associated with age-related diseases, including cardiovascular disease and metabolic disease. Adiponectin is mediated via the activation of AMPK, and adiponectin stimulates GLUT4 translocation and glucose uptake. Moreover, binding between adiponectin and adiponectin receptors activates AMPK, PPAR-$\alpha$, and p38 MAPK to increase insulin sensitivity. In addition, in clinical studies, an association between decreased adiponectin and diabetes was demonstrated. In conclusion, adiponectin improves impaired insulin signaling and improves cognitive decline as a typical feature of vascular dementia.

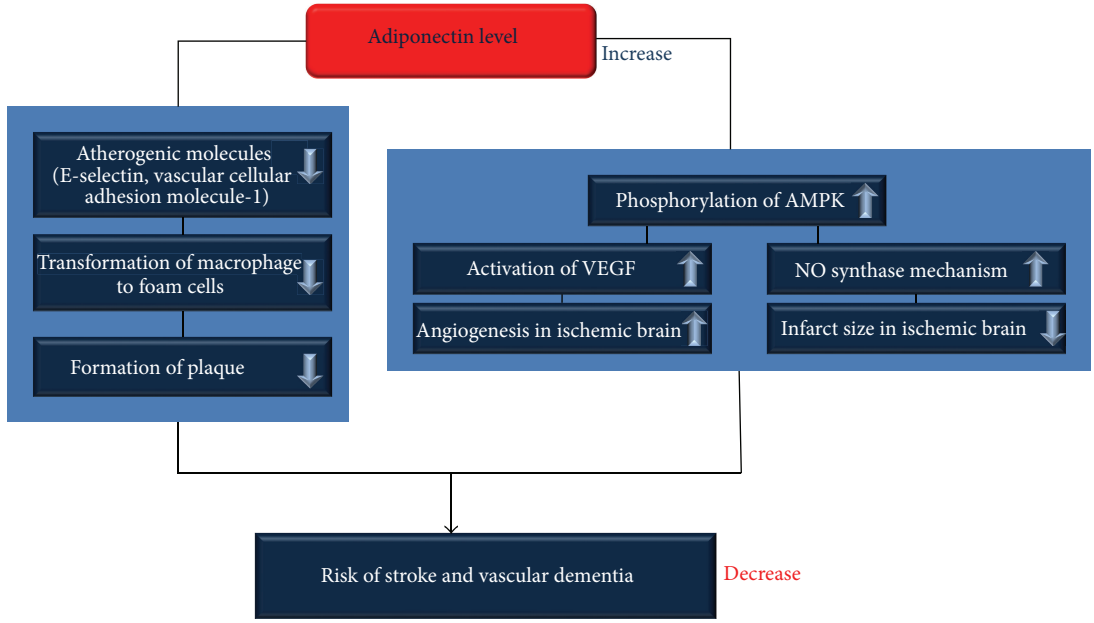



Figure 4: Adiponectin attenuates the risk of stroke and vascular dementia. Adiponectin decreases the expression of atherogenic molecules and formation of form cells in blood vessels. Adiponectin attenuates the risk of stroke by decreasing plaque formation in blood vessels. In addition, adiponectin binds with AdipoR1 and AdipoR2 and then activates the phosphorylation of AMPK. Increased AMPK phosphorylation promotes the activation of VEGF and NO synthase mechanism. As a result, adiponectin ameliorates angiogenesis in ischemic brain and reduces infarct size in ischemic brain. Consequentially, adiponectin decreases the risk of stroke and vascular dementia. AMPK: AMP-activated kinase, VEGF: vascular endothelial growth factor, and NO: nitric oxide.

(2) Vascular dementia characterized by cerebrovascular dysfunction is associated with hypertension, atherosclerosis, and stroke. Adiponectin stimulates NO production through the AMPK signaling pathway. Adiponectin also plays the role of an antiatherogenic modulator. Adiponectin inhibits atherogenic molecules and attenuates the transformation of macrophages to foam cells. In conclusion, adiponectin improves vascular dysfunction and alleviates the progression of hypertension, atherosclerosis, and stroke as risk factors for vascular dementia.

(3) Taken together, adiponectin attenuates the risk of vascular dementia and ameliorates vascular dementia-related pathologies including cerebrovascular dysfunction and cognitive decline which resulted from impaired insulin transduction and neuroinflammation.

In this review, we summarized the current research regarding the association between risk factors for vascular dementia and adiponectin. Considering the relationship between adiponectin and risk factors for vascular dementia including aging, diabetes, hypertension, atherosclerosis, and stroke, we suggest that further studies are necessary to examine the role of adiponectin in vascular dementia. Moreover, we emphasize that the regulation of adiponectin levels and receptors of adiponectin would be important for the prevention and treatment of vascular dementia.

## Conflict of Interests

The authors declare no conflict of interests.

## Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean government (MEST) (2012-0005440). This work was also supported by the Brain Korea 21 Plus Project for Medical Science, Yonsei University.

## References

[1] A. Ruitenberg, A. Ott, J. C. van Swieten, A. Hofman, and M. M. B. Breteler, "Incidence of dementia: does gender make a difference?" *Neurobiology of Aging*, vol. 22, no. 4, pp. 575–580, 2001.

[2] P. B. Gorelick, A. Scuteri, S. E. Black et al., "Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 42, no. 9, pp. 2672–2713, 2011.

[3] M. Wortmann, "Dementia: a global health priority—highlights from an ADI and World Health Organization report," *Alzheimer's Research & Therapy*, vol. 4, no. 5, article 40, 2012.

[4] Alzheimer's Association, "2012 Alzheimer's disease facts and figures," *Alzheimer's & Dementia*, vol. 8, no. 2, pp. 131–168, 2012.

[5] S. Craft, "The role of metabolic disorders in Alzheimer disease and vascular dementia: two roads converged," *Archives of Neurology*, vol. 66, no. 3, pp. 300–305, 2009.

[6] H. Fillit, D. T. Nash, T. Rundek, and A. Zuckerman, "Cardiovascular risk factors and dementia," *The American Journal of Geriatric Pharmacotherapy*, vol. 6, no. 2, pp. 100–118, 2008.

[7] K. Honjo, S. E. Black, and N. P. Verhoeff, "Alzheimer's disease, cerebrovascular disease, and the $\beta$-amyloid cascade," *The Canadian Journal of Neurological Sciences*, vol. 39, no. 6, pp. 712–728, 2012.

[8] C. Purnell, S. Gao, C. M. Callahan, and H. C. Hendrie, "Cardiovascular risk factors and incident alzheimer disease: a systematic review of the literature," *Alzheimer Disease and Associated Disorders*, vol. 23, no. 1, pp. 1–10, 2009.

[9] J. A. Schneider, Z. Arvanitakis, W. Bang, and D. A. Bennett, "Mixed brain pathologies account for most dementia cases in community-dwelling older persons," *Neurology*, vol. 69, no. 24, pp. 2197–2204, 2007.

[10] J. B. Toledo, S. E. Arnold, K. Raible et al., "Contribution of cerebrovascular disease in autopsy confirmed neurodegenerative disease cases in the National Alzheimer's Coordinating Centre," *Brain*, vol. 136, no. 9, pp. 2697–2706, 2013.

[11] G. C. Roman, T. K. Tatemichi, T. Erkinjuntti et al., "Vascular dementia: diagnostic criteria for research studies: report of the NINDS-AIREN International Workshop," *Neurology*, vol. 43, no. 2, pp. 250–260, 1993.

[12] V. C. Hachinski, J. V. Bowler, C. Loeb, G. C. Roman, and T. K. Tatemichi, "Vascular dementia," *Neurology*, vol. 43, no. 10, pp. 2159–2161, 1993.

[13] UK Prospective Diabetes Study (UKPDS) Group, "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

[14] T. Kadowaki, T. Yamauchi, N. Kubota, K. Hara, K. Ueki, and K. Tobe, "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome," *The Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1784–1792, 2006.

[15] M. Shimabukuro, N. Higa, T. Asahi et al., "Hypoadiponectinemia is closely linked to endothelial dysfunction in man," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 7, pp. 3236–3240, 2003.

[16] M. P. Chen, J. C. Tsai, F. M. Chung et al., "Hypoadiponectinemia is associated with ischemic cerebrovascular disease," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 4, pp. 821–826, 2005.

[17] M. B. Snijder, R. J. Heine, J. C. Seidell et al., "Associations of adiponectin levels with incident impaired glucose metabolism and type 2 diabetes in older men and women the hoorn study," *Diabetes Care*, vol. 29, no. 11, pp. 2498–2503, 2006.

[18] P.-A. Jansson, F. Pellmé, A. Hammarstedt et al., "A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin," *The FASEB Journal*, vol. 17, no. 11, pp. 1434–1440, 2003.

[19] K. Dunajska, A. Milewicz, D. Jędrzejuk et al., "Plasma adiponectin concentration in relation to severity of coronary atherosclerosis and cardiovascular risk factors in middle-aged men," *Endocrine*, vol. 25, no. 3, pp. 215–221, 2004.

[20] T. Shoji, H. Koyama, S. Fukumoto et al., "Platelet activation is associated with hypoadiponectinemia and carotid atherosclerosis," *Atherosclerosis*, vol. 188, no. 1, pp. 190–195, 2006.

[21] J. Saltevo, M. Laakso, J. Jokelainen, S. Keinänen-Kiukaanniemi, E. Kumpusalo, and M. Vanhala, "Levels of adiponectin, C-reactive protein and interleukin-1 receptor antagonist are associated with insulin sensitivity: a population-based study," *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 5, pp. 378–383, 2008.

[22] S. G. Wannamethee, G. D. O. Lowe, A. Rumley, L. Cherry, P. H. Whincup, and N. Sattar, "Adipokines and risk of type 2 diabetes in older men," *Diabetes Care*, vol. 30, no. 5, pp. 1200–1205, 2007.

[23] C. Heidemann, Q. Sun, R. M. van Dam et al., "Total and high-molecular-weight adiponectin and resistin in relation to the risk for type 2 diabetes in women," *Annals of Internal Medicine*, vol. 149, no. 5, pp. 307–316, 2008.

[24] S. H. Ley, S. B. Harris, P. W. Connelly et al., "Adipokines and incident type 2 diabetes in a Canadian Aborigine population: the Sandy Lake Health and Diabetes Project," *Diabetes Care*, vol. 31, no. 7, pp. 1410–1415, 2008.

[25] A. G. Tabák, E. J. Brunner, M. A. Miller et al., "Low serum adiponectin predicts 10-year risk of type 2 diabetes and hba1c independently of obesity, lipids, and inflammation: Whitehall II study," *Hormone and Metabolic Research*, vol. 41, no. 8, pp. 626–629, 2009.

[26] W. Koenig, N. Khuseyinova, J. Baumert, C. Meisinger, and H. Löwel, "Serum concentrations of adiponectin and risk of type 2 diabetes mellitus and coronary heart disease in apparently healthy middle-aged men: results from the 18-year follow-up of a large cohort from southern Germany," *Journal of the American College of Cardiology*, vol. 48, no. 7, pp. 1369–1377, 2006.

[27] D. R. Thal, L. T. Grinberg, and J. Attems, "Vascular dementia: different forms of vessel disorders contribute to the development of dementia in the elderly brain," *Experimental Gerontology*, vol. 47, no. 11, pp. 816–824, 2012.

[28] R. A. Cohen and X. Tong, "Vascular oxidative stress: the common link in hypertensive and diabetic vascular disease," *Journal of Cardiovascular Pharmacology*, vol. 55, no. 4, pp. 308–316, 2010.

[29] C. Iadecola and R. L. Davisson, "Hypertension and cerebrovascular dysfunction," *Cell Metabolism*, vol. 7, no. 6, pp. 476–484, 2008.

[30] K. F. Yates, V. Sweat, P. L. Yau, M. M. Turchiano, and A. Convit, "Impact of metabolic syndrome on cognition and brain: a selected review of the literature," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 9, pp. 2060–2067, 2012.

[31] F. M. Faraci, "Protecting against vascular disease in brain," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 300, no. 5, pp. H1566–H1582, 2011.

[32] R. Sahathevan, A. Brodtmann, and G. A. Donnan, "Dementia, stroke, and vascular risk factors; a review," *International Journal of Stroke*, vol. 7, no. 1, pp. 61–73, 2012.

[33] M. M. Corrada, R. Brookmeyer, D. Berlau, A. Paganini-Hill, and C. H. Kawas, "Prevalence of dementia after age 90: results from the 90+ study," *Neurology*, vol. 71, no. 5, pp. 337–343, 2008.

[34] J. A. H. R. Claassen, R. Diaz-Arrastia, K. Martin-Cook, B. D. Levine, and R. Zhang, "Altered cerebral hemodynamics in early alzheimer disease: a pilot study using transcranial doppler," *Journal of Alzheimer's Disease*, vol. 17, no. 3, pp. 621–629, 2009.

[35] Y. Z. Gao, J. J. Zhang, H. Liu, G. Y. Wu, L. Xiong, and M. Shu, "Regional cerebral blood flow and cerebrovascular reactivity in Alzheimer's disease and vascular dementia assessed by arterial spinlabeling magnetic resonance imaging," *Current Neurovascular Research*, vol. 10, no. 1, pp. 49–53, 2013.

[36] C. Luckhaus, M. O. Flüß, H.-J. Wittsack et al., "Detection of changed regional cerebral blood flow in mild cognitive

impairment and early Alzheimer's dementia by perfusion-weighted magnetic resonance imaging," *NeuroImage*, vol. 40, no. 2, pp. 495–503, 2008.

[37] M. J. Mentis, B. Horwitz, C. L. Grady et al., "Visual cortical dysfunction in Alzheimer's disease evaluated with a temporally graded "stress test" during PET," *American Journal of Psychiatry*, vol. 153, no. 1, pp. 32–40, 1996.

[38] E. Niedermeyer, "Alzheimer disease: caused by primary deficiency of the cerebral blood flow," *Clinical EEG and Neuroscience*, vol. 37, no. 3, pp. 175–177, 2006.

[39] A. Ruitenberg, T. den Heijer, S. L. M. Bakker et al., "Cerebral hypoperfusion and clinical onset of dementia: the Rotterdam study," *Annals of Neurology*, vol. 57, no. 6, pp. 789–794, 2005.

[40] B. Sabayan, S. Jansen, A. M. Oleksik et al., "Cerebrovascular hemodynamics in Alzheimer's disease and vascular dementia: a meta-analysis of transcranial Doppler studies," *Ageing Research Reviews*, vol. 11, no. 2, pp. 271–277, 2012.

[41] M. Tanaka, H. Fukuyama, H. Yamauchi et al., "Regional cerebral blood flow abnormalities in nondemented patients with memory impairment," *Journal of Neuroimaging*, vol. 12, no. 2, pp. 112–118, 2002.

[42] C. Iadecola, "Neurovascular regulation in the normal brain and in Alzheimer's disease," *Nature Reviews Neuroscience*, vol. 5, no. 5, pp. 347–360, 2004.

[43] F. I. Achike, N.-H. P. To, H. Wang, and C.-Y. Kwan, "Obesity, metabolic syndrome, adipocytes and vascular function: a holistic viewpoint," *Clinical and Experimental Pharmacology and Physiology*, vol. 38, no. 1, pp. 1–10, 2011.

[44] Y. Okamoto, S. Kihara, T. Funahashi, Y. Matsuzawa, and P. Libby, "Adiponectin: a key adipocytokine in metabolic syndrome," *Clinical Science*, vol. 110, no. 3, pp. 267–278, 2006.

[45] P. E. Scherer, S. Williams, M. Fogliano, G. Baldini, and H. F. Lodish, "A novel serum protein similar to C1q, produced exclusively in adipocytes," *The Journal of Biological Chemistry*, vol. 270, no. 45, pp. 26746–26749, 1995.

[46] L. Shapiro and P. E. Scherer, "The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor," *Current Biology*, vol. 8, no. 6, pp. 335–338, 1998.

[47] H. Ruan and H. F. Lodish, "Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-$\alpha$," *Cytokine and Growth Factor Reviews*, vol. 14, no. 5, pp. 447–455, 2003.

[48] A. H. Kissebah, G. E. Sonnenberg, J. Myklebust et al., "Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 26, pp. 14478–14483, 2000.

[49] Y. Mori, S. Otabe, C. Dina et al., "Genome-wide search for type 2 diabetes in Japanese affected sib-pairs confirms susceptibility genes on 3q, 15q, and 20q and identifies two new candidate loci on 7p and 11p," *Diabetes*, vol. 51, no. 4, pp. 1247–1255, 2002.

[50] F. Vasseur, N. Helbecque, C. Dina et al., "Single-nucleotide polymorphism haplotypes in the both proximal promoter and exon 3 of the *APM1* gene modulate adipocyte-secreted adiponectin hormone levels and contribute to the genetic risk for type 2 diabetes in French Caucasians," *Human Molecular Genetics*, vol. 11, no. 21, pp. 2607–2614, 2002.

[51] H.-B. Leu, C.-M. Chung, S.-J. Lin, Y.-S. Jong, W.-H. Pan, and J.-W. Chen, "Adiponectin gene polymorphism is selectively associated with the concomitant presence of metabolic syndrome and essential hypertension," *PLoS ONE*, vol. 6, no. 5, Article ID e19999, 2011.

[52] M. Stumvoll, O. Tschritter, A. Fritsche et al., "Association of the T-G polymorphism in adiponectin (exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes," *Diabetes*, vol. 51, no. 1, pp. 37–41, 2002.

[53] A. Kollias, P. C. Tsiotra, I. Ikonomidis et al., "Adiponectin levels and expression of adiponectin receptors in isolated monocytes from overweight patients with coronary artery disease," *Cardiovascular Diabetology*, vol. 10, article 14, 2011.

[54] K. Hara, P. Boutin, Y. Mori et al., "Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population," *Diabetes*, vol. 51, no. 2, pp. 536–540, 2002.

[55] V. J. Bermudez, E. Rojas, A. Toledo et al., "Single-nucleotide polymorphisms in adiponectin, AdipoR1, and AdipoR2 genes: insulin resistance and type 2 diabetes mellitus candidate genes," *American Journal of Therapeutics*, vol. 20, no. 4, pp. 414–421, 2013.

[56] C. Menzaghi, T. Ercolino, R. D. Paola et al., "A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome," *Diabetes*, vol. 51, no. 7, pp. 2306–2312, 2002.

[57] Z. Zhang, H. Tian, Y. Zhao et al., "Meta-analysis of the association between adiponectin gene +276 g/T polymorphisms and coronary atherosclerotic heart disease," *Wei Sheng Yan Jiu*, vol. 42, no. 4, pp. 693–697, 2013.

[58] T. Yamauchi, J. Kamon, H. Waki et al., "Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis," *The Journal of Biological Chemistry*, vol. 278, no. 4, pp. 2461–2468, 2003.

[59] F. Ziemke and C. S. Mantzoros, "Adiponectin in insulin resistance: lessons from translational research," *The American Journal of Clinical Nutrition*, vol. 91, no. 1, pp. 258S–261S, 2010.

[60] J. Spranger, S. Verma, I. Göhring et al., "Adiponectin does not cross the blood-brain barrier but modifies cytokine expression of brain endothelial cells," *Diabetes*, vol. 55, no. 1, pp. 141–147, 2006.

[61] N. Kubota, W. Yano, T. Kubota et al., "Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake," *Cell Metabolism*, vol. 6, no. 1, pp. 55–68, 2007.

[62] A. Psilopanagioti, H. Papadaki, E. F. Kranioti, T. K. Alexandrides, and J. N. Varakis, "Expression of adiponectin and adiponectin receptors in human pituitary gland and brain," *Neuroendocrinology*, vol. 89, no. 1, pp. 38–47, 2009.

[63] E. Zoico, V. di Francesco, G. Mazzali et al., "Adipocytokines, fat distribution, and insulin resistance in elderly men and women," *The Journals of Gerontology A: Biological Sciences and Medical Sciences*, vol. 59, no. 9, pp. 935–939, 2004.

[64] M. Cnop, P. J. Havel, K. M. Utzschneider et al., "Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex," *Diabetologia*, vol. 46, no. 4, pp. 459–469, 2003.

[65] A. M. Kanaya, T. Harris, B. H. Goodpaster, F. Tylavsky, and S. R. Cummings, "Adipocytokines attenuate the association between visceral adiposity and diabetes in older adults," *Diabetes Care*, vol. 27, no. 6, pp. 1375–1380, 2004.

[66] S. Ahmadizad, A. H. Haghighi, and M. R. Hamedinia, "Effects of resistance versus endurance training on serum adiponectin and insulin resistance index," *European Journal of Endocrinology*, vol. 157, no. 5, pp. 625–631, 2007.

[67] M. Blüher, C. J. Williams, N. Klöting et al., "Gene expression of adiponectin receptors in human visceral and subcutaneous

adipose tissue is related to insulin resistance and metabolic parameters and is altered in response to physical training," *Diabetes Care*, vol. 30, no. 12, pp. 3110–3115, 2007.

[68] M. Blüher, J. W. Bullen Jr., J. H. Lee et al., "Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 6, pp. 2310–2316, 2006.

[69] H. Tilg and A. R. Moschen, "Adipocytokines: mediators linking adipose tissue, inflammation and immunity," *Nature Reviews Immunology*, vol. 6, no. 10, pp. 772–783, 2006.

[70] P. M. Ridker, J. E. Buring, N. R. Cook, and N. Rifai, "C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women," *Circulation*, vol. 107, no. 3, pp. 391–397, 2003.

[71] U. B. Pajvani and P. E. Scherer, "Adiponectin: systemic contributor to insulin sensitivity," *Current Diabetes Reports*, vol. 3, no. 3, pp. 207–213, 2003.

[72] S. Lim, H. C. Sung, I.-K. Jeong et al., "Insulin-sensitizing effects of exercise on adiponectin and retinol-binding protein-4 concentrations in young and middle-aged women," *The Journal of Clinical Endocrinology & Metabolism*, vol. 93, no. 6, pp. 2263–2268, 2008.

[73] T. Yamauchi, J. Kamon, Y. Minokoshi et al., "Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase," *Nature Medicine*, vol. 8, no. 11, pp. 1288–1295, 2002.

[74] N. Stefan, B. Vozarova, T. Funahashi et al., "Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans," *Diabetes*, vol. 51, no. 6, pp. 1884–1888, 2002.

[75] A. S. Lihn, S. B. Pedersen, and B. Richelsen, "Adiponectin: action, regulation and association to insulin sensitivity," *Obesity Reviews*, vol. 6, no. 1, pp. 13–21, 2005.

[76] B. Iglseder, V. Mackevics, A. Stadlmayer, G. Tasch, G. Ladurner, and B. Paulweber, "Plasma adiponectin levels and sonographic phenotypes of subclinical carotid artery atherosclerosis: data from the SAPHIR study," *Stroke*, vol. 36, no. 12, pp. 2577–2582, 2005.

[77] W.-Q. Wang, H.-F. Zhang, G.-X. Gao, Q.-X. Bai, R. Li, and X.-M. Wang, "Adiponectin inhibits hyperlipidemia-induced platelet aggregation via attenuating oxidative/nitrative stress," *Physiological Research*, vol. 60, no. 2, pp. 347–354, 2011.

[78] P. Restituto, I. Colina, J. J. Varo, and N. Varo, "Adiponectin diminishes platelet aggregation and sCD40L release. Potential role in the metabolic syndrome," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 298, no. 5, pp. E1072–E1077, 2010.

[79] M. Prince, R. Bryce, E. Albanese, A. Wimo, W. Ribeiro, and C. P. Ferri, "The global prevalence of dementia: a systematic review and metaanalysis," *Alzheimer's & Dementia*, vol. 9, no. 1, pp. 63.e2–75.e2, 2013.

[80] G. J. Brewer, "Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories," *Experimental Gerontology*, vol. 45, no. 3, pp. 173–179, 2010.

[81] A. I. Duarte, P. I. Moreira, and C. R. Oliveira, "Insulin in central nervous system: more than just a peripheral hormone," *Journal of Aging Research*, vol. 2012, Article ID 384017, 21 pages, 2012.

[82] L. Kappeler, C. D. M. Filho, J. Dupont et al., "Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism," *PLoS Biology*, vol. 6, no. 10, Article ID e254, 2008.

[83] C. Franceschi, F. Olivieri, F. Marchegiani et al., "Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: the lesson of centenarians," *Mechanisms of Ageing and Development*, vol. 126, no. 2, pp. 351–361, 2005.

[84] A. Lorenzini, A. B. Salmon, C. Lerner et al., "Mice producing reduced levels of insulin-like growth factor type 1 display an increase in maximum, but not mean, life span," *The Journals of Gerontology A: Biological Sciences and Medical Sciences*, vol. 69, no. 4, pp. 410–419, 2014.

[85] C. Selman, S. Lingard, A. I. Choudhury et al., "Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice," *The FASEB Journal*, vol. 22, no. 3, pp. 807–818, 2008.

[86] M. Blüher, B. B. Kahn, and C. R. Kahn, "Extended longevity in mice lacking the insulin receptor in adipose tissue," *Science*, vol. 299, no. 5606, pp. 572–574, 2003.

[87] M. Holzenberger, J. Dupont, B. Ducos et al., "IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice," *Nature*, vol. 421, no. 6919, pp. 182–187, 2003.

[88] A. Besson, S. Salemi, S. Gallati et al., "Reduced longevity in untreated patients with isolated growth hormone deficiency," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 8, pp. 3664–3667, 2003.

[89] E. S. Ford, W. H. Giles, and W. H. Dietz, "Prevalence of the metabolic syndrome among US adults: findings from the Third National Health and Nutrition Examination Survey," *The Journal of the American Medical Association*, vol. 287, no. 3, pp. 356–359, 2002.

[90] J. Kobayashi, K. Nishimura, M. Matoba, N. Maekawa, and H. Mabuchi, "Generation and gender differences in the components contributing to the diagnosis of the metabolic syndrome according to the Japanese criteria," *Circulation Journal*, vol. 71, no. 11, pp. 1734–1737, 2007.

[91] T. Fulop, A. Larbi, and N. Douziech, "Insulin receptor and ageing," *Pathologie Biologie*, vol. 51, no. 10, pp. 574–580, 2003.

[92] A. P. Muller, A. M. Fernandez, C. Haas, E. Zimmer, L. V. Portela, and I. Torres-Aleman, "Reduced brain insulin-like growth factor I function during aging," *Molecular and Cellular Neuroscience*, vol. 49, no. 1, pp. 9–12, 2012.

[93] J.-A. Kim, Y. Wei, and J. R. Sowers, "Role of mitochondrial dysfunction in insulin resistance," *Circulation Research*, vol. 102, no. 4, pp. 401–414, 2008.

[94] M. A. Abdul-Ghani and R. A. DeFronzo, "Mitochondrial dysfunction, insulin resistance, and type 2 diabetes mellitus," *Current Diabetes Reports*, vol. 8, no. 3, pp. 173–178, 2008.

[95] K. F. Petersen, D. Befroy, S. Dufour et al., "Mitochondrial dysfunction in the elderly: possible role in insulin resistance," *Science*, vol. 300, no. 5622, pp. 1140–1142, 2003.

[96] R. Barazzoni, K. R. Short, and K. S. Nair, "Effects of aging on mitochondrial DNA copy number and cytochrome *c* oxidase gene expression in rat skeletal muscle, liver, and heart," *The Journal of Biological Chemistry*, vol. 275, no. 5, pp. 3343–3347, 2000.

[97] G. A. Cortopassi, D. Shibata, N.-W. Soong, and N. Arnheim, "A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues," *Proceedings of the National*

*Academy of Sciences of the United States of America*, vol. 89, no. 16, pp. 7370–7374, 1992.

[98] C. A. Benbassat, K. C. Maki, and T. G. Unterman, "Circulating levels of insulin-like growth factor (IGF) binding protein-1 and -3 in aging men: relationships to insulin, glucose, IGF, and dehydroepiandrosterone sulfate levels and anthropometric measures," *The Journal of Clinical Endocrinology & Metabolism*, vol. 82, no. 5, pp. 1484–1491, 1997.

[99] E. Corpas, S. M. Harman, and M. R. Blackman, "Human growth hormone and human aging," *Endocrine Reviews*, vol. 14, no. 1, pp. 20–39, 1993.

[100] D. Rudman, A. G. Feller, H. S. Nagraj et al., "Effects of human growth hormone in men over 60 years old," *The New England Journal of Medicine*, vol. 323, no. 1, pp. 1–6, 1990.

[101] N. E. Gulcelik, M. Halil, S. Ariogul, and A. Usman, "Adipocytokines and aging: adiponectin and leptin," *Minerva Endocrinologica*, vol. 38, no. 2, pp. 203–210, 2013.

[102] T. Minamino, M. Orimo, I. Shimizu et al., "A crucial role for adipose tissue p53 in the regulation of insulin resistance," *Nature Medicine*, vol. 15, no. 9, pp. 1082–1087, 2009.

[103] R. J. Schulingkamp, T. C. Pagano, D. Hung, and R. B. Raffa, "Insulin receptors and insulin action in the brain: review and clinical implications," *Neuroscience and Biobehavioral Reviews*, vol. 24, no. 8, pp. 855–872, 2000.

[104] L. P. van der Heide, G. M. J. Ramakers, and M. P. Smidt, "Insulin signaling in the central nervous system: learning to survive," *Progress in Neurobiology*, vol. 79, no. 4, pp. 205–221, 2006.

[105] A. R. Cole, A. Astell, C. Green, and C. Sutherland, "Molecular connexions between dementia and diabetes," *Neuroscience and Biobehavioral Reviews*, vol. 31, no. 7, pp. 1046–1063, 2007.

[106] L. Plum, M. Schubert, and J. C. Brüning, "The role of insulin receptor signaling in the brain," *Trends in Endocrinology and Metabolism*, vol. 16, no. 2, pp. 59–65, 2005.

[107] L. Gasparini, W. J. Netzer, P. Greengard, and H. Xu, "Does insulin dysfunction play a role in Alzheimer's disease?" *Trends in Pharmacological Sciences*, vol. 23, no. 6, pp. 288–293, 2002.

[108] D. Bosco, A. Fava, M. Plastino, T. Montalcini, and A. Pujia, "Possible implications of insulin resistance and glucose metabolism in Alzheimer's disease pathogenesis," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 9, pp. 1807–1821, 2011.

[109] X. Fang, S. X. Yu, Y. Lu, R. C. Bast Jr., J. R. Woodgett, and G. B. Mills, "Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 11960–11965, 2000.

[110] B. Kim and E. L. Feldman, "Insulin resistance in the nervous system," *Trends in Endocrinology and Metabolism*, vol. 23, no. 3, pp. 133–141, 2012.

[111] S. Cunnane, S. Nugent, M. Roy et al., "Brain fuel metabolism, aging, and Alzheimer's disease," *Nutrition*, vol. 27, no. 1, pp. 3–20, 2011.

[112] E. C. McNay and A. K. Recknagel, "Brain insulin signaling: a key component of cognitive processes and a potential basis for cognitive impairment in type 2 diabetes," *Neurobiology of Learning and Memory*, vol. 96, no. 3, pp. 432–442, 2011.

[113] Y. T. Wang and M. W. Salter, "Regulation of NMDA receptors by tyrosine kinases and phosphatases," *Nature*, vol. 369, no. 6477, pp. 233–235, 1994.

[114] L. P. van der Heide, A. Kamal, A. Artola, W. H. Gispen, and G. M. J. Ramakers, "Insulin modulates hippocampal activity-dependent synaptic plasticity in a *N*-methyl-D-aspartate receptor and phosphatidyl-inositol-3-kinase-dependent manner," *Journal of Neurochemistry*, vol. 94, no. 4, pp. 1158–1166, 2005.

[115] S. C. Correia, R. X. Santos, G. Perry, X. Zhu, P. IMoreira, and M. A. Smith, "Insulin-resistant brain state: the culprit in sporadic Alzheimer's disease?" *Ageing Research Reviews*, vol. 10, no. 2, pp. 264–273, 2011.

[116] E. Steen, B. M. Terry, E. J. Rivera et al., "Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes?" *Journal of Alzheimer's Disease*, vol. 7, no. 1, pp. 63–80, 2005.

[117] E. J. Rivera, A. Goldin, N. Fulmer, R. Tavares, J. R. Wands, and S. M. de la Monte, "Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine," *Journal of Alzheimer's Disease*, vol. 8, no. 3, pp. 247–268, 2005.

[118] S. Craft, S. Asthana, J. W. Newcomer et al., "Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose," *Archives of General Psychiatry*, vol. 56, no. 12, pp. 1135–1140, 1999.

[119] V. Vingtdeux, P. Davies, D. W. Dickson, and P. Marambaud, "AMPK is abnormally activated in tangle-and pre-tangle-bearing neurons in Alzheimer's disease and other tauopathies," *Acta Neuropathologica*, vol. 121, no. 3, pp. 337–349, 2011.

[120] C. R. Park, R. J. Seeley, S. Craft, and S. C. Woods, "Intracerebroventricular insulin enhances memory in a passive-avoidance task," *Physiology and Behavior*, vol. 68, no. 4, pp. 509–514, 2000.

[121] S. Craft, S. Asthana, G. Schellenberg et al., "Insulin metabolism in Alzheimer's disease differs according to apolipoprotein E genotype and gender," *Neuroendocrinology*, vol. 70, no. 2, pp. 146–152, 1999.

[122] M. A. Reger, G. S. Watson, P. S. Green et al., "Intranasal insulin improves cognition and modulates $\beta$-amyloid in early AD," *Neurology*, vol. 70, no. 6, pp. 440–448, 2008.

[123] M.-A. Cornier, D. Dabelea, T. L. Hernandez et al., "The metabolic syndrome," *Endocrine Reviews*, vol. 29, no. 7, pp. 777–822, 2008.

[124] V. Vu, P. Bui, M. Eguchi, A. Xu, and G. Sweeney, "Globular adiponectin induces LKB1/AMPK-dependent glucose uptake via actin cytoskeleton remodeling," *Journal of Molecular Endocrinology*, vol. 51, no. 1, pp. 155–165, 2013.

[125] J. Song and J. E. Lee, "Adiponectin as a new paradigm for approaching Alzheimer's disease," *Anatomy & Cell Biology*, vol. 46, no. 4, pp. 229–234, 2013.

[126] X. Mao, C. K. Kikani, R. A. Riojas et al., "APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function," *Nature Cell Biology*, vol. 8, no. 5, pp. 516–523, 2006.

[127] T. Yamauchi, J. Kamon, H. Waki et al., "The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity," *Nature Medicine*, vol. 7, no. 8, pp. 941–946, 2001.

[128] M. K. S. Leow, C. L. Addy, and C. S. Mantzoros, "Clinical review 159: human immunodeficiency virus/highly active antiretroviral therapy-associated metabolic syndrome: clinical presentation, pathophysiology, and therapeutic strategies," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 5, pp. 1961–1976, 2003.

[129] N. Kubota, Y. Terauchi, T. Yamauchi et al., "Disruption of adiponectin causes insulin resistance and neointimal formation," *The Journal of Biological Chemistry*, vol. 277, no. 29, pp. 25863–25866, 2002.

[130] M. Okamoto, M. Ohara-Imaizumi, N. Kubota et al., "Adiponectin induces insulin secretion in vitro and in vivo at a low glucose concentration," *Diabetologia*, vol. 51, no. 5, pp. 827–835, 2008.

[131] N. Yamaguchi, T. Kukita, Y.-J. Li et al., "Adiponectin inhibits osteoclast formation stimulated by lipopolysaccharide from *Actinobacillus actinomycetemcomitans*," *FEMS Immunology & Medical Microbiology*, vol. 49, no. 1, pp. 28–34, 2007.

[132] A. Hofman, A. Ott, M. M. B. Breteler et al., "Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam study," *The Lancet*, vol. 349, no. 9046, pp. 151–154, 1997.

[133] F. A. McAlister, K. B. Zarnke, N. R. C. Campbell et al., "The 2001 Canadian recommendations for the management of hypertension—part two: therapy," *Canadian Journal of Cardiology*, vol. 18, no. 6, pp. 625–641, 2002.

[134] K. Jandeleit-Dahm and M. E. Cooper, "Hypertension and diabetes," *Current Opinion in Nephrology and Hypertension*, vol. 11, no. 2, pp. 221–228, 2002.

[135] R. W. Schrier, R. O. Estacio, A. Esler, and P. Mehler, "Effects of aggressive blood pressure control in normotensive type 2 diabetic patients on albuminuria, retinopathy and strokes," *Kidney International*, vol. 61, no. 3, pp. 1086–1097, 2002.

[136] E. C. McNay, "The impact of recurrent hypoglycemia on cognitive function in aging," *Neurobiology of Aging*, vol. 26, supplement 1, pp. S76–S79, 2005.

[137] C. Weyer, R. L. Hanson, P. A. Tataranni, C. Bogardus, and R. E. Pratley, "A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia," *Diabetes*, vol. 49, no. 12, pp. 2094–2101, 2000.

[138] D. J. Cox, B. P. Kovatchev, L. A. Gonder-Frederick et al., "Relationships between hyperglycemia and cognitive performance among adults with type 1 and type 2 diabetes," *Diabetes Care*, vol. 28, no. 1, pp. 71–77, 2005.

[139] K. Yaffe, T. Blackwell, A. M. Kanaya, N. Davidowitz, E. Barrett-Connor, and K. Krueger, "Diabetes, impaired fasting glucose, and development of cognitive impairment in older women," *Neurology*, vol. 63, no. 4, pp. 658–663, 2004.

[140] A. Imagawa, T. Funahashi, T. Nakamura et al., "Elevated serum concentration of adipose-derived factor, adiponectin, in patients with type 1 diabetes," *Diabetes Care*, vol. 25, no. 9, pp. 1665–1666, 2002.

[141] M. Zhu, J. Miura, L. X. Lu et al., "Circulating adiponectin levels increase in rats on caloric restriction: the potential for insulin sensitization," *Experimental Gerontology*, vol. 39, no. 7, pp. 1049–1059, 2004.

[142] N. Stefan, J. C. Bunt, A. D. Salbe, T. Funahashi, Y. Matsuzawa, and P. Antonio Tataranni, "Plasma adiponectin concentrations in children: relationships with obesity and insulinemia," *The Journal of Clinical Endocrinology & Metabolism*, vol. 87, no. 10, pp. 4652–4656, 2002.

[143] J. Krakoff, T. Funahashi, C. D. A. Stehouwer et al., "Inflammatory markers, adiponectin, and risk of type 2 diabetes in the Pima Indian," *Diabetes Care*, vol. 26, no. 6, pp. 1745–1751, 2003.

[144] R. S. Lindsay, T. Funahashi, R. L. Hanson et al., "Adiponectin and development of type 2 diabetes in the Pima Indian population," *The Lancet*, vol. 360, no. 9326, pp. 57–58, 2002.

[145] D. C. W. Lau, B. Dhillon, H. Yan, P. E. Szmitko, and S. Verma, "Adipokines: molecular links between obesity and atheroslcerosis," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 288, no. 5, pp. H2031–H2041, 2005.

[146] K. Hotta, T. Funahashi, Y. Arita et al., "Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 6, pp. 1595–1599, 2000.

[147] A. J. Hanley, L. E. Wagenknecht, J. M. Norris et al., "Adiponectin and the incidence of type 2 diabetes in Hispanics and African Americans: the IRAS Family study," *Diabetes Care*, vol. 34, no. 10, pp. 2231–2236, 2011.

[148] K. Hotta, T. Funahashi, N. L. Bodkin et al., "Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys," *Diabetes*, vol. 50, no. 5, pp. 1126–1133, 2001.

[149] J. V. Tu, "Reducing the global burden of stroke: INTERSTROKE," *The Lancet*, vol. 376, no. 9735, pp. 74–75, 2010.

[150] S. MacMahon, R. Peto, J. Cutler et al., "Blood pressure, stroke, and coronary heart disease—part 1: prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias," *The Lancet*, vol. 335, no. 8692, pp. 765–774, 1990.

[151] M. F. O'Rourke and M. E. Safar, "Relationship between aortic stiffening and microvascular disease in brain and kidney: cause and logic of therapy," *Hypertension*, vol. 46, no. 1, pp. 200–204, 2005.

[152] C. Qiu, B. Winblad, and L. Fratiglioni, "The age-dependent relation of blood pressure to cognitive function and dementia," *The Lancet Neurology*, vol. 4, no. 8, pp. 487–499, 2005.

[153] J. A. Staessen, T. Richart, and W. H. Birkenhäger, "Less atherosclerosis and lower blood pressure for a meaningful life perspective with more brain," *Hypertension*, vol. 49, no. 3, pp. 389–400, 2007.

[154] M. Nagai, S. Hoshide, and K. Kario, "Hypertension and dementia," *American Journal of Hypertension*, vol. 23, no. 2, pp. 116–124, 2010.

[155] L. J. Launer, G. W. Ross, H. Petrovitch et al., "Midlife blood pressure and dementia: the Honolulu-Asia Aging study," *Neurobiology of Aging*, vol. 21, no. 1, pp. 49–55, 2000.

[156] L. J. Launer, K. Masaki, H. Petrovitch, D. Foley, and R. J. Havlik, "The association between midlife blood pressure levels and late-life cognitive function: the Honolulu-Asia Aging study," *The Journal of the American Medical Association*, vol. 274, no. 23, pp. 1846–1851, 1995.

[157] K. A. Jellinger and J. Attems, "Prevalence of dementia disorders in the oldest-old: an autopsy study," *Acta Neuropathologica*, vol. 119, no. 4, pp. 421–433, 2010.

[158] P. T. Nelson, E. Head, F. A. Schmitt et al., "Alzheimer's disease is not "brain aging": neuropathological, genetic, and epidemiological human studies," *Acta Neuropathologica*, vol. 121, no. 5, pp. 571–587, 2011.

[159] S. R. Waldstein, S. C. Rice, J. F. Thayer, S. S. Najjar, A. Scuteri, and A. B. Zonderman, "Pulse pressure and pulse wave velocity are related to cognitive decline in the Baltimore Longitudinal Study of Aging," *Hypertension*, vol. 51, no. 1, pp. 99–104, 2008.

[160] C. Qiu, B. Winblad, M. Viitanen, and L. Fratiglioni, "Pulse pressure and risk of alzheimer disease in persons aged 75 years and older: a community-based, longitudinal study," *Stroke*, vol. 34, no. 3, pp. 594–599, 2003.

[161] A. Y. Lee, S.-H. Jeong, B. H. Choi, E. H. Sohn, and H. Chui, "Pulse pressure correlates with leukoaraiosis in Alzheimer disease," *Archives of Gerontology and Geriatrics*, vol. 42, no. 2, pp. 157–166, 2006.

[162] D. A. Nation, C. E. Wierenga, L. Delano-Wood et al., "Elevated pulse pressure is associated with age-related decline in language ability," *Journal of the International Neuropsychological Society*, vol. 16, no. 5, pp. 933–938, 2010.

[163] I. Skoog, B. Lernfelt, S. Landahl et al., "15-year longitudinal study of blood pressure and dementia," *The Lancet*, vol. 347, no. 9009, pp. 1141–1145, 1996.

[164] L. Molander, Y. Gustafson, and H. Lövheim, "Longitudinal associations between blood pressure and dementia in the very old," *Dementia and Geriatric Cognitive Disorders*, vol. 30, no. 3, pp. 269–276, 2010.

[165] P. L. Huang, Z. Huang, H. Mashimo et al., "Hypertension in mice lacking the gene for endothelial nitric oxide synthase," *Nature*, vol. 377, no. 6546, pp. 239–242, 1995.

[166] T. J. Guzik, E. Black, and N. E. West, "Relationship between the G894T polymorphism (Glu 298 Asp variant) in endothelial nitric oxide synthase and nitric oxide-mediated endothelial function in human atherosclerosis," *American Journal of Medical Genetics*, vol. 100, no. 2, pp. 130–137, 2001.

[167] W. Zhu, K. K. Y. Cheng, P. M. Vanhoutte, K. S. L. Lam, and A. Xu, "Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention," *Clinical Science*, vol. 114, no. 5-6, pp. 361–374, 2008.

[168] R. Ouedraogo, Y. Gong, B. Berzins et al., "Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo," *The Journal of Clinical Investigation*, vol. 117, no. 6, pp. 1718–1726, 2007.

[169] R. Shibata, N. Ouchi, S. Kihara, K. Sato, T. Funahashi, and K. Walsh, "Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of AMP-activated protein kinase signaling," *The Journal of Biological Chemistry*, vol. 279, no. 27, pp. 28670–28674, 2004.

[170] H. Chen, M. Montagnani, T. Funahashi, I. Shimomura, and M. J. Quon, "Adiponectin stimulates production of nitric oxide in vascular endothelial cells," *The Journal of Biological Chemistry*, vol. 278, no. 45, pp. 45021–45026, 2003.

[171] N. Ouchi, H. Kobayashi, S. Kihara et al., "Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells," *The Journal of Biological Chemistry*, vol. 279, no. 2, pp. 1304–1309, 2004.

[172] H. Kobayashi, N. Ouchi, S. Kihara et al., "Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin," *Circulation Research*, vol. 94, no. 4, pp. e27–e31, 2004.

[173] A. Takahashi, Y. Kureishi, J. Yang et al., "Myogenic Akt signaling regulates blood vessel recruitment during myofiber growth," *Molecular and Cellular Biology*, vol. 22, no. 13, pp. 4803–4814, 2002.

[174] K. Mahadev, X. Wu, S. Donnelly, R. Ouedraogo, A. D. Eckhart, and B. J. Goldstein, "Adiponectin inhibits vascular endothelial growth factor-induced migration of human coronary artery endothelial cells," *Cardiovascular Research*, vol. 78, no. 2, pp. 376–384, 2008.

[175] B. J. Goldstein, R. G. Scalia, and X. L. Ma, "Protective vascular and myocardial effects of adiponectin," *Nature Clinical Practice: Cardiovascular Medicine*, vol. 6, no. 1, pp. 27–35, 2009.

[176] M. Takaoka, D. Nagata, S. Kihara et al., "Periadventitial adipose tissue plays a critical role in vascular remodeling," *Circulation Research*, vol. 105, no. 9, pp. 906–911, 2009.

[177] B. J. Goldstein and R. Scalia, "Adiponectin: a novel adipokine linking adipocytes and vascular function," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 6, pp. 2563–2568, 2004.

[178] T. G. Beach, J. R. Wilson, L. I. Sue et al., "Circle of Willis atherosclerosis: association with Alzheimer's disease, neuritic plaques and neurofibrillary tangles," *Acta Neuropathologica*, vol. 113, no. 1, pp. 13–21, 2007.

[179] H. C. Stary, "Natural history and histological classification of atherosclerotic lesions an update," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 20, no. 5, pp. 1177–1178, 2000.

[180] H. C. Stary, A. B. Chandler, R. E. Dinsmore et al., "A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association," *Circulation*, vol. 92, no. 5, pp. 1355–1374, 1995.

[181] E. S. C. Korf, L. R. White, P. Scheltens, and L. J. Launer, "Midlife blood pressure and the risk of hippocampal atrophy: the Honolulu Asia Aging study," *Hypertension*, vol. 44, no. 1, pp. 29–34, 2004.

[182] J.-O. Deguchi, E. Aikawa, P. Libby et al., "Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques," *Circulation*, vol. 112, no. 17, pp. 2708–2715, 2005.

[183] J.-O. Deguchi, M. Aikawa, C.-H. Tung et al., "Inflammation in atherosclerosis: visualizing matrix metalloproteinase action in macrophages in vivo," *Circulation*, vol. 114, no. 1, pp. 55–62, 2006.

[184] C. M. Dollery, C. A. Owen, G. K. Sukhova, A. Krettek, S. D. Shapiro, and P. Libby, "Neutrophil elastase in human atherosclerotic plaques production by macrophages," *Circulation*, vol. 107, no. 22, pp. 2829–2836, 2003.

[185] S. Larionov, O. Dedeck, G. Birkenmeier, and D. R. Thal, "Expression of $\alpha$2-macroglobulin, neutrophil elastase, and interleukin-1$\alpha$ differs in early-stage and late-stage atherosclerotic lesions in the arteries of the circle of Willis," *Acta Neuropathologica*, vol. 113, no. 1, pp. 33–43, 2007.

[186] J. Liang, E. Liu, Y. Yu et al., "Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits," *Circulation*, vol. 113, no. 16, pp. 1993–2001, 2006.

[187] S. H. Han, M. J. Quon, J.-A. Kim, and K. K. Koh, "Adiponectin and cardiovascular disease: response to therapeutic interventions," *Journal of the American College of Cardiology*, vol. 49, no. 5, pp. 531–538, 2007.

[188] N. Ouchi, S. Kihara, Y. Arita et al., "Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-$\kappa$B signaling through a cAMP-dependent pathway," *Circulation*, vol. 102, no. 11, pp. 1296–1301, 2000.

[189] N. Ouchi, S. Kihara, Y. Arita et al., "Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin," *Circulation*, vol. 100, no. 25, pp. 2473–2476, 1999.

[190] N. Ouchi, S. Kihara, Y. Arita et al., "Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages," *Circulation*, vol. 103, no. 8, pp. 1057–1063, 2001.

[191] L. M. Allan, E. N. Rowan, M. J. Firbank et al., "Long term incidence of dementia, predictors of mortality and pathological

diagnosis in older stroke survivors," *Brain*, vol. 134, no. 12, pp. 3716–3727, 2011.

[192] D. Leys, H. Hénon, M.-A. Mackowiak-Cordoliani, and F. Pasquier, "Poststroke dementia," *The Lancet Neurology*, vol. 4, no. 11, pp. 752–759, 2005.

[193] S. T. Pendlebury and P. M. Rothwell, "Prevalence, incidence, and factors associated with pre-stroke and post-stroke dementia: a systematic review and meta-analysis," *The Lancet Neurology*, vol. 8, no. 11, pp. 1006–1018, 2009.

[194] C. Reitz, M. J. Bos, A. Hofman, P. J. Koudstaal, and M. M. B. Breteler, "Prestroke cognitive performance, incident stroke, and risk of dementia: the Rotterdam study," *Stroke*, vol. 39, no. 1, pp. 36–41, 2008.

[195] P. Schaapsmeerders, N. A. Maaijwee, E. J. van Dijk et al., "Long-term cognitive impairment after first-ever ischemic stroke in young adults," *Stroke*, vol. 44, no. 6, pp. 1621–1628, 2013.

[196] E. Kokmen, J. P. Whisnant, W. M. O'Fallon, C.-P. Chu, and C. M. Beard, "Dementia after ischemic stroke: a population-based study in Rochester, Minnesota (1960–1984)," *Neurology*, vol. 46, no. 1, pp. 154–159, 1996.

[197] I. Grant, J. Rochford, T. Fleming, and A. Stunkard, "A neuropsychological assessment of the effects of moderate marihuana use," *Journal of Nervous and Mental Disease*, vol. 156, no. 4, pp. 278–280, 1973.

[198] D. J. Stott, P. Welsh, A. Rumley et al., "Adipocytokines and risk of stroke in older people: a nested case-control study," *International Journal of Epidemiology*, vol. 38, no. 1, pp. 253–261, 2009.

[199] C. Prugger, G. Luc, B. Haas et al., "Adipocytokines and the risk of ischemic stroke: the PRIME study," *Annals of Neurology*, vol. 71, no. 4, pp. 478–486, 2012.

[200] C. Savopoulos, K. Michalakis, M. Apostolopoulou, A. Miras, and A. Hatzitolios, "Adipokines and stroke: a review of the literature," *Maturitas*, vol. 70, no. 4, pp. 322–327, 2011.

[201] B. J. Kim, S.-H. Lee, W.-S. Ryu, C. K. Kim, and B.-W. Yoon, "Adipocytokines and ischemic stroke: differential associations between stroke subtypes," *Journal of the Neurological Sciences*, vol. 312, no. 1-2, pp. 117–122, 2012.

[202] Y. Nakamura, K. Shimada, D. Fukuda et al., "Implications of plasma concentrations of adiponectin in patients with coronary artery disease," *Heart*, vol. 90, no. 5, pp. 528–533, 2004.

[203] F. Otsuka, S. Sugiyama, S. Kojima et al., "Plasma adiponectin levels are associated with coronary lesion complexity in men with coronary artery disease," *Journal of the American College of Cardiology*, vol. 48, no. 6, pp. 1155–1162, 2006.

[204] M. Kumada, S. Kihara, S. Sumitsuji et al., "Association of hypoadiponectinemia with coronary artery disease in men," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 1, pp. 85–89, 2003.

[205] R. Shibata, K. Sato, D. R. Pimentel et al., "Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms," *Nature Medicine*, vol. 11, no. 10, pp. 1096–1103, 2005.

[206] L. Tao, X. Jiao, E. Gao et al., "Nitrative inactivation of thioredoxin-1 and its role in postischemic myocardial apoptosis," *Circulation*, vol. 114, no. 13, pp. 1395–1402, 2006.

[207] R. Shibata, N. Ouchi, M. Ito et al., "Adiponectin-mediated modulation of hypertrophic signals in the heart," *Nature Medicine*, vol. 10, no. 12, pp. 1384–1389, 2004.

[208] Y. S. Jung, S. K. Ha, S. D. Kim, S. H. Kim, D. J. Lim, and J. I. Choi, "The role of adiponectin in secondary inflammatory reaction in cerebral ischemia," *Journal of Cerebrovascular and Endovascular Neurosurgery*, vol. 15, no. 3, pp. 171–176, 2013.

[209] M. Nishimura, Y. Izumiya, A. Higuchi et al., "Adiponectin prevents cerebral ischemic injury through endothelial nitric oxide synthase-dependent mechanisms," *Circulation*, vol. 117, no. 2, pp. 216–223, 2008.

[210] W. Song, T. Huo, F. Guo et al., "Globular adiponectin elicits neuroprotection by inhibiting NADPH oxidase-mediated oxidative damage in ischemic stroke," *Neuroscience*, vol. 248, pp. 136–144, 2013.

[211] L. Shen, J. Miao, F. Yuan et al., "Overexpression of adiponectin promotes focal angiogenesis in the mouse brain following middle cerebral artery occlusion," *Gene Therapy*, vol. 20, no. 1, pp. 93–101, 2013.

[212] D. Nagata, M. Mogi, and K. Walsh, "AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress," *The Journal of Biological Chemistry*, vol. 278, no. 33, pp. 31000–31006, 2003.

[213] N. Ouchi, R. Shibata, and K. Walsh, "AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle," *Circulation Research*, vol. 96, no. 8, pp. 838–846, 2005.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 601084, 12 pages
http://dx.doi.org/10.1155/2014/601084

*Review Article*

# Potential Therapeutic Effects of Neurotrophins for Acute and Chronic Neurological Diseases

**Junying Cai,[1] Fuzhou Hua,[1] Linhui Yuan,[1] Wei Tang,[2] Jun Lu,[1] Shuchun Yu,[1] Xifeng Wang,[3] and Yanhui Hu[1]**

[1] *Department of Anesthesiology, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China*
[2] *Department of Urology, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China*
[3] *Department of Anesthesiology, The first Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China*

Correspondence should be addressed to Yanhui Hu; juce102323@163.com

Received 25 February 2014; Accepted 25 March 2014; Published 9 April 2014

Academic Editor: Jian Xiao



The neurotrophins (NTs) nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 are proteins that regulate cell proliferation, differentiation, and survival in both the developing and mature central nervous system (CNS) by binding to two receptor classes, Trk receptors and p75 NTR. Motivated by the broad growth- and survival-promoting effects of these proteins, numerous studies have attempted to use exogenous NTs to prevent the death of cells that are associated with neurological disease or promote the regeneration of severed axons caused by mechanical injury. Indeed, such neurotrophic effects have been repeatedly demonstrated in animal models of stroke, nerve injury, and neurodegenerative disease. However, limitations, including the short biological half-lives and poor blood-brain permeability of these proteins, prevent routine application from treating human disease. In this report, we reviewed evidence for the neuroprotective efficacy of NTs in animal models, highlighting outstanding technical challenges and discussing more recent attempts to harness the neuroprotective capacity of endogenous NTs using small molecule inducers and cell transplantation.

## 1. Introduction: Current Challenges in the Clinical Use of Neurotrophins

Preclinical research over the past 3 decades has described in immense detail the signaling pathways that lead to neuronal cell death [1, 2]. Moreover, a plethora of neuroprotective strategies have been developed for ameliorating brain damage and preserving or restoring neurological function in animal models of stroke, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and other neurological disorders [3–5]. Such treatments include the administration of neurotrophic factors, which are endogenous proteins critical for proliferation, differentiation, and survival during development and neuroplasticity throughout life [6, 7]. Indeed, brain delivery of exogenous neurotrophic factors, such as the neurotrophin nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), can reduce infarct volumes by 60–90% with near total restoration of behavioral function in experimental stroke models.

Despite this success in the laboratory, there are currently no broadly effective treatments to reverse or halt the progression of these diseases in patients.

Numerous theories have been advanced to explain the failure of neurotrophins in clinical trials, including limited access of peripherally administered neurotrophins to the central nerve system (CNS), the short biological half-lives of neurotrophins, the multimodal nature of disease progression, and the relatively short temporal window in which such treatments are effective (at least for acute neural insults such as stroke) [8]. For example, antistroke therapies must be instituted within 1–6 h of the event for significant efficacy, and few stroke patients can be treated within this timeframe. Thus, prophylactic strategies that upregulate endogenous protective capacity may be needed.

In recent years, methods for delivery of neurotrophins across the blood brain barrier (BBB) have advanced rapidly, as has the development of smaller neurotrophin receptor (Trk) agonists with significantly longer biological half-lives and BBB permeability than native neurotrophins like BDNF (serum half-life of ≈10 min) [9]. Perhaps the most difficult problem is that of delivering these molecules to appropriate targets, such as astrocyte cells, microglia cells, and vascular endothelial cells as well as neurons, while limiting exposure to undamaged brain tissue.

Despite these challenges, we may be close to developing neurotrophin receptor agonists and delivery systems that allow for rapid brain penetration to protect vulnerable human neurons from cell death. This goal may be facilitated by the fact that the many distinct pathogenic processes associated with stroke, trauma, and neurodegenerative diseases appear to converge on several mutually reinforcing final common pathways: excitotoxicity by excess glutamate receptor stimulation, intracellular calcium overload, oxidative stress, mitochondrial failure, and apoptosis. Here, we review evidence that neurotrophic factors can prevent neuronal death in acute and chronic brain diseases and highlight the remaining problems that have thus far prevented clinical application.

## 2. Neurotrophin-Mediated Protection against Acute Neural Insult

*2.1. Direct Neurotrophin Administration Reduces Stroke Damage.* Numerous studies have demonstrated that neurotrophins, particularly BDNF, reduce infarct volume in rodents when administered before, during, and/or after experimental stroke [10]. These studies have used various strategies to enhance the level of BDNF in brain. Intraventricular injection of BDNF for 8 days at the beginning of 24 hours before permanent middle cerebral artery occlusion (MCAO) in Wistar rats was found to improve neurological deficits [11]. Similarly, BDNF was delivered into the territory of the MCA by an osmotic minipump shortly after occlusion reduced cortical infarct volume by 37% [12]. Infusion of recombinant BDNF into Sprague-Dawley rat neocortex by an implanted osmotic minipump 2.5–14 days before, during, and for 2 days following MCAO reduced infarct volume as measured 2 days after stroke without affecting cerebral blood flow [13]. Continuous BDNF infusion immediately following 2 h right MCAO in rats reduced both cortical and subcortical infarct volume as well as the number of neurons in the ischemic penumbra expressing the proapoptotic factor Bax, while increasing the number of neurons expressing antiapoptotic Bcl-2 [14]. It was found that hippocampal CA1 pyramidal neurons were rescued by intraventricular injection of a viral vector encoding BDNF, as well as similar vectors encoding glial-derived neurotrophic factor (GDNF), NGF, insulin-like growth factor-1 (IGF-1), or vascular endothelial growth factor (VEGF) 30 min after the ischemic insult [15]. Transplantation of fresh bone marrow (BM) together with BDNF into the ischemic boundary zone (IBZ) of the rat brain following MCAO facilitated functional recovery of sensory and motor functions [16]. The effects of BDNF on infarct size were synergistic with brain hypothermia, another well-described antistroke treatment. Furthermore, postischemic striatal glutamate concentrations were reduced to a greater extent by combining BDNF and hypothermia than for either treatment alone [17].

Transplantation of choroid plexus (CP), which is known to secrete a variety of growth factors including BDNF, GDNF, and NGF, reduced infarct volume in rats [18]. Injection of neural progenitors into the hippocampus immediately after cerebral ischemia increased spatial memory performance in the Morris water maze (MWM) 12–28 days after cerebral ischemia and reduced infarct volume. Injection of neural progenitor cells (NPCs) also reversed the postischemic decline in BDNF expression [19]. Sodium orthovanadate protected cortical and hippocampal neurons after experimental subarachnoid hemorrhage by increasing BDNF and inhibiting BDNF receptor TrkB inactivation, effects that were abolished by pretreatment with the TrkB inhibitor K252a [20].

While most of these studies examined infarct formation or neurological recovery in the early period following ischemia, a more recent study demonstrated that transplantation of neural stem cells (NSCs) overexpressing BDNF improved neurological function as late as 12 weeks after MCAO [21]. In addition to grey matter damage, BDNF may also reduce damage caused by lacunar-type stroke in primates following occlusion of the deep subcortical penetrating arteries. Moreover, expression of BDNF was stimulated in the affected white matter following ischemic injury [22]. In a similar study, BDNF did not reduce infarct volume but still resulted in greater functional recovery following ischemia compared to controls. In addition, this treatment reduced astrogliosis, a process that may impede long-term recovery by blocking regrowth of axons [23].

Conversely, mice lacking a single BDNF allele or both neurotrophin-4 alleles ($nt4^{-}/^{-}$) exhibited larger cerebral infarcts than wild-type littermates [24]. Transgenic mice overexpressing the dominant-negative truncated variant of the BDNF receptor (TrkB.T1) specifically in cortical and hippocampal neurons exhibited greater damage in the cortex but not in regions (striatum) without TrkB.T1 transgene expression [25]. Infusion of an antisense BDNF oligonucleotide for 28 days after unilateral cortical ischemia blocked BDNF mRNA expression and negated the beneficial effects of motor rehabilitation on the recovery of skilled reaching by the contralateral limb but had no effect on motor function of the ipsilateral limb [26].

While neurotrophins are broadly neuroprotective against experimental stoke, several studies have shown greater efficacy of one particular neurotrophin over others as well as superior protection in specific brain regions. Infusion of BDNF into the substantia nigra (SN) had no effect on survival of nigrostriatal projection neurons after stroke and even exacerbated death of some striatal cholinergic and GABAergic interneurons [27]. Conversely, BDNF may be more effective than other neurotrophins against stroke-induced damage in the cortex. Mesenchymal stem cells (MSCs) overexpressing either BDNF or GDNF improved functional outcome as revealed by MRI one and two weeks after MCAO, while MSCs overexpressing the neurotrophin NT-3 did not [28]. Thus, region-specific administration may be necessary for full

efficacy, and this constitutes a major hurdle for noninvasive peripheral administration.

*2.2. Neurotrophins Mediate the Protective Efficacy of Other Treatments.* The neuroprotective efficacies of many other manipulations that rescue neurons from stroke damage are mediated at least in part by enhanced expression of neurotrophins, particularly BDNF. For example, brief daily treadmill running for 3 weeks prior to stroke reduced infarct volumes in the frontoparietal cortex and dorsolateral striatum, and these effects were correlated with upregulation of NGF and BDNF in cortical neurons and striatal glia. In addition, there was a significant increase in microvessel density in the striatum [29]. The neuroprotective effects of fibroblast growth factor 2 (FGF-2) following MCAO may also be mediated in part by BDNF; FGF-2 null mice exhibited dramatically larger infarct volumes than wild-type littermates and a diminished postischemic induction of hippocampal BDNF and TrkB mRNA [30]. Cortical spreading depression (SD), which is a wave of depolarization followed by neuronal quiescence, can induce a preconditioning effect involving BDNF expression. Induced SD for 6 or 9 days prior to experimental focal ischemia reduced infarct volume and resulted in less severe neurological deficits both at one and 14 days after ischemia. This preconditioning effect was not observed in heterozygous BDNF-deficient mice [31]. Preconditioning can also be achieved by sublethal doses of N-methyl D-asparate (NMDA), which increased hippocampal CA1 neuron survival after ischemia, an effect blocked by the TrkB antagonist K252a [32]. Lithium is another widely studied neuroprotectant that requires BDNF/TrkB signaling [33]. Lithium-mediated neuroprotection involves GSK-3 inhibition, which in turn leads to enhanced BDNF expression [34]. Atorvastatin (Lipitor) treatment starting 24 h after MCAO also improved functional recovery and concomitantly increased BDNF expression (as well as VEGF and VEGFR2) at the ischemic border. In addition, atorvastatin treatment increased the number of migrating neurons, synaptophysin-positive cells, and angiogenesis around the infarct. *In vitro*, atorvastatin enhanced the migration of subventricular zone (SVZ) cells, an effect inhibited by a BDNF antibody [35].

In addition to invasive methods like cell therapy, brain concentrations of BDNF can be elevated by orally administered agents. The rennin-angiotensin system inhibitor candesartan was found to reduce stroke volume, improve neurological outcome, and promote TrkB expression [36]. Inhibitors of nitric oxide production may also be effective. The endothelial cell isoform of nitric oxide synthase (eNOS) protected against stroke through BDNF upregulation [37]; eNOS knockout mice exhibited more severe deficits than wild-type mice following permanent right MCAO, as well as reduced progenitor cell proliferation and migration from the SVZ and decreased angiogenesis at the ischemic boarder. These changes were associated with lower postischemic BDNF (but no change in VEGF and bFGF expression). Moreover, neurosphere formation and proliferation and neurite outgrowth were reduced in cultured SVZ cells derived from eNOS$^{-/-}$ mice, characteristics reversed by BDNF administration [37]. Treadmill exercise prior to experimental ischemia improved motor performance and upregulated BDNF and TrkB expression in the contralateral hemisphere [38]. Significantly, enhanced BDNF only occurred following voluntary exercise, possibly because "forced" exercise enhances serum corticosterone, which may reduce neurotrophin expression [39]. Allosteric AMPAR modulators enhanced limb control when administered several days after stroke, but impaired recovery when administered immediately. Inhibition of BDNF function in the cortex preinfarct blocked this motor rescue [40].

Note, however, that other neuroprotective effects appear independently of neurotrophin expression. Preconditioning by sublethal MCAO 3 days prior to (normally) neurotoxic MCAO protected rats but did not alter expression of most neurotrophins, though BDNF expression was transiently reduced [41]. Housing rats in an enriched environment after experimental stroke was found to enhance recovery, but this was associated with a decrease in BDNF mRNA and protein expression, and enriched BDNF(+/−) mice showed improved motor function on the rotorod two weeks after transient MCAO compared to wild-type mice [42]. Enriched environments thus appear to enhance functional recovery after ischemic stroke independently of BDNF [43].

*2.3. Novel Neurotrophin Delivery, Induction, and Targeting Methods.* Several novel delivery systems may allow for noninvasive neurotrophin administration [44]. A single systemic administration of BDNF conjugated to the OX26 murine monoclonal antibody against the transferrin receptor, which facilitates transport across the BBB via the transferrin receptor transcytosis system, reduced infarct volume by 70% (although it had no effect on subcortical stroke volume) [45]. In another study, BDNF conjugated to a transferrin receptor antibody reduced infarct volume by 65–70% and improved rotorod performance [46]. High-voltage electric potential (HELP) stimulation of adult mice also enhanced BDNF levels, increased resistance to cerebral infarction, and improved escape latency in the MWM [47]. BDNF engineered to bind laminin, which is highly expressed in the ischemic brain, reduced infarct volume and improved neurological function as well as neurogenesis in the dentate gyrus [48]. Intranasal BDNF 2 h after reperfusion reduced the number of TUNEL-positive (apoptotic) neurons (but not infarct volume). This BDNF application also enhanced microglial activation (as indicated by OX-42 expression) and the number of phagocytotic microglia (ED1-positive), suppressed tumor necrosis factor-$\alpha$ expression, and enhanced expression of the anti-inflammatory cytokine interleukin-10 [49].

In addition to novel delivery/targeting systems for native BDNF, there have been extensive efforts to develop small mimetics with superior pharmacokinetic properties and BBB permeability [9]. For example, 7,8-dihydroxyflavone, a high-affinity TrkB agonist, was found to inhibit kainic acid-induced toxicity, decrease infarct volume following experimental stroke in a TrkB-dependent manner, and be

neuroprotective in an animal model of Parkinson's disease [50].

*2.4. Neuroprotection by Natural Neurotrophin Inducers.* Several natural compounds have demonstrated neuroprotective efficacy mediated in part by BDNF upregulation. The plant flavonoids baicalin and jasminoidin in combination promoted BDNF expression and inhibited the apoptotic effector caspase-3 [51]. Niacin also increased BDNF and TrkB expression in neurons and reduced infarct volume *in vivo* following experimental stroke [52]. Infusion of galectin-1, an endogenous mammalian lectin, enhanced the expression and secretion of astrocytic BDNF, reduced the number of apoptotic neurons at the ischemic boundary, and improved functional recovery after experimental stroke [53]. p-Hydroxybenzyl alcohol (HBA), an active component of gastrodia elata blume, administered 1 h before MCAO, decreased infarct volume, promoted functional recovery as measured by the modified neurological severity score, and enhanced Nrf2, BDNF, GDNF, and MBP expression levels [54].

*2.5. Mechanisms of Neurotrophin-Mediated Neuroprotection.* Oxidative stress and intracellular-free calcium dysregulation are mutually reinforcing early events that lead to neuronal death following stroke [2]. Thus, much stroke research has focused on developing strategies to reduce free radical accumulation, augment cellular antioxidant defenses, chelate intracellular calcium, or interfere with upstream events such as excess glutamate receptor stimulation (excitotoxicity) in the ischemic brain [3]. There is an emerging consensus that inflammation is also critical in stroke pathogenesis [5], indicating that anti-inflammatory drugs are feasible therapeutic options. It is thus noteworthy that neurotrophins like BDNF can stabilize intracellular calcium, induce antioxidant enzyme expression, and suppress the release of proinflammatory cytokines [4, 55]. One mechanism of BDNF-induced protection relevant to both acute and chronic models of neurological disease is preservation of mitochondrial function. BDNF (but not GDNF or NGF) substantially increased the respiratory control index (RCI, a measure of respiratory coupling efficiency and ATP synthesis) in synaptosomes [56] but not in pure mitochondria nor in synaptosomes treated with an inhibitor of complex I, suggesting that activity of a TrkB-associated signaling pathway is necessary. Indeed, the effect was also blocked by inhibitors of the MEK-Bcl-2 pathway, the major pathway implicated in BDNF-TrkB-dependent neuroprotection [56].

Another possible mechanism for these neuroprotective effects is enhanced neurogenesis and migration of neural precursor cells (NPCs) from the SVZ. Daily intravenous bolus applications of BDNF after stroke resulted in significantly improved sensorimotor scores 6 weeks after stoke as well as enhanced neurogenesis in the DG and SVZ [57]. Continuous intraventricular BDNF infusion for two weeks enhanced the number of proliferating (doublecortin-positive) SVZ cells surrounding the lesion 14 and 42 days after stoke [58]. Mobilization of this endogenous cell pool reduces stroke damage. Blockade of NPC proliferation by infusion of cytosine arabinoside (Ara-C) for the first 7 days after brain ischemia enlarged the infarct volume and exacerbated postischemic neurological deficits. Moreover, if BDNF and VEGF were inactivated, NSCs cultured in the ischemic brain conditioned media lost neuroprotective efficacy [59]. Transplantation of human bone marrow stem cells (BMSCs) in rats after MCAO resulted in elevated BDNF, NT-3, and VEGF, enhanced endogenous NPC proliferation in both the SVZ and hippocampal subgranular zone (SGZ), and promoted NPC migration to the ischemic boundary [60].

*2.6. Endogenous Signaling Pathways for Neurotrophin Induction.* BDNF is widely expressed throughout the CNS and by most cell types, including neurons, astrocytes, microglia, and vascular endothelial cells. The classical induction mechanism involves calcium influx and phosphoactivation of the transcription factor CREB. CREB can be activated by factors released by the ischemic brain, thereby coupling BDNF synthesis and release to metabolic status. The phosphodiesterase inhibitor cilostazol increased the number of doublecortin-positive NPCs expressing phosphoactivated CREB (pCREB) and BDNF-expressing astrocytes in the ipsilateral SVZ and peri-infarct area [61]. Bone-marrow-derived stem cells cultured in ischemic brain-conditioned medium for 12 h—but not BMCs cultured in media from normoxic brain—were transformed from polygonal to fibroblast-like. This morphological change was associated with induction of 7 neurotrophic and growth factor genes and 5 receptor genes—that is, FGF-2, ILG-1, VEGF A, NGFb, and BDNF—and epidermal growth factor [62].

Therefore, many factors released from the ischemic brain may be compensatory protective signals that could potentially be exploited for neuroprotection. Epithelial cells release neurotrophic factors, including BDNF, in response to these factors. Conditioned media from cerebral endothelial cells protected neurons against oxygen-glucose deprivation *in vitro*, and this effect was blocked when condition medium was treated with free TrkB-Fc [63]. Human MSCs cultured in ischemic rat brain extract also exhibited increased production of BDNF, VEGF, and hepatocyte growth factor [64]. Stimulated microglial cells are also a source of BDNF; downregulation of the microglial response to ischemia by inhibiting the poly(ADP-ribose) polymerase-1 (PARP-1) pathway reduced BDNF production as well as markers of synaptogenesis [65]. Moreover, transplantation of a human microglial cell line—HMO6—48 hours after MCAO was found to reduce infarct volume and the number of apoptotic cells in the penumbra, while also reducing the number of host microglia/macrophages and reactive astrocytes. Transplanted HMO6 microglia also exhibited elevated GDNF, BDNF, VEGF, BMP7, and anti-inflammatory cytokines [66].

*2.7. Summary.* Enhanced neurotrophin expression in the CNS is broadly neuroprotective against acute neurological insults, including ischemia and hemorrhage. Moreover, there are a plethora of ways to increase brain neurotrophin activity, including direct infusion, transplantation of cells that express

neurotrophins when exposed to the CNS environment or that are engineered to overexpress neurotrophins constitutively, small molecule mimetics, and neurotrophin inducers. The latter category of molecules presents particularly promising targets for future research, both to enhance basal ischemic tolerance and to aid in recovery following transient ischemia. Extracellular signals released by the ischemic brain also enhance neurotrophin expression. These as yet unidentified signaling pathways may also be exploited for neuroprotection. It should be noted, however, that neuroplastic changes associated with acute and chronic diseases may reduce the efficacy of these treatments (see Section 3.2).

## 3. Neurotrophins for Treatment of Chronic Neurodegenerative Diseases

There is strong evidence that many of the pathogenic mechanisms implicated in acute neurodegenerative diseases, such as oxidative stress and inflammation, are equally involved in the slower neuronal loss characteristic of chronic neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), various tauopathies, and Huntington's disease (HD). Therefore, the therapeutic promise of neurotrophins for the treatment of chronic neurodegenerative diseases is based in part on studies demonstrating suppression of these basic pathogenic mechanisms. Indeed, exogenous application of neurotrophins protects against neurodegeneration in animal models of AD, PD, and HD. A multitude of studies have also demonstrated that expression of neurotrophins and/or Trk receptors is reduced in the afflicted brain regions of patients and animal models. In fact, this reduction may be observed prior to symptom expression, suggesting that insufficient trophic activity may initiate or accelerate disease progression. Moreover, neurotrophins often restore or improve function in animal models in the absence of observable neuroprotection, indicating that these agents or mimetics may be therapeutically useful even in patients with advanced disease.

*3.1. Therapeutic Efficacy of NTs, Mimetics, and Inducers in Animal Models of Chronic Neurodegenerative Disease.* Animal models of chronic neurodegenerative diseases have demonstrated the therapeutic efficacy of neurotrophins, particularly BDNF. Alzheimer's disease, the most common form of age-related dementia, is characterized by accumulation of extracellular amyloid plaques of beta-amyloids (A$\beta$) and intracellular neurofibrillary tangles containing hyperphosphorylated variants of the cytoskeleton-associated protein tau. BDNF was found to protect cultured neurons against the cytotoxic A$\beta$ fragments A$\beta$(1–42) and A$\beta$(25–35), and this protection was inhibited by the TrkB inhibitor K252a [67]. One of the most comprehensive studies on the protective efficacy of BDNF in animal models of AD found that injection of a lentivirus vector encoding BDNF into the entorhinal cortex (EC) improved spatial and fear memory in J20 transgenic mice expressing the mutant human amyloid precursor protein (APP), rescued EC neurons in monkeys subjected to perforant path lesions, and improved learning in delayed matching object-and-location task in aged monkeys. In this same study, direct infusion of BDNF into the EC also improved spatial memory in aged rats [68]. Infection with a viral vector encoding BDNF was found to enhance BDNF expression and improve memory deficits in the Tg2576 transgenic mouse model of AD. Significantly, the vector used—Sendai virus (SeV)—is highly infective but does not integrate into the host genome and is nonpathogenic in humans. Thus, SeV vectors hold great potential for the safe delivery of neuroprotective peptides to the brain [69]. In addition to neuroprotection, BDNF may slow disease progression by inhibiting amyloid production. BDNF was found to induce the expression of sorting protein-related receptor with A-type repeats (SORLA), a protein that reduces APP processing and plaque formation, and no longer reduce A$\beta$ in SORLA-deficient mice [70].

In aged 3xTg-AD mice, a model exhibiting amyloid plaques, neurofibrillary tangles, and age-associated cognitive decline, the injection of NSCs into the bilateral hippocampus improved spatial memory in the MWM but did not reduce amyloid or tau pathology. Preservation of cognitive function effect was absent when BDNF expression was knocked down using a targeted shRNA [71]. The fact that BDNF can improve cognitive function without reducing neuropathology suggests that this strategy may be useful in advanced AD patients.

Cognitive dysfunction in AD arises in part from the loss of septal cholinergic neurons that are project to the hippocampus. Toxic A$\beta$(1–42) induced the death of septal neurons *in vitro*, and this effect was reduced by BDNF (as well as by IGF-1 and GDNF) and by coculture of septal neurons with NSCs overexpressing neurotrophins [72]. The cholinergic neurotoxin (192) IgG-saporin reduced both MWM performance in rats and BDNF expression, while the reversal of these effects by the clinical cholinesterase inhibitor galantamine was associated with enhanced hippocampal BDNF levels [73].

Transgenic mice overexpressing APP and infected with a lentiviral BDNF vector at 2 months of age showed significantly reduced neurodegeneration in the EC and improved fear memory at 7 months of age [74], indicating that these therapeutic effects are relatively long-lasting. BDNF may also preserve neuronal viability and function in AD and other tauopathies by reducing tau phosphorylation. Indeed, BDNF reduced phosphorylation at multiple sites, an effect blocked by inhibition of TrkB, PI-3 kinase, and GSK 3$\beta$ [75]. The TrkB agonist 7,8-dihydroxyflavone rescued deficient spatial working memory in 5XFAD mice and reduced expression levels of $\beta$-secretase (BACE1), A$\beta$(1–40), and A$\beta$(1–42) [76].

In accord with the aforementioned results on neuroprotective strategies against stroke-induced neuronal damage, many potentially effective treatments for AD may be mediated by enhanced BDNF signaling. A curcumin derivative, J147, increased BDNF expression and reduced A$\beta$(1–42) and A$\beta$(1–40) in aged AD mice (APP/swePS1$\Delta$E9). Furthermore, these changes were associated with enhanced long-term potentiation, a putative neurocellular mechanism for associative learning, and a reduction in oxidative stress [77]. In another study, J147 improved both spatial and fear memory in aged AD mice and upregulated NGF, BDNF, and several

synaptic proteins induced by BDNF, including homer-1, a protein critical for the trafficking of glutamate receptors. Moreover, J147 was shown to be nontoxic at millimolar serum concentrations while demonstrating neuroprotection in nanomolar doses *in vitro* [78]. Elevated A$\beta$ reduced the colocalization of the synaptic marker synaptophysin with AMPA receptor subunits GluR1 and GluR4. As trafficking of AMPA receptors underlies forms of synaptic plasticity linked to learning, this disruption may explain the learning deficits associated with AD. Furthermore, exogenous BDNF was found to restore synaptic AMPA receptor trafficking [79].

Sildenafil (Viagra) also rescued cognitive function in the AD model mice, but without reducing the amyloid burden [80]. The neuroprotective effects of NPY *in vitro* against toxic A$\beta$ fragments were also found to be associated with neurotrophin upregulation [81]. The antidepressant amitriptyline enhanced BDNF and TrkB expression in the hippocampus, promoted neurogenesis, and improved spatial and object recognition memory in aged 3xTg mice [82]. The multiple sclerosis drug fingolimod phosphate (a sphingosine-1-phosphate receptor agonist) enhanced BDNF expression and protected mouse cortical neurons against A$\beta$ toxicity, an effect blocked by inhibition of BDNF-TrkB-ERK signaling [83]. Neuropep-1 is a new BDNF inducer that rescued spatial memory deficits in 3xTg-AD mice. In addition, Neuropep-1 also reduced the A$\beta$ burden, possibly by enhancing expression of the insulin/A$\beta$-degrading enzyme [84]. Sodium phenylbutyrate (NaPB) induced BDNF and NT-3 expression in astrocytes by activating the PKC-CREB signaling pathway and improved spatial learning and memory in a mouse model of AD [85].

Significant neuroprotective effects of neurotrophins, particularly BDNF, have also been demonstrated in animal models of PD. Intrathecal infusion of BDNF suppressed Parkinsonian signs in monkeys one week after treatment with the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which selectively destroys dopaminergic neurons and is widely used to model PD pathology. Moreover, BDNF infusion reduced neuronal loss in the SN and the extent of rescue was correlated with the preservation of function [86]. Transplantation of astrocytes expressing tyrosine hydroxylase (TH) and/or BDNF blocked 6-OHDA-induced degeneration and the effect of TH plus BDNF was more effective than either alone [87]. Injection of a BDNF viral vector did not increase TH-positive SN neurons six months after unilateral 6-hydroxydopamine (6-OHDA) injection but did block amphetamine-induced ipsilateral rotation [88], a PD-like behavior. Transplantation of rat fibroblasts secreting human BDNF near the SN prior to striatal MPP+ administration enhanced neuronal survival compared to transplantation of wild-type fibroblasts [89].

Other PD treatment strategies are associated with BDNF induction. Repetitive transcranial magnetic stimulation (rTMS) rescued DA neurons, improved locomotor function, and induced expression of BDNF, GDNF, PDNF, and VEGF in the 6-OHDA model of PD [90]. Similar to results in stroke models, prior exercise reduced Parkinsonian behavior (apomorphine-induced rotations), rescued dopaminergic neurons, and reversed the early reduction in BDNF expression observed following PD model induction. Neuronal rescue was reduced by inhibition of BDNF signaling [91]. Prior exercise (4 weeks) also reduced the loss of dopaminergic neurons and improved motor function following intraperitoneal injection of the activator of inflammation lipopolysaccharide (LPS), an effect blocked by a TrkB antagonist and mimicked by intracerebral perfusion of BDNF [92]. Successful motor rehabilitation also increased serum BDNF in PD patients [93]. The therapeutic efficacy of deep brain stimulation (DBS) targeting the subthalamic nucleus (STN-DBS), a widely used PD treatment, may also depend on BDNF induction; STN-DBS increased BDNF but not GDNF expression in the SN, striatum, motor cortex (M1), and hippocampus—an effect that was blocked by an NMDA receptor antagonist [94]. A diet high in omega-3 polyunsaturated fatty acids (n-3 PUFAs) also protected against MPTP-induced death of dopaminergic neurons and induced striatal expression of BDNF [95]. Pretreatment with the ampakine and BDNF inducer CX614 significantly reduced MPP(+)-induced toxicity in brain slices, an effect blocked by an inhibitor of TrkB [96]. Another novel neurotrophin inducer, PYM50028, also reduced the loss of SN dopaminergic neurons in mice when administered after MPTP [97].

*3.2. Is Dysregulation of Neurotrophin Involved in the Pathogenesis of Chronic Neurodegenerative Disease?* Deficient neurotrophin signaling has also been implicated in neurodegenerative disease progression. A$\beta$ reduction in neurotrophin expression in vulnerable brain regions has been widely reported, while genetic association studies have identified specific neurotrophin gene alleles as risk factors [98]. As discussed below, however, many of these studies also indicate that pathogenic processes may interfere with neurotrophin signaling and thereby reduce the effects of exogenous neurotrophin administration.

Expression levels of neurotrophins and Trk receptors are altered in neurodegenerative diseases, but the functional implications are currently unclear. The large-scale Framingham Study found a specific association between low-serum BDNF and dementia/AD risk in elderly women [99]. Reduced BDNF levels were also observed in the cortex of female APdE8 AD model mice [100]. A comparison of several transgenic AD model mice revealed that cortical BDNF mRNA expression was reduced in two models—namely, APP(NLh) and TgCRND8—compared to wild types, and all models studies exhibited an inverse correlation between toxic A$\beta$ levels and BDNF expression [101]. In aged model mice, however, BDNF immunoreactivity was elevated around amyloid plaques [100]. It was suggested that this elevation could act to sequester BDNF and reduce BDNF-TrkB signaling. Alternatively, it may represent a reactive response to damage. *In situ* hybridization also showed a several-fold increase in BDNF mRNA around amyloid plaques due to elevated microglial and astrocytic expression [102].

Despite uncertainties concerning brain neurotrophin levels during the different phases of neurodegenerative disorders, there is substantial evidence that neurotrophin

signaling is disrupted in AD and PD. For instance, A$\beta$ can also alter the expression of TrkB isoforms [103]. In cultured neurons, sublethal doses of A$\beta$ inhibited BDNF-MAPK/ERK and BDNF-PI3-K signaling by interfering with insulin receptor substrate-1 and Shc, two docking proteins that link TrkB to the activation of downstream kinase cascades [104]. In addition, A$\beta$ treatment increased truncated TrkB.T1 and decreased full-length TrkB.TK expression in cultured neurons; furthermore, overexpression of TrkB.T1 in APdE9 mice enhanced cognitive deficits [105]. The AD-linked gene presenilin 1 can also regulate surface expression of TrkB [106]. Amyloid-$\beta$ accumulation also interfered with CREB activation, while viral delivery of CREB-binding protein (CBP) restored CREB function and improved memory deficits in an AD model. These changes were associated with elevated BDNF but no reduction in A$\beta$ or tau pathology [107]. Finally, the Tg2576 mouse exhibited reduced retrograde axonal transport of BDNF that was mitigated by inhibition of $\gamma$-secretase, implicating A$\beta$ accumulation in reduced BDNF-nuclear signaling. Moreover, the administration of A$\beta$ alone reduced retrograde transport [108]. This deficit may stem from deficient expression of ubiquitin C-terminal hydrolase L1 (UCH-L1), a deubiquitinating enzyme reduced in AD, as a UCH-IL inhibitor also reduced retrograde transport of BDNF [109]. Inhibition of BDNF signaling independent of BDNF expression levels may be a major impediment to the clinical application of techniques that only augment endogenous BDNF or TrkB.

This disruption of neurotrophin signaling could further exacerbate neuronal injury. AD patients expressing the Met allele of BDNF showed enhanced atrophy in several brain regions, including anterior cingulate cortex, posterior cingulate cortex, and precuneus, compared to Val/Val carriers [110]. Several SNPs in the BDNF gene were associated with more rapid brain atrophy over two years [111]. Carriers of both the BDNF Met allele and the AD risk factor APOE $\varepsilon$4 exhibited enhanced degeneration in the precuneus, orbitofrontal cortex, gyrus rectus, and lateral prefrontal cortex, as well as episodic memory deficits that were correlated with amyloid burden [112]. Moreover, a meta-analysis of studies on the BDNF 196A/G polymorphism and AD risk reported a significant association in females [113]. Carriers of the Met allele also showed faster cognitive decline and greater brain atrophy over 36 months compared to Val/Val carriers; however, this only occurred in elderly adults with high basal A$\beta$ at the start of the study, suggesting that neurotrophin dysregulation accelerates rather than initiates neurodegeneration in AD [114].

Deficient BDNF signaling is also implicated in PD and HD progression. In the substantia nigra of PD patients, BDNF expression is reduced, even in surviving neurons [115, 116]. BDNF is also reduced in Huntington's disease, and BDNF expression is necessary for the survival of medium-sized spiny striatal neurons lost during HD progression [117]. Continuous infusion of a BDNF antisense oligonucleotide into the SN of rats induced Parkinsonian behaviors, including apomorphine-evoked rotation, and resulted in the loss of TH-positive neurons and dopamine uptake [118]. Mice lacking BDNF expression in the mid- and hindbrain demonstrated motor impairments and reduced numbers of TH-positive neurons, while no reduction was observed in the calbindin- and calretinin-positive neurons spared in PD [119]. Exercise mitigated Parkinsonian signs, improved mitochondrial function, and elevated BDNF/GDNF expression in a mouse PD model [120]. Reduced TrkB expression reduced the number of SN neurons in elderly mice (21–23 months old) and resulted in accumulation of alpha-synuclein in surviving neurons [121]. Aged TrkB hypomorphic mice were hypersensitive to MPTP and showed enhanced age-related loss of dopaminergic neurons in the SN [122]. Finally, nigral dopaminergic neurons expressing TrkB were more resistant to MPTP than neurons expressing mainly TrkC [123].

## 4. Conclusions and Future Directions

These studies indicate that neurotrophins can protect neurons against a variety of pathological insults, including ischemia and neurotoxins such as A$\beta$. Moreover, a variety of innovative methods have been developed to noninvasively enhance neurotrophin expression in the brain. Despite these advances, neither neurotrophin delivery nor induction is used in the clinic as a routine treatment. All of these disorders are associated with multiple pathogenic processes, so it is possible that effective treatment will require multiple complementary strategies. Furthermore, these disorders appear to disrupt neurotrophin signaling, so future work should concentrate on the targeted delivery of neurotrophins to vulnerable tissues and methods of preserving neurotrophin function in the diseased brain.

## Abbreviations

AD: Alzheimer's disease
PD: Parkinson's disease
HD: Huntington's disease
NGF: Nerve growth factor
BDNF: Brain-derived neurotrophic factor
CNS: Central nerve system
BBB: Blood brain barrier
Trk: Neurotrophin receptor
MCAO: Middle cerebral artery occlusion
GDNF: Glial-derived neurotrophic factor
IGF-1: Insulin-like growth factor-1
VEGF: Vascular endothelial growth factor
NSC: Neural stem cell
MSC: Mesenchymal stem cell
BM: Bone marrow
IBZ: Ischemic boundary zone
MWM: Morris water maze
NPC: Neural progenitor cells
CP: Choroid plexus
SN: Substantia nigra
NMDA: N-methyl-D-asparate
FGF-2: Fibroblast growth factor-2
SVZ: Subventricular zone
NOS: Nitric oxide synthase
HELP: High-voltage electric potential

RCI: Respiratory control index
SGZ: Subgranular zone
PARP-1: poly(ADP-ribose) polymerase-1
A$\beta$: Beta-amyloids
APP: Amyloid precursor protein
EC: Entorhinal cortex
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
TH: Tyrosine hydroxylase
LPS: Lipopolysaccharide.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Junying Cai and Fuzhou Hua contributed equally to this paper.

## References

[1] T. Numakawa, T. Matsumoto, Y. Numakawa, M. Richards, S. Yamawaki, and H. Kunugi, "Protective action of neurotrophic factors and estrogen against oxidative stress-mediated neurodegeneration," *Journal of Toxicology*, vol. 2011, Article ID 405194, 12 pages, 2011.

[2] K. Dasuri, L. Zhang, and J. N. Keller, "Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis," *Free Radical Biology and Medicine*, vol. 62, pp. 170–185, 2013.

[3] R. Rodrigo, R. Fernandez-Gajardo, R. Gutierrez et al., "Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities," *CNS & Neurological Disorders Drug Targets*, vol. 12, no. 5, pp. 698–714, 2013.

[4] M. P. Mattson, "Glutamate and neurotrophic factors in neuronal plasticity and disease," *Annals of the New York Academy of Sciences*, vol. 1144, pp. 97–112, 2008.

[5] S. E. Lakhan, A. Kirchgessner, and M. Hofer, "Inflammatory mechanisms in ischemic stroke: therapeutic approaches," *Journal of Translational Medicine*, vol. 7, article 97, 2009.

[6] L. F. Reichardt, "Neurotrophin-regulated signalling pathways," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 361, no. 1473, pp. 1545–1564, 2006.

[7] A. H. Nagahara and M. H. Tuszynski, "Potential therapeutic uses of BDNF in neurological and psychiatric disorders," *Nature Reviews Drug Discovery*, vol. 10, no. 3, pp. 209–219, 2011.

[8] J. N. Stankowski and R. Gupta, "Therapeutic targets for neuroprotection in acute ischemic stroke: lost in translation?" *Antioxidants and Redox Signaling*, vol. 14, no. 10, pp. 1841–1851, 2011.

[9] N. J. G. Webster and M. C. Pirrung, "Small molecule activators of the Trk receptors for neuroprotection," *BMC Neuroscience*, vol. 9, no. 2, article S1, 2008.

[10] D. Wu, "Neuroprotection in experimental stroke with targeted neurotrophins," *NeuroRx*, vol. 2, no. 1, pp. 120–128, 2005.

[11] W.-R. Schäbitz, S. Schwab, M. Spranger, and W. Hacke, "Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats," *Journal of Cerebral Blood Flow and Metabolism*, vol. 17, no. 5, pp. 500–506, 1997.

[12] K. Yamashita, C. Wiessner, D. Lindholm, H. Thoenen, and K.-A. Hossmann, "Post-occlusion treatment with BDNF reduces infarct size in a model of permanent occlusion of the middle cerebral artery in rat," *Metabolic Brain Disease*, vol. 12, no. 4, pp. 271–280, 1997.

[13] H. Yanamoto, I. Nagata, M. Sakata et al., "Infarct tolerance induced by intra-cerebral infusion of recombinant brain-derived neurotrophic factor," *Brain Research*, vol. 859, no. 2, pp. 240–248, 2000.

[14] W.-R. Schabitz, C. Sommer, W. Zoder et al., "Intravenous brain-derived neurotrophic factor reduces infarct size and counterregulates Bax and Bcl-2 expression after temporary focal cerebral ischemia," *Stroke*, vol. 31, no. 9, pp. 2212–2217, 2000.

[15] M. Shirakura, M. Inoue, S. Fujikawa et al., "Postischemic administration of Sendai virus vector carrying neurotrophic factor genes prevents delayed neuronal death in gerbils," *Gene Therapy*, vol. 11, no. 9, pp. 784–790, 2004.

[16] J. Chen, Y. Li, and M. Chopp, "Intracerebral transplantation of bone marrow with BDNF after MCAo in rat," *Neuropharmacology*, vol. 39, no. 5, pp. 711–716, 2000.

[17] C. Berger, W.-R. Schabitz, M. Wolf, H. Mueller, C. Sommer, and S. Schwab, "Hypothermia and brain-derived neurotrophic factor reduce glutamate synergistically in acute stroke," *Experimental Neurology*, vol. 185, no. 2, pp. 305–312, 2004.

[18] C. V. Borlongan, S. J. M. Skinner, M. Geaney, A. V. Vasconcellos, R. B. Elliott, and D. F. Emerich, "Intracerebral transplantation of porcine choroid plexus provides structural and functional neuroprotection in a rodent model of stroke," *Stroke*, vol. 35, no. 9, pp. 2206–2210, 2004.

[19] N. Mochizuki, N. Takagi, K. Kurokawa et al., "Injection of neural progenitor cells improved learning and memory dysfunction after cerebral ischemia," *Experimental Neurology*, vol. 211, no. 1, pp. 194–202, 2008.

[20] Y. Hasegawa, H. Suzuki, O. Altay, and J. H. Zhang, "Preservation of tropomyosin-related kinase B (TrkB) signaling by sodium orthovanadate attenuates early brain injury after subarachnoid hemorrhage in rats," *Stroke*, vol. 42, no. 2, pp. 477–483, 2011.

[21] J. M. Zhu, Y. Y. Zhao, S. D. Chen, W. H. Zhang, L. Lou, and X. Jin, "Functional recovery after transplantation of neural stem cells modified by brain-derived neurotrophic factor in rats with cerebral ischaemia," *Journal of International Medical Research*, vol. 39, no. 2, pp. 488–498, 2011.

[22] Y. Sato, Y. Chin, T. Kato et al., "White matter activated glial cells produce BDNF in a stroke model of monkeys," *Neuroscience Research*, vol. 65, no. 1, pp. 71–78, 2009.

[23] W.-R. Schäbitz, C. Berger, R. Kollmar et al., "Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia," *Stroke*, vol. 35, no. 4, pp. 992–997, 2004.

[24] M. Endres, G. Fan, L. Hirt et al., "Ischemic brain damage in mice after selectively modifying BDNF or NT4 gene expression," *Journal of Cerebral Blood Flow and Metabolism*, vol. 20, no. 1, pp. 139–144, 2000.

[25] T. Saarelainen, J. A. Lukkarinen, S. Koponen et al., "Transgenic mice overexpressing truncated trkB neurotrophin receptors in neurons show increased susceptibility to cortical injury after focal cerebral ischemia," *Molecular and Cellular Neurosciences*, vol. 16, no. 2, pp. 87–96, 2000.

[26] M. Ploughman, V. Windle, C. L. MacLellan, N. White, J. J. Doré, and D. Corbett, "Brain-derived neurotrophic factor contributes

to recovery of skilled reaching after focal ischemia in rats," *Stroke*, vol. 40, no. 4, pp. 1490–1495, 2009.

[27] E. Gustafsson, G. Andsberg, V. Darsalia et al., "Anterograde delivery of brain-derived neurotrophic factor to striatum via nigral transduction of recombinant adeno-associated virus increases neuronal death but promotes neurogenic response following stroke," *European Journal of Neuroscience*, vol. 17, no. 12, pp. 2667–2678, 2003.

[28] K. Kurozumi, K. Nakamura, T. Tamiya et al., "Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model," *Molecular Therapy*, vol. 11, no. 1, pp. 96–104, 2005.

[29] Y. Ding, J. Li, X. Luan et al., "Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin," *Neuroscience*, vol. 124, no. 3, pp. 583–591, 2004.

[30] I. Kiprianova, K. Schindowski, O. Von Bohlen Und Halbach et al., "Enlarged infarct volume and loss of BDNF mRNA induction following brain ischemia in mice lacking FGF-2," *Experimental Neurology*, vol. 189, no. 2, pp. 252–260, 2004.

[31] H. Yanamoto, J.-H. Xue, S. Miyamoto et al., "Spreading depression induces long-lasting brain protection against infarcted lesion development via BDNF gene-dependent mechanism," *Brain Research*, vol. 1019, no. 1-2, pp. 178–188, 2004.

[32] J. Xu, Q.-G. Zhang, C. Li, and G.-Y. Zhang, "Subtoxic N-methyl-D-aspartate delayed neuronal death in ischemic brain injury through TrkB receptor- and calmodulin-mediated PI-3K/Akt pathway activation," *Hippocampus*, vol. 17, no. 7, pp. 525–537, 2007.

[33] D.-M. Chuang, "Neuroprotective and neurotrophic actions of the mood stabilizer lithium: can it be used to treat neurodegenerative diseases?" *Critical Reviews in Neurobiology*, vol. 16, no. 1-2, pp. 83–90, 2004.

[34] D. M. Chuang, Z. Wang, and C. T. Chiu, "GSK-3 as a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke," *Frontiers in Molecular Neuroscience*, vol. 4, p. 15, 2011.

[35] J. Chen, C. Zhang, H. Jiang et al., "Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice," *Journal of Cerebral Blood Flow and Metabolism*, vol. 25, no. 2, pp. 281–290, 2005.

[36] M. Krikov, C. Thone-Reineke, S. Müller, A. Villringer, and T. Unger, "Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BNDF/TrkB system in rats," *Journal of Hypertension*, vol. 26, no. 3, pp. 544–552, 2008.

[37] J. Chen, A. Zacharek, C. Zhang et al., "Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice," *Journal of Neuroscience*, vol. 25, no. 9, pp. 2366–2375, 2005.

[38] M.-W. Kim, M.-S. Bang, T.-R. Han et al., "Exercise increased BDNF and trkB in the contralateral hemisphere of the ischemic rat brain," *Brain Research*, vol. 1052, no. 1, pp. 16–21, 2005.

[39] Z. Ke, S. P. Yip, L. Li, X.-X. Zheng, and K.-Y. Tong, "The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model," *PLoS ONE*, vol. 6, no. 2, Article ID e16643, 2011.

[40] A. N. Clarkson, J. J. Overman, S. Zhong, R. Mueller, G. Lynch, and S. T. Carmichael, "AMPA receptor-induced local brain-derived neurotrophic factor signaling mediates motor recovery after stroke," *Journal of Neuroscience*, vol. 31, no. 10, pp. 3766–3775, 2011.

[41] J. Pera, M. Zawadzka, B. Kaminska, and A. Szczudlik, "Neurotrophic factor expression after focal brain ischemia preceded by different preconditioning strategies," *Cerebrovascular Diseases*, vol. 19, no. 4, pp. 247–252, 2005.

[42] J. Nygren, M. Kokaia, and T. Wieloch, "Decreased expression of brain-derived neurotrophic factor in $BDNF^{+/-}$ mice is associated with enhanced recovery of motor performance and increased neuroblast number following experimental stroke," *Journal of Neuroscience Research*, vol. 84, no. 3, pp. 626–631, 2006.

[43] K. Hirata, Y. Kuge, C. Yokota et al., "Gene and protein analysis of brain derived neurotrophic factor expression in relation to neurological recovery induced by an enriched environment in a rat stroke model," *Neuroscience Letters*, vol. 495, no. 3, pp. 210–215, 2011.

[44] C. Geral, A. Angelova, and S. Lesieur, "From molecular to nanotechnology strategies for delivery of neurotrophins: emphasis on brain-derived neurotrophic factor (BDNF)," *Pharmaceutics*, vol. 5, no. 1, pp. 127–167, 2013.

[45] Y. Zhang and W. M. Pardridge, "Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system," *Stroke*, vol. 32, no. 6, pp. 1378–1383, 2001.

[46] Y. Zhang and W. M. Pardridge, "Blood-brain barrier targeting of BDNF improves motor function in rats with middle cerebral artery occlusion," *Brain Research*, vol. 1111, no. 1, pp. 227–229, 2006.

[47] H. Yanamoto, S. Miyamoto, Y. Nakajo et al., "Repeated application of an electric field increases BDNF in the brain, enhances spatial learning, and induces infarct tolerance," *Brain Research*, vol. 1212, pp. 79–88, 2008.

[48] Q. Han, B. Li, H. Feng et al., "The promotion of cerebral ischemia recovery in rats by laminin-binding BDNF," *Biomaterials*, vol. 32, no. 22, pp. 5077–5085, 2011.

[49] Y. Jiang, N. Wei, T. Lu, J. Zhu, G. Xu, and X. Liu, "Intranasal brain-derived neurotrophic factor protects brain from ischemic insult via modulating local inflammation in rats," *Neuroscience*, vol. 172, pp. 398–405, 2011.

[50] S.-W. Jang, X. Liu, M. Yepes et al., "A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 6, pp. 2687–2692, 2010.

[51] Z.-J. Zhang, P. Li, Z. Wang et al., "A comparative study on the individual and combined effects of baicalin and jasminoidin on focal cerebral ischemia-reperfusion injury," *Brain Research*, vol. 1123, no. 1, pp. 188–195, 2006.

[52] X. Cui, M. Chopp, A. Zacharek et al., "Niacin treatment of stroke increases synaptic plasticity and axon growth in rats," *Stroke*, vol. 41, no. 9, pp. 2044–2049, 2010.

[53] W.-S. Qu, Y.-H. Wang, J.-P. Wang et al., "Galectin-1 enhances astrocytic BDNF production and improves functional outcome in rats following ischemia," *Neurochemical Research*, vol. 35, no. 11, pp. 1716–1724, 2010.

[54] K.-Y. Kam, S. J. Yu, N. Jeong et al., "p-hydroxybenzyl alcohol prevents brain injury and behavioral impairment by activating Nrf2, PDI, and neurotrophic factor genes in a rat model of brain ischemia," *Molecules and Cells*, vol. 31, no. 3, pp. 209–215, 2011.

[55] S. M. Rothman and M. P. Mattson, "Activity-dependent, stress-responsive BDNF signaling and the quest for optimal brain

health and resilience throughout the lifespan," *Neuroscience*, vol. 239, pp. 228–240, 2013.

[56] A. Markham, I. Cameron, R. Bains et al., "Brain-derived neurotrophic factor-mediated effects on mitochondrial respiratory coupling and neuroprotection share the same molecular signalling pathways," *European Journal of Neuroscience*, vol. 35, no. 3, pp. 366–374, 2012.

[57] W.-R. Schäbitz, T. Steigleder, C. M. Cooper-Kuhn et al., "Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis," *Stroke*, vol. 38, no. 7, pp. 2165–2172, 2007.

[58] S. Keiner, O. W. Witte, and C. Redecker, "Immunocytochemical detection of newly generated neurons in the perilesional area of cortical infarcts after intraventricular application of brain-derived neurotrophic factor," *Journal of Neuropathology and Experimental Neurology*, vol. 68, no. 1, pp. 83–93, 2009.

[59] B. Li, C.-S. Piao, X.-Y. Liu et al., "Brain self-protection: the role of endogenous neural progenitor cells in adult brain after cerebral cortical ischemia," *Brain Research*, vol. 1327, pp. 91–102, 2010.

[60] X. Bao, J. Wei, M. Feng et al., "Transplantation of human bone marrow-derived mesenchymal stem cells promotes behavioral recovery and endogenous neurogenesis after cerebral ischemia in rats," *Brain Research*, vol. 1367, pp. 103–113, 2011.

[61] Y. Tanaka, R. Tanaka, M. Liu, N. Hattori, and T. Urabe, "Cilostazol attenuates ischemic brain injury and enhances neurogenesis in the subventricular zone of adult mice after transient focal cerebral ischemia," *Neuroscience*, vol. 171, no. 4, pp. 1367–1376, 2010.

[62] R. Qu, Y. Li, Q. Gao et al., "Neurotrophic and growth factor gene expression profiling of mouse bone marrow stromal cells induced by ischemic brain extracts," *Neuropathology*, vol. 27, no. 4, pp. 355–363, 2007.

[63] S. Guo, W. J. Kim, J. Lok et al., "Neuroprotection via matrix-trophic coupling between cerebral endothelial cells and neurons," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 21, pp. 7582–7587, 2008.

[64] Y. J. Choi, W. Y. Li, G. J. Moon et al., "Enhancing trophic support of mesenchymal stem cells by ex vivo treatment with trophic factors," *Journal of the Neurological Sciences*, vol. 298, no. 1-2, pp. 28–34, 2010.

[65] A. Madinier, N. Bertrand, C. Mossiat et al., "Microglial involvement in neuroplastic changes following focal brain ischemia in rats," *PloS one*, vol. 4, no. 12, p. e8101, 2009.

[66] D. Narantuya, A. Nagai, M. Abdullah et al., "Human microglia transplanted in rat focal ischemia brain induce neuroprotection and behavioral improvement," *PLoS ONE*, vol. 5, no. 7, Article ID e11746, 2010.

[67] S. Arancibia, M. Silhol, F. Moulière et al., "Protective effect of BDNF against beta-amyloid induced neurotoxicity in vitro and in vivo in rats," *Neurobiology of Disease*, vol. 31, no. 3, pp. 316–326, 2008.

[68] A. H. Nagahara, D. A. Merrill, G. Coppola et al., "Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease," *Nature Medicine*, vol. 15, no. 3, pp. 331–337, 2009.

[69] Y. Iwasaki, T. Negishi, M. Inoue, T. Tashiro, T. Tabira, and N. Kimura, "Sendai virus vector-mediated brain-derived neurotrophic factor expression ameliorates memory deficits and synaptic degeneration in a transgenic mouse model of Alzheimer's disease," *Journal of Neuroscience Research*, vol. 90, no. 5, pp. 981–989, 2012.

[70] M. Rohe, M. Synowitz, R. Glass, S. M. Paul, A. Nykjaer, and T. E. Willnow, "Brain-derived neurotrophic factor reduces amyloidogenic processing through control of SORLA gene expression," *Journal of Neuroscience*, vol. 29, no. 49, pp. 15472–15478, 2009.

[71] M. Blurton-Jones, M. Kitazawa, H. Martinez-Coria et al., "Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 32, pp. 13594–13599, 2009.

[72] N. Kitiyanant, Y. Kitiyanant, C. N. Svendsen, and W. Thangnipon, "BDNF-, IGF-1- and GDNF-secreting human neural progenitor cells rescue amyloid $\beta$-induced toxicity in cultured rat septal neurons," *Neurochemical Research*, vol. 37, no. 1, pp. 143–152, 2012.

[73] F. J. Gil-Bea, M. Solas, L. Mateos, B. Winblad, M. J. Ramírez, and A. Cedazo-Mínguez, "Cholinergic hypofunction impairs memory acquisition possibly through hippocampal Arc and BDNF down regulation," *Hippocampus*, vol. 21, no. 9, pp. 999–1009, 2011.

[74] A. H. Nagahara, M. Mateling, I. Kovacs et al., "Early BDNF treatment ameliorates cell loss in the entorhinal cortex of APP transgenic mice," *Journal of Neuroscience*, vol. 33, no. 39, pp. 15596–15602, 2013.

[75] E. Elliott, R. Atlas, A. Lange, and I. Ginzburg, "Brain-derived neurotrophic factor induces a rapid dephosphorylation of tau protein through a PI-3Kinase signalling mechanism," *European Journal of Neuroscience*, vol. 22, no. 5, pp. 1081–1089, 2005.

[76] L. Devi and M. Ohno, "7,8-dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of alzheimer's disease," *Neuropsychopharmacology*, vol. 37, no. 2, pp. 434–444, 2012.

[77] Q. Chen, M. Prior, R. Dargusch et al., "A novel neurotrophic drug for cognitive enhancement and Alzheimer's disease," *PLoS ONE*, vol. 6, no. 12, Article ID e27865, 2011.

[78] M. Prior, R. Dargusch, J. L. Ehren, C. Chiruta, and D. Schubert, "The neurotrophic compound J147 reverses cognitive impairment in aged Alzheimer's disease mice," *Alzheimer's Research & Therapy*, vol. 5, no. 3, p. 25, 2013.

[79] Z. Zheng, B. Sabirzhanov, and J. Keifer, "Oligomeric amyloid-$\beta$ inhibits the proteolytic conversion of brain-derived neurotrophic factor (BDNF), AMPA receptor trafficking, and classical conditioning," *Journal of Biological Chemistry*, vol. 285, no. 45, pp. 34708–34717, 2010.

[80] M. Cuadrado-Tejedor, I. Hervias, A. Ricobaraza et al., "Sildenafil restores cognitive function without affecting $\beta$-amyloid burden in a mouse model of Alzheimer's disease," *British Journal of Pharmacology*, vol. 164, no. 8, pp. 2029–2041, 2011.

[81] N. Croce, V. Dinallo, V. Ricci et al., "Neuroprotective effect of neuropeptide Y against beta-amyloid 25-35 toxicity in SH-SY5Y neuroblastoma cells is associated with increased neurotrophin production," *Neurodegenerative Diseases*, vol. 8, no. 5, pp. 300–309, 2011.

[82] W. Chadwick, N. Mitchell, J. Caroll et al., "Amitriptyline-mediated cognitive enhancement in aged 3×Tg Alzheimer's disease mice is associated with neurogenesis and neurotrophic activity," *PLoS ONE*, vol. 6, no. 6, Article ID e21660, 2011.

[83] Y. Doi, H. Takeuchi, H. Horiuchi et al., "Fingolimod phosphate attenuates oligomeric amyloid beta-induced neurotoxicity via increased brain-derived neurotrophic factor expression in neurons," *PloS ONE*, vol. 8, no. 4, Article ID e61988.

[84] M. K. Shin, H. G. Kim, S. H. Baek et al., "Neuropep-1 ameliorates learning and memory deficits in an Alzheimer's disease mouse model, increases brain-derived neurotrophic factor expression in the brain, and causes reduction of amyloid beta plaques," *Neurobiology of Aging*, vol. 35, no. 5, pp. 990–1001, 2014.

[85] G. T. Corbett, A. Roy, and K. Pahan, "Sodium phenylbutyrate enhances astrocytic neurotrophin synthesis via protein kinase C (PKC)-mediated activation of cAMP-response element-binding protein (CREB): implications for Alzheimer disease therapy," *Journal of Biological Chemistry*, vol. 288, no. 12, pp. 8299–8312, 2013.

[86] T. Tsukahara, M. Takeda, S. Shimohama et al., "Effects of brain-derived neurotrophic factor on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in monkeys," *Neurosurgery*, vol. 37, no. 4, pp. 733–741, 1995.

[87] Z. H. Wang, Y. Ji, W. Shan et al., "Therapeutic effects of astrocytes expressing both tyrosine hydroxylase and brain-derived neurotrophic factor on a rat model of Parkinson's disease," *Neuroscience*, vol. 113, no. 3, pp. 629–640, 2002.

[88] R. L. Klein, M. H. Lewis, N. Muzyczka, and E. M. Meyer, "Prevention of 6-hydroxydopamine-induced rotational behavior by BDNF somatic gene transfer," *Brain Research*, vol. 847, no. 2, pp. 314–320, 1999.

[89] D. M. Frim, T. A. Uhler, W. R. Galpern, M. F. Beal, X. O. Breakefield, and O. Isacson, "Implanted fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevent 1-methyl-4-phenylpyridinium toxicity to dopaminergic neurons in the rat," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 11, pp. 5104–5108, 1994.

[90] J. Y. Lee, S. H. Kim, A. R. Ko et al., "Therapeutic effects of repetitive transcranial magnetic stimulation in an animal model of Parkinson's disease," *Brain Research*, vol. 1537, pp. 290–302, 2013.

[91] C. C. Real, A. F. Ferreira, G. P. Chaves-Kirsten, A. S. Torrao, R. S. Pires, and L. R. Britto, "BDNF receptor blockade hinders the beneficial effects of exercise in a rat model of Parkinson's disease," *Neuroscience*, vol. 237, pp. 118–129, 2013.

[92] S.-Y. Wu, T.-F. Wang, L. Yu et al., "Running exercise protects the substantia nigra dopaminergic neurons against inflammation-induced degeneration via the activation of BDNF signaling pathway," *Brain, Behavior, and Immunity*, vol. 25, no. 1, pp. 135–146, 2011.

[93] G. Frazzitta, G. Bertotti, G. Riboldazzi et al., "Effectiveness of intensive inpatient rehabilitation treatment on disease progression in parkinsonian patients: a randomized controlled trial with 1-year follow-up," *Neurorehabilitation and Neural Repair*, vol. 26, no. 2, pp. 144–150, 2012.

[94] A. L. Spieles-Engemann, K. Steece-Collier, M. M. Behbehani et al., "Subthalamic nucleus stimulation increases brain derived neurotrophic factor in the nigrostriatal system and primary motor cortex," *Journal of Parkinson's Disease*, vol. 1, no. 1, pp. 123–136, 2011.

[95] M. Bousquet, C. Gibrat, M. Saint-Pierre, C. Julien, F. Calon, and F. Cicchetti, "Modulation of brain-derived neurotrophic factor as a potential neuroprotective mechanism of action of omega-3 fatty acids in a parkinsonian animal model," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 33, no. 8, pp. 1401–1408, 2009.

[96] H. Jourdi, L. Hamo, T. Oka, A. Seegan, and M. Baudry, "BDNF mediates the neuroprotective effects of positive AMPA receptor modulators against MPP+-induced toxicity in cultured hippocampal and mesencephalic slices," *Neuropharmacology*, vol. 56, no. 5, pp. 876–885, 2009.

[97] N. P. Visanji, A. Orsi, T. H. Johnston et al., "PYM50028, a novel, orally active, nonpeptide neurotrophic factor inducer, prevents and reverses neuronal damage induced by $MPP^+$ in mesencephalic neurons and by MPTP in a mouse model of Parkinson's disease," *FASEB Journal*, vol. 22, no. 7, pp. 2488–2497, 2008.

[98] C. Zuccato and E. Cattaneo, "Role of brain-derived neurotrophic factor in Huntington's disease," *Progress in Neurobiology*, vol. 81, no. 5-6, pp. 294–330, 2007.

[99] G. Weinstein, A. S. Beiser, S. H. Choi et al., "Serum brain-derived neurotrophic factor and the risk for dementia: the framingham heart study," *JAMA Neurology*, vol. 71, no. 1, pp. 55–61, 2014.

[100] T. Rantamaki, S. Kemppainen, H. Autio et al., "The impact of Bdnf gene deficiency to the memory impairment and brain pathology of APPswe/PS1dE9 mouse model of Alzheimer's disease," *PloS ONE*, vol. 8, no. 7, Article ID e68722, 2013.

[101] S. Peng, D. J. Garzon, M. Marchese et al., "Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease," *Journal of Neuroscience*, vol. 29, no. 29, pp. 9321–9329, 2009.

[102] G. J. Burbach, R. Hellweg, C. A. Haas et al., "Induction of brain-derived neurotrophic factor in plaque-associated glial cells of aged APP23 transgenic mice," *Journal of Neuroscience*, vol. 24, no. 10, pp. 2421–2430, 2004.

[103] J. Wong, M. Higgins, G. Halliday, and B. Garner, "Amyloid beta selectively modulates neuronal TrkB alternative transcript expression with implications for Alzheimer's disease," *Neuroscience*, vol. 210, pp. 363–374, 2012.

[104] L. Tong, R. Balazs, P. L. Thornton, and C. W. Cotman, "$\beta$-amyloid peptide at sublethal concentrations downregulates brain-derived neurotrophic factor functions in cultured cortical neurons," *Journal of Neuroscience*, vol. 24, no. 30, pp. 6799–6809, 2004.

[105] S. Kemppainen, T. Rantamäki, A. Jerónimo-Santos et al., "Impaired TrkB receptor signaling contributes to memory impairment in APP/PS1 mice," *Neurobiology of Aging*, vol. 33, no. 6, pp. 1122.e23–1122.e39, 2012.

[106] G. Barthet, J. Dunys, Z. Shao et al., "Presenilin mediates neuroprotective functions of ephrinB and brain-derived neurotrophic factor and regulates ligand-induced internalization and metabolism of EphB2 and TrkB receptors," *Neurobiology of Aging*, vol. 34, no. 2, pp. 499–510, 2012.

[107] A. Caccamo, M. A. Maldonado, A. F. Bokov, S. Majumder, and S. Oddo, "CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 52, pp. 22687–22692, 2010.

[108] W. W. Poon, M. Blurton-Jones, C. H. Tu et al., "$\beta$-Amyloid impairs axonal BDNF retrograde trafficking," *Neurobiology of Aging*, vol. 32, no. 5, pp. 821–833, 2011.

[109] W. W. Poon, A. J. Carlos, B. L. Aguilar et al., "beta-amyloid (Abeta) oligomers impair brain-derived neurotrophic factor retrograde trafficking by down-regulating ubiquitin C-terminal hydrolase, UCH-L1," *Journal of Biological Chemistry*, vol. 288, no. 23, pp. 16937–16948, 2013.

[110] R. Hashimoto, Y. Hirata, T. Asada et al., "Effect of the brain-derived neurotrophic factor and the apolipoprotein E polymorphisms on disease progression in preclinical Alzheimer's disease," *Genes, Brain and Behavior*, vol. 8, no. 1, pp. 43–52, 2009.

[111] R. A. Honea, C. Cruchaga, R. D. Perea et al., "Characterizing the role of brain derived neurotrophic factor genetic variation in Alzheimer's disease neurodegeneration," *PloS ONE*, vol. 8, no. 9, Article ID e76001, 2013.

[112] K. Adamczuk, A. S. De Weer, N. Nelissen et al., "Polymorphism of brain derived neurotrophic factor influences beta amyloid load in cognitively intact apolipoprotein E epsilon4 carriers," *NeuroImage: Clinical*, vol. 2, pp. 512–520, 2013.

[113] J. Chen, X. Liang, B. Li, X. Jiang, and Z. Xu, "Gender-related association of brain-derived neurotrophic factor gene 196A/G polymorphism with Alzheimer's disease—a meta-analysis including 6854 cases and 6868 controls," *International Journal of Neuroscience*, 2014.

[114] Y. Y. Lim, V. L. Villemagne, S. M. Laws et al., "BDNF Val66Met, Abeta amyloid, and cognitive decline in preclinical Alzheimer's disease," *Neurobiology of Aging*, vol. 34, no. 11, pp. 2457–2464, 2013.

[115] D. W. Howells, M. J. Porritt, J. Y. F. Wong et al., "Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra," *Experimental Neurology*, vol. 166, no. 1, pp. 127–135, 2000.

[116] T. Nagatsu and M. Sawada, "Biochemistry of postmortem brains in Parkinson's disease: historical overview and future prospects," *Journal of Neural Transmission, Supplementa*, no. 72, pp. 113–120, 2007.

[117] C. Zuccato and E. Cattaneo, "Role of brain-derived neurotrophic factor in Huntington's disease," *Progress in Neurobiology*, vol. 81, no. 5-6, pp. 294–330, 2007.

[118] M. J. Porritt, P. E. Batchelor, and D. W. Howells, "Inhibiting BDNF expression by antisense oligonucleotide infusion causes loss of nigral dopaminergic neurons," *Experimental Neurology*, vol. 192, no. 1, pp. 226–234, 2005.

[119] Z. C. Baquet, P. C. Bickford, and K. R. Jones, "Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta," *Journal of Neuroscience*, vol. 25, no. 26, pp. 6251–6259, 2005.

[120] Y.-S. Lau, G. Patki, K. Das-Panja, W.-D. Le, and S. O. Ahmad, "Neuroprotective effects and mechanisms of exercise in a chronic mouse model of Parkinson's disease with moderate neurodegeneration," *European Journal of Neuroscience*, vol. 33, no. 7, pp. 1264–1274, 2011.

[121] O. von Bohlen und Halbach, L. Minichiello, and K. Unsicker, "Haploin-sufficiency for trkB and trkC receptors induces cell loss and accumulation of $\alpha$-synuclein in the substantia nigra," *FASEB Journal*, vol. 19, no. 12, pp. 1740–1742, 2005.

[122] M. Baydyuk, M. T. Nguyen, and B. Xu, "Chronic deprivation of TrkB signaling leads to selective late-onset nigrostriatal dopaminergic degeneration," *Experimental Neurology*, vol. 228, no. 1, pp. 118–125, 2011.

[123] Y.-X. Ding, Y. Xia, X.-Y. Jiao et al., "The TrkB-positive dopaminergic neurons are less sensitive to MPTP insult in the substantia nigra of adult C57/BL mice," *Neurochemical Research*, vol. 36, no. 10, pp. 1759–1766, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 579632, 9 pages
http://dx.doi.org/10.1155/2014/579632

*Research Article*

# Recombinant Keratinocyte Growth Factor 1 in Tobacco Potentially Promotes Wound Healing in Diabetic Rats

**Zhi-Guo Feng,[1] Shi-Feng Pang,[2] Ding-Jiong Guo,[3] Yue-Tao Yang,[4] Bin Liu,[4] Ji-Wei Wang,[4] Ke-Qin Zheng,[2] and Yi Lin[1]**

[1] *College of Life Science, Anhui Agricultural University, Hefei 230036, China*
[2] *Department of Biology, Guangdong Medical College, Dongguang 523808, China*
[3] *Department of General Surgery, Cixi People's Hospital, Ningbo 315300, China*
[4] *Traumatic Medicine Center, Lishui People's Hospital, Lishui 315300, China*

Correspondence should be addressed to Yi Lin; xiaozhang8183@126.com

Received 13 December 2013; Accepted 20 February 2014; Published 24 March 2014

Academic Editor: Jian Xiao



Keratinocyte growth factor 1 (KGF1) is a growth factor that promotes epidermal cell proliferation, migration, differentiation, and wound repair. It is expressed at low levels in a form of inclusion body in *E. coli*. In order to increase its expression and activity, we produced tobacco plants expressing KGF1 via *Agrobacterium-mediated* transformation using a *potato virus X (PVX)*-based vector (pgR107). The vector contained the sequence encoding the KGF1 gene fused with a green florescence protein. The recombinant plasmid was introduced into leaf cells of *Nicotiana benthamiana* (a wild Australian tobacco) via *Agrobacterium-mediated* agroinfiltration. As determined by fluorescence and Western blot of leaf extracts, the KGF1 gene was correctly translated into the tobacco plants. The recombinant KGF1 was purified from plant tissues by heparin affinity chromatography, and cell proliferation in NIH/3T3 cells was stimulated by the purified KGF1. The purified KGF1 was also applied to the wounds of type-II diabetic rats. KGF1 had accumulated to levels as high as 530 $\mu$g/g fresh weight in the leaves of agroinfected plants. We show that plant-derived KGF1 can promote the proliferation of NIH/3T3 cells and have significant effects on the type-II diabetic rat. The present findings indicated that KGF1 from tobacco maintains its biological activity, implying prospective industrial production in a plant bioreactor.

## 1. Introduction

Keratinocyte growth factor (KGF1) is a member of the fibroblast growth factor family (also denoted as FGF7). KGF1 is produced by cells of mesenchymal origin and secreted via a paracrine secretion mechanism. It binds to epithelial cell-specific receptors and plays important roles in wound repair, embryonic development, tumor generation, and immunologic reconstitution [1–7]. KGF1 promotes cell proliferation and is expressed at high levels during wound repair. KGF1 can also promote epidermal cell proliferation, migration, and differentiation and can increase proliferation and differentiation of skin hair follicles and sebaceous gland precursor cells [6, 8–10]. Previously, we expressed KGF1 in *E. coli* and found that KGF1 expression levels were low and mainly confined to a form of inclusion body, which causes difficulties in KGF1 purification and limits the usefulness of KGF1 in basic and applied pharmacology and pathology researches. In this study, we employed a tobacco viral expression system to improve the expression efficiency and activity of KGF1.

Plants have been studied extensively for the production of pharmaceutical proteins, as they represent an inexpensive and scalable alternative to common expression systems. Plants offer advantages over microbial or mammalian host systems: the bioprocessing is more effective, they have all of the cellular machinery needed to complete posttranslational modifications of proteins, and they are intrinsically safe. The use of the *Potato virus X* (PVX) vector in such systems is also safe, as there is no evidence that PVX is transmitted by naturally occurring vectors, for example, insects, nematodes, and fungi. Moreover, the virus is not transmitted through

seeds or pollen, so risk of its accidental release into the environment is extremely small. Therefore, environmentally safe conditions are attainable with only a moderate investment in infrastructure. The combination of a plant production system and a PVX-based vector is a promising platform for the production of recombinant proteins [11–15].

Distinct from stable transgenic plants, plant RNA viruses provide a temporary, transient expression system. These viruses are engineered to carry and replicate foreign genes in susceptible host plants. The sequences delivered by viruses into the infected plant cell remain part of the virus genomic RNA and do not integrate into the plant genome. Moreover, the foreign transcripts are amplified by the viral replicase in the cell cytoplasm and are not inherited. Plant viruses are plausible transient expression vectors since the introduced genes are expressed at high levels and the purification of the products from the plants is simple and inexpensive.

## 2. Methods

*2.1. Vector Construction.* Plasmids pgR107 and pgR107-smGFP and KGF1 DNA were generously provided by Dr. Xingzhi Wang (Institute of Genetics and Cytology, Northeast Normal University, Changchun, China). The parental plasmid pUCKGF1 contains a ClaI/SmaI/SalI multiple cloning site surrounded by two subgenomic viral coat protein (CP) promoters. The pgR107 plasmid contains a 35S promoter, which drives synthesis of infectious PVX transcripts in plants [16]. The KGF1 gene (Gene ID: 14178) was inserted into the cloning site of the PVX within pgR107, and its expression was controlled by one of the CP promoters. The *KGF1-GFP* sequence flanked by ClaI and SalI digestion sites was amplified by PCR and cloned into the pgR107 vector, resulting in pgR107-*KGF1-GFP*.

*2.2. Plant Material and Inoculation Conditions.* The transfection of pgR107-*KGF1-GFP* and pgR107-smGFP plasmids was conducted by a freeze-thaw method using *A. tumefaciens* GV3101 with *N. benthamiana* plants (4-5 weeks of age) at approximately the ten-leaf stage [17]. The recombinant agrobacteria were separately cultured in 50 mL LB (Luria-Bertani) medium (supplemented with kanamycin (50 mg/L), rifampicin (50 mg/L), tetracycline (12.5 mg/L), 10 mM MES, and 20 $\mu$M acetosyringone) overnight at 28°C. The cells were collected by centrifugation at 1,500 g for 10 min and resuspended and incubated in liquid minimal medium agar without shaking (10 mM $MgCl_2$, 10 mM MES, and 100 $\mu$M acetosyringone) for 2-3 h at room temperature before infiltration. After agrobacterial infiltration onto the underside of the tobacco leaves, the leaves were placed in a dark humid atmosphere for 24 h to recover from the treatment. The recombinant protein expression was confirmed by green fluorescence illumination with an ultraviolet light as well as by TCS SP5 confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Germany).

*2.3. Protein Extraction and Purification.* Seven days after infection, the *N. benthamiana* leaves were harvested and frozen in liquid nitrogen. The leaves were then ground into an extraction buffer (50 mM PBS, 1 mM PMSF, and pH 9.5), at a ratio of 3 mL per gram leaf material. Extracts were collected by centrifugation at 13,520 g for 20 min at 4°C. *KGF1-GFP* fusion protein was purified by heparin affinity chromatography and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fusion protein was further cleaved with bovine enterokinase (rbEK) to remove the end of the *C*-terminal peptide for obtaining active KGF-1 protein; KGF-1 was separated from smGFP by heparin affinity chromatography.

*2.4. SDS-PAGE and Western Blot.* For Western blot analysis, cellular lysates were transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Perkin Elmer, Boston, MA, USA) and immunoblotted with a monoclonal mouse anti-KGF1 antibody (Abcam Inc., USA) (diluted to 1 : 1,500 in 5% DM/PBST) according to the manufacturer's instructions. Immunoreactive bands were visualized with Western blotting luminal reagents (Western Blotting Luminol Reagent sc-2048 Trial Kit, Santa Cruz Biotechnology Inc., CA, USA).

*2.5. ELISA Assay of KGF1-GFP in N. benthamiana Plants.* The powder of ground leaves (0.5 g fresh weight) was homogenized with 1.5 mL of 50 mM PBS (pH 9.5) and centrifuged for 20 min at 13,520 g to obtain total protein extracts from transfected and control plants. The concentration of total soluble protein was estimated by the Bradford assay method. For enzyme-linked immunosorbent assay (ELISA), serial dilutions of total protein extracts from 1 : 50 to 1 : 1,000 in phosphate-buffered saline (PBS) were incubated in 96-well polyvinyl chloride microtiter plates for 2 h at 37°C. The plates were then incubated with 1% fat-free dry milk (DM) for 1 h at 37°C. After the plates were washed three times with PBS containing 0.05% Tween 20 (PBST), the mouse anti-KGF1 antibodies (diluted to 1 : 2,000 in 1% DM/PBST) were added (50 $\mu$L per well) to the testing wells and the plates were incubated for 2 h at 37°C. Next, a rabbit anti-mouse IgG-horseradish peroxidase conjugate (Sigma; 1 : 2,000 in 1% DM/PBST) was added to the testing wells and the plate was incubated for 1 h at 37°C. The plate was then developed with Slow TMB substrate (Pierce) for 15 min at 23°C, and the reaction was subsequently terminated by adding an equal volume of 0.5 M $H_2SO_4$. To construct the standard curve, bacteria-derived KGF1 was diluted with PBS to concentrations ranging from 0 to 10 $\mu$g/mL and processed as above. All of the ELISA assays results are means of triplicated experiments.

*2.6. NIH/3T3 Cell Proliferation Assay.* For cell counting and the construction of growth curves, NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. At 80% confluence, cells were collected and split. Cells ($10^5$/mL) were seeded in 96-well plates (50 $\mu$L/well) and incubated at 37°C, in a 5% $CO_2$ atmosphere for 24 h. The media were replaced with keratinocyte serum-free medium (K-SFM, Sigma). Cells were divided into 4 groups with 6 replicates in each group. The control group received only K-SFM (free of KGF1),

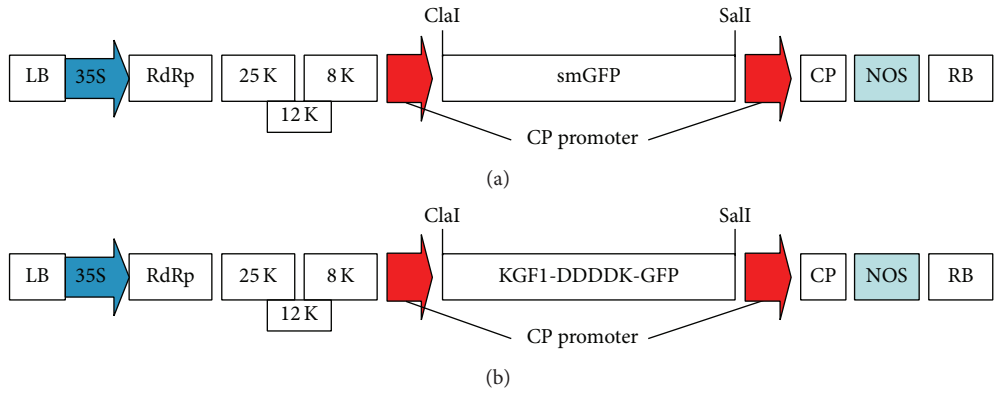



FIGURE 1: (a) Schematic map of plasmid pgR107-smGFP. (b) Schematic map of plasmid pgR107-KGF1-GFP. LB and RB, left and right border sequences, respectively, of T-DNA of *A. tumefaciens*; NOS, transcriptional terminator of nopaline synthase gene of *A. tumefaciens*; RdRp, viral RNA-dependent RNA polymerase; K, kDa; 25 K, 12 K, and 8 K, viral movement proteins; CP, viral coat protein; 35S, cauliflower mosaic virus 35S promoter.

and the positive control group received medium containing 100 ng/mL commercial standard KGF-1. The experimental groups received media containing 100 ng/mL purified plant KGF1. Cells were incubated at 37°C in a 5% $CO_2$ atmosphere for 24, 36, 48, 60, and 72 h, respectively. After the treatments, cells were collected by mild trypsinization and counted for cell numbers to construct a growth curve. Cell morphology was monitored at 72 h.

*2.7. Animal Model for Wound Healing.* We took care of all animals according to the Guiding Principles in the Care and Use of Animals. Female Sprague-Dawley rats (~ 200 g) were subjected for induction of type-II diabetes by a single intraperitoneal injection of streptozotocin (STZ, Sigma; 30 mg/kg in citrate buffer). Diabetic rats were injected intraperitoneally with 8% chloral hydrate as an anaesthetic and then were fixed on a simple platform to facilitate cutting of hair and disinfection of the back skin with iodophor. Each rat was given two wounds (2 cm in diameter) by application of weights, heated to 95°C in a hot water bath, to its back bilateral to the spinal axis. Plant KGF-1 (500 ng/mL in saline) was applied to the right wound, and an identical amount of saline was applied on the left as in the control group. Application of 500 ng/mL of standard KGF1 served as the positive control. The administration method consisted of applying either 200 $\mu$L KGF-1 solution or saline once every other day for two weeks. Photographic records were obtained throughout the experiments, for a total period of 28 d.

*2.8. Pathology Evaluation.* Rat skin tissue samples were collected from three groups: plant KGF1, positive control, and control. Samples were embedded in paraffin, sliced, subjected to Masson's staining, and examined with light microscopy.

*2.9. Statistical Analysis.* The data were expressed as the mean ± SEM. Statistical significance was determined by Student's $t$-test when there were two experimental groups. For more than two groups, statistical evaluation of the data was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, with $P < 0.05$ considered significant.

## 3. Results

*3.1. Plasmid Construction.* We constructed binary virus vectors pgR107-*KGF1-GFP*. The DNA coding sequences for *KGF1-GFP* were controlled by a strong subgenomic promoter of the PVX coat protein. Therefore, the genes could be expressed rapidly in *N. benthamiana* concurrently with viral replication. To increase the expression of KGF1, we inserted the Kozak sequence at the 5′ end of KGF1 and changed some of the native KGF1 sequence codons to plant preferred codons without altering the amino acid composition of the protein. Furthermore, the DDDDK enterokinase cleavage site was inserted into the *KGF1-GFP* gene (Figure 1(b)), which allowed complete removal of the smGFP portion from *KGF1-GFP* via a cleavage reaction.

*3.2. Agroinfection of N. benthamiana Plants.* The *N. benthamiana* plants agroinfected with *A. tumefaciens* containing pgR107-smGFP (Figure 1(a)) and pgR107-*KGF1-GFP* (Figure 1(b)) developed the characteristic mosaic symptoms of PVX infection. Expression of the recombinant protein was visualized by fluorescence in plants agroinfected with GV3101/pgR107-*KGF1-GFP*. Under ultraviolet illumination, green fluorescent spots were clearly visible on agroinfected leaves of *N. benthamiana* at 3-4 days postagroinfection (dpi). Diffuse green fluorescence of smGFP and *KGF1-GFP* was visible in stems and leaves above the infected leaf on the day after agroinfection. The highest accumulation of smGFP (Figure 2(d)) or *KGF1-GFP* (Figure 2(c)) was in leaves and stems at approximately 7 dpi, after which the amounts of smGFP and *KGF1-GFP* tended to decrease. Confocal microscopy analysis revealed that the *KGF1-GFP* and smGFP proteins were localized in the cytoplasm (Figure 3). These results demonstrate that agroinfection is an efficient method of initiating PVX infection and that the PVX-based

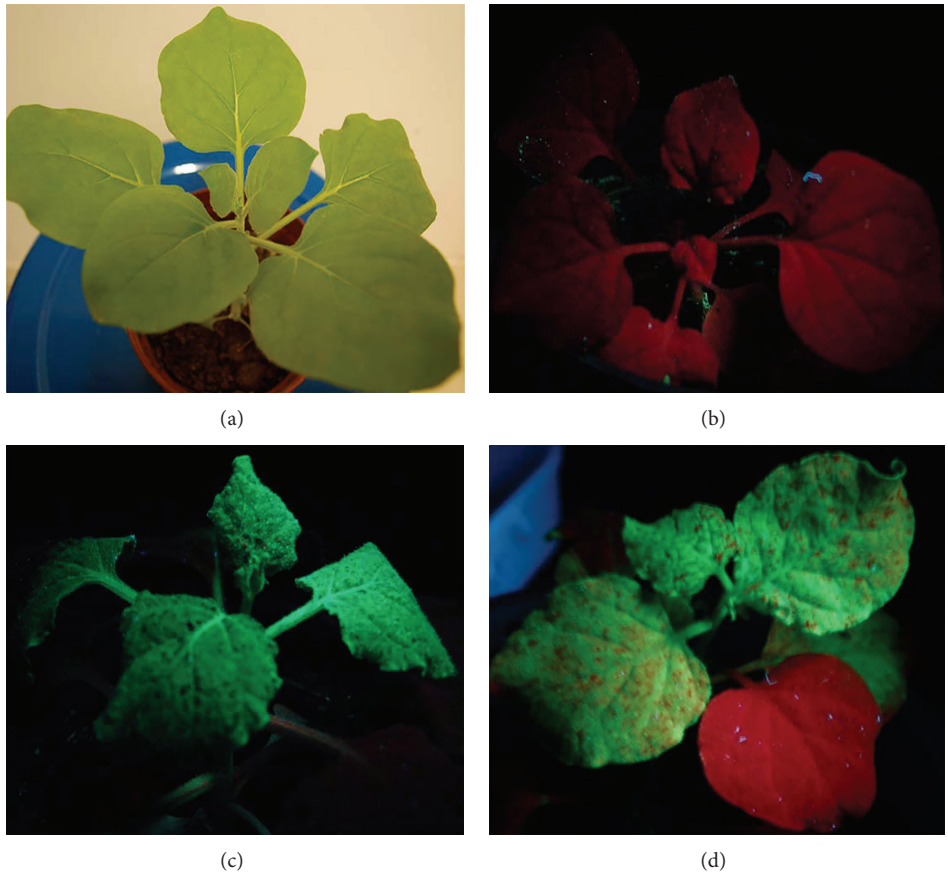



Figure 2: Green fluorescent images produced by UV illumination. (a) Control plants (noninfected) under visible light. (b) Noninfected plant under UV illumination. (c) Expressed KGF1-GFP visualized at 7 dpi under UV illumination. (d) Expressed smGFP visualized at 7 dpi under UV illumination.

binary vector is an efficient transient expression vector for production of *KGF1-GFP* in *N. benthamiana*.

*3.3. SDS PAGE and Western Blot.* The analysis of KGF1-GFP (49 kDa) expression by SDS-PAGE showed that *KGF1-GFP* (Figure 4) was expressed at high levels. The KGF1 protein was cleaved from the *KGF1-GFP* fusion protein by bovine enterokinase. *KGF1-GFP* and KGF1 (Figure 4) were purified from the cellular lysates of *N. benthamiana* leaves. Western blots of proteins purified from *N. benthamiana* leaves further confirmed the expression of *KGF1-GFP* fusion protein and KGF1. The results showed that *KGF1-GFP* was expressed (Figure 5).

To reliably quantify the level of expression, leaf extracts were analyzed by direct ELISA. The ELISA results showed that *KGF1-GFP* was expressed at high levels in the cytosol, accumulating to approximately 530 $\mu$g per gram fresh leaf weight. The stability of the *KGF1-GFP* fusion protein may well protect its KGF1 portion from degradation by hydrolase. Native KGF1 can be obtained after cleavage of the GFP. In other systems using plant virus-mediated transient expression, the reported yields of recombinant pharmaceutical protein purified from tobacco leaves ranged from 0.00003 to 2 mg/g leaf fresh weight.

*3.4. NIH/3T3 Cell Proliferation Assay Treated by KGF1.* We used K-SFM as the control and KGF1 standard protein as the positive control. Cell count results show that the KGF-1 standard and plant-derived KGF1 promote significantly more NIH/3T3 cell proliferation than that observed in the control group, especially in the time period from 36 h to 72 h after treatment (Figure 6). The proliferation effect of KGF-1 standard and plant-derived KGF1 seems to be of no difference. After 72 h, both two kinds of KGF-1 stimulated cell proliferate for 3-fold more than the cell number in 24 h. These results confirm that the plant-derived KGF1 protein can promote cell proliferation. When compared with the standard KGF1 protein from bacteria, there is no significant difference.

*3.5. Wound Healing Evaluation Assay.* In order to evaluate the wound healing activity of plant-derived KGF1, we performed a healing evaluation assay in SD rats. Results are shown in Figure 7. We used 0.9% normal saline solution as the control. It was seen that the wounds treated by KGF-1 and KGF-1 standard recovered much more quickly with better skin appearance (Figure 7). After the 28th day, the wounds treated with plant KGF-1 showed wound closured mostly, while the wounds in the control had obvious wound surface. The healing time of the control group was 35.80 ± 0.37 days,

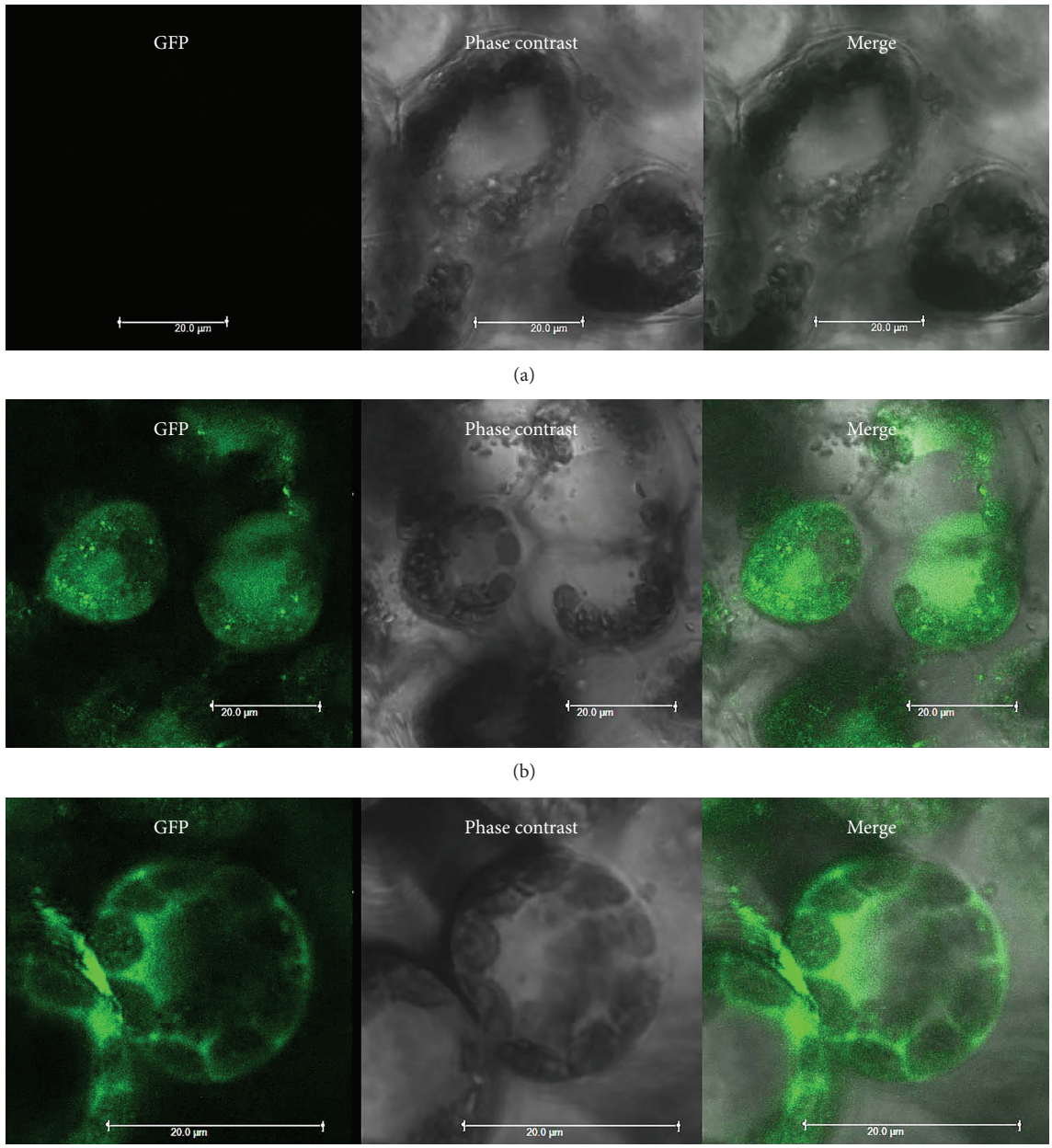



Figure 3: Confocal microscopy images. (a) Noninfected control tobacco cells. (b) KGF1-GFP fusion gene expression in tobacco cells. (c) smGFP expression in tobacco cells. Bar = 20 $\mu$m.

while that of the plant-derived KGF1 group was 28.19 ± 0.62 days and that of the KGF1 standard protein group was 28.84 ± 0.77 days. These results show that the wound healing time of the plant-derived KGF1 group was significantly shorter than that of the control group, while there was no significant difference between the plant-derived KGF1 group and the KGF1 standard control group.

*3.6. Masson's Staining.* Masson's staining stained the collagen fibers of the tissue green. After 3 days, we observed that the rat skin epithelia were deeply stained in all groups. Figure 8 shows increased signs of necrosis, thickening layers, formation of blood crust on the surface, and dermal fiber degeneration. Particularly, KGF1 groups (the KGF1 standard group and recombinant KGF1 group) showed more serious dermis denaturing accompanied by vacuolar degeneration. At the 7th and 14th days, the KGF1 groups showed severe edema, more profound necrosis, severe structural damage, increased necrotic degeneration, and pyknosis more than the control group. In the meantime, the necrosis organization also began to be cleared, and this is conducive to the remodeling of latter tissue. On the 21st day, tissue remodeling had

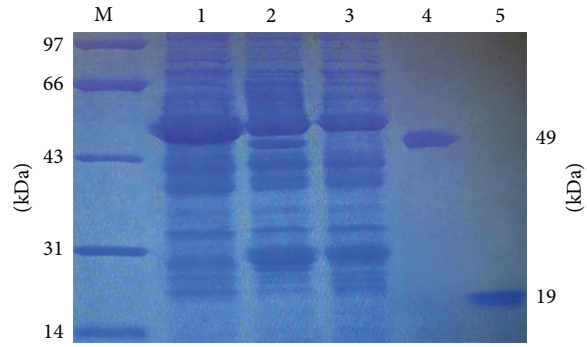



Figure 4: SDS-PAGE analysis. M: molecular weight standard. 1: WT noninoculated control tobacco leaves. 2: pgR107-KGF1-GFP. 3: pgR107. 4: KGF1-GFP (49 KDa) purified from tobacco leaves at 7 dpi. 5: KGF1 (19 KDa) after cleavage and separation from KGF1-GFP fusion protein.

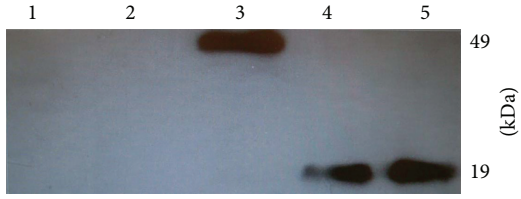



Figure 5: Western blot of purified KGF1-GFP and KGF1. KGF1-GFP and KGF1 from tobacco leaves were resolved by SDS-PAGE, blotted, and probed with mouse anti-KGF1 antibody. 1: noninoculated control tobacco leaves. 2: agroinfected with GV3101/pgR107. 3: agroinfected with GV3101/pgR107-KGF1-GFP(49 KDa). 4: KGF1 (19 KDa) after cleavage and separation from KGF1-GFP fusion protein. 5: *E. coli* recombinant KGF1.

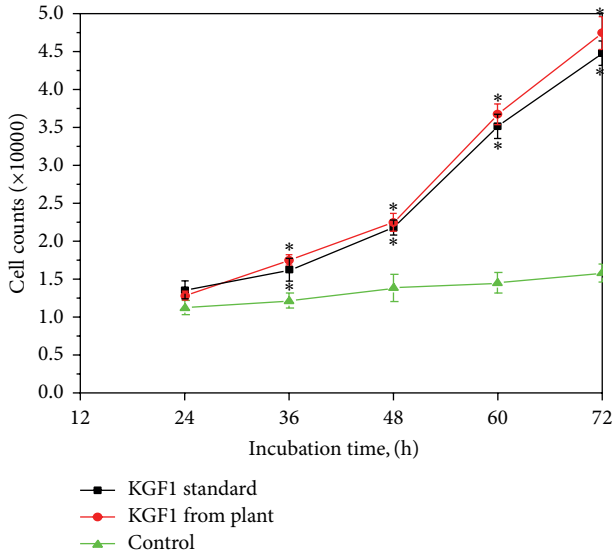



Figure 6: Growth curve of NIH/3T3 cells. KGF1 standard protein and plant-derived KGF1 protein have the same effect on proliferation of NIH/3T3 cell. Both promote faster cell proliferation than control (K-SFM). Values are mean ± SD of three independent experiments. $^{*}P < 0.05$ versus control group.

been completed, and collagen formation was also observed. The administration groups' skin collagen was better arranged than that of the control group. Also, much more neovascularization was observed in the administration groups, which is favorable for supplying nutrients to the tissue. By the 28th day, the tissue had completed epithelization. The collagen fibers in the plant-derived KGF1 treated group and KGF-1 standard protein treated group were in good arrangement. On the contrary, the control group displayed disordered collagen.

## 4. Discussion

Here, we report an expression system using *Nicotiana benthamiana* to produce KGF-1. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strains containing pgR107 binary vectors that were designed to combine the advantages of *A. tumefaciens*-mediated transfection and viral infection. We inserted the coding sequence of KGF-1 and smGFP into the cloning sites of the viral binary vector to construct the experimental plasmids pgR107-KGF1-GFP. Viral replication and expression of the recombinant fusion gene occurred concurrently in agroinfiltrated *N. benthamiana*.

A strong subgenomic promoter of the PVX coat protein controlled the DNA coding sequences for *KGF1-GFP*. This assures that the genes will be expressed rapidly in *N. benthamiana* concurrently with viral replication. Also, the Kozak sequence at the 5′ end of KGF1 is useful for increasing the expression of KGF1. Furthermore, alternating some of the native KGF1 sequence DNA codons to plant-preferred codons without changing the amino acid composition of the protein may increase the expression as well.

KGF1 is an epithelial cell-specific growth factor that promotes epidermal cell proliferation, migration, and differentiation, skin wound healing, and improvement of the quality of wound healing. Wound base fibroblasts are able to synthesize and release KGF1, which induces epidermal cell proliferation and migration toward the wound.

Traditional methods of KGF1 production involve animal tissue extraction and prokaryotic expression in *E. coli*. The first method, however, suffers from low yield and is expensive. The second method has the problem of unstable expression in

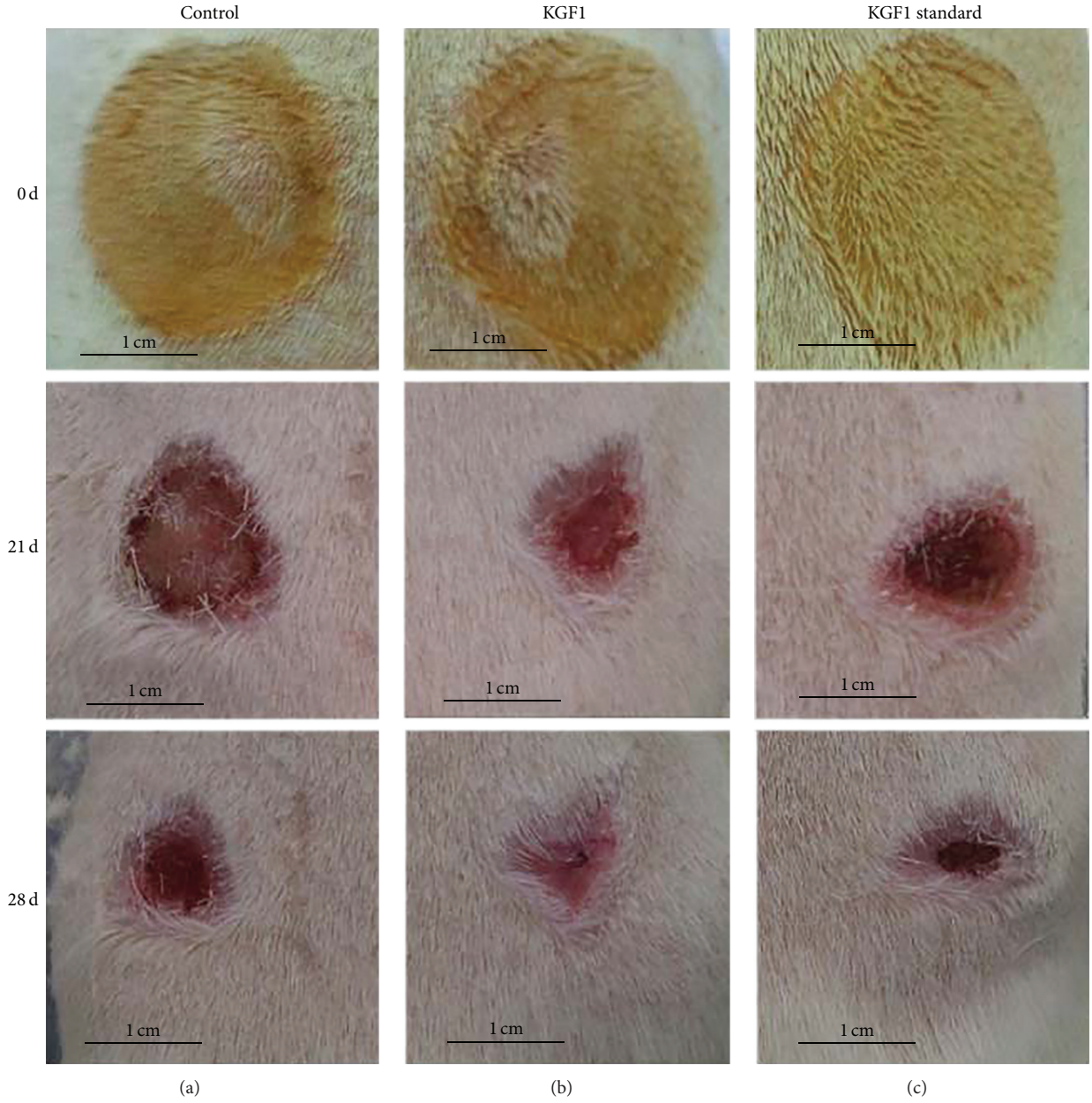



Figure 7: Wound healing assay. Left column: normal saline control group; center column: plant KGF1 group; right column: KGF1 standard protein group.

*E. coli*, mainly because the exogenous protein is expressed in inclusion bodies and so requires renaturation of the inclusion body protein. The biological activity of this product is very low. We expressed KGF1 in tobacco by using *Potato virus X* (pGR107), thereby seeking a low cost, simple method with a high yield of KGF1. In the present study, we constructed a plant viral expression vector pGR107-*KGF1-GFP* and then transiently expressed this construct in tobacco. We observed clear green fluorescence in infected tobacco leaves by ultraviolet excitation. Laser confocal microscopy scanning of the tobacco leaf cells revealed expression of the fusion protein containing KGF1. These pieces of data confirm that the fusion protein can be highly expressed in the tobacco leaf and can be excited to produce green fluorescence. After grinding the leaf and purifying the fusion protein, we validated that the *KGF1-GFP* fusion protein was successfully expressed in tobacco by SDS-PAGE and Western blot.

NIH/3T3 cell proliferation assay results show that plant-derived KGF1 can significantly promote the proliferation of NIH/3T3 cells, with the same activity of the KGF1 standard protein. Studies have shown that KGF-1 has a therapeutic effect on diabetic ulcers [18, 19]. In the present study, we used plant-derived KGF1 in the treatment of wounds in diabetic rats. The results show that the plant-derived fusion protein *KGF1-GFP*, after cleavage by erepsin, yields KGF1 that can significantly accelerate the speed of wound healing of diabetic

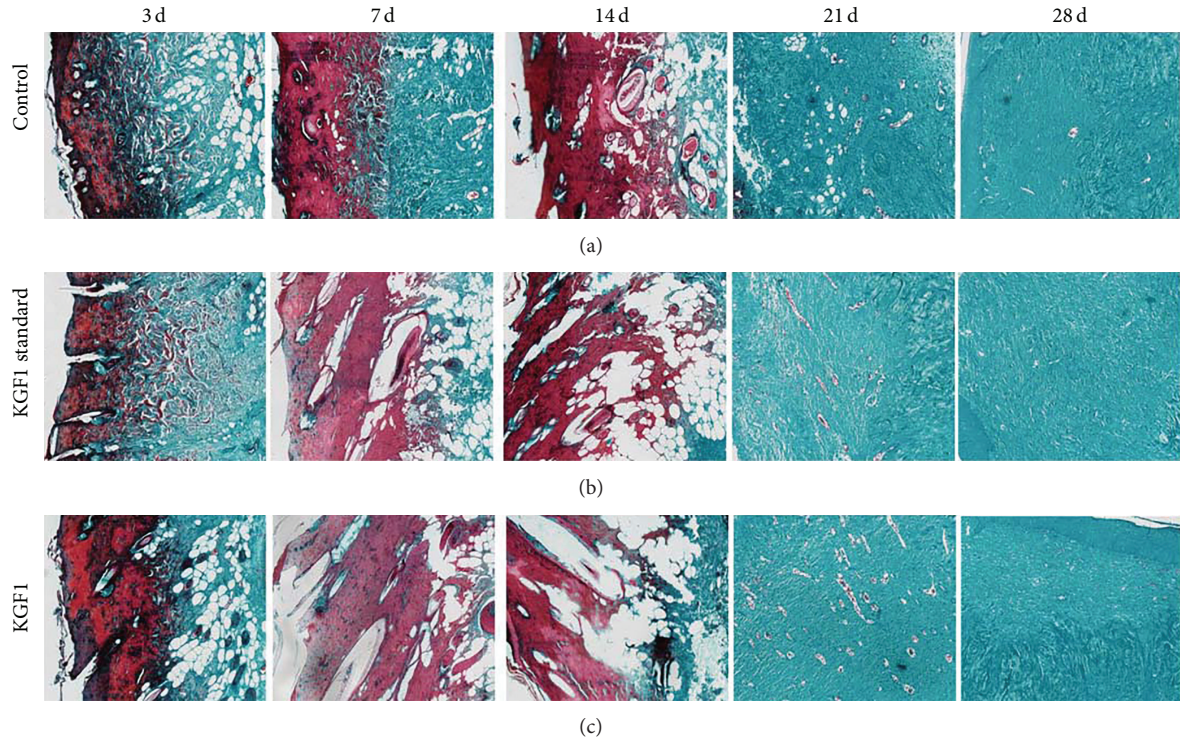



Figure 8: Masson's staining assay imaging. Top row: normal saline control group; center row: KGF-1 standard protein group; bottom row: plant-derived KGF-1 group. Magnification: 200x.

rats compared with rats treated with normal saline. Masson's staining shows that the plant-derived KGF1 has the same effects as KGF-1 standard protein, significantly increasing wound epithelialization and promoting necrotic tissue clearance and collagen synthesis compared with normal saline. These results indicate that KGF1 has positive effects on the treatment of difficult type-II diabetic ulcers, and the results also provide a theoretical basis for the application of KGF-1 in clinical type-II diabetes therapy.

## 5. Conclusion

In conclusion, the evidence demonstrated that tobacco plants expressed KGF1 via *Agrobacterium*-mediated transformation using a *Potato virus X*- (PVX-) based vector (pgR107). The plant-derived KGF1 promotes the proliferation of NIH/3T3 cells and significantly stimulates wound healing in the diabetic wounded rat model. Therefore, KGF1 is suggested as a prospective industrial production factor in plant bioreactors and a promising candidate for further development as a therapeutic agent for wound healing.

## Conflict of Interests

The authors confirm that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Zhi-Guo Feng and Shi-Feng Pang contributed equally to this work.

## Acknowledgments

This work was supported by Science & Technology Plan Fund of the Dongguan (Grant no. 201010815209), Ninbo City Natural Science Foundation (Grant no. 2012A610254), and Zhejiang Provincial Project of Key Group (Grant no. 2010R50042).

## References

[1] H. M. Takase, T. Itoh, S. Ino et al., "FGF7 is a functional niche signal required for stimulation of adult liver progenitor cells that support liver regeneration," *Genes & Development*, vol. 27, no. 2, pp. 169–181, 2013.

[2] I. Kakizaki, N. Itano, K. Kimata et al., "Up-regulation of hyaluronan synthase genes in cultured human epidermal keratinocytes by UVB irradiation," *Archives of Biochemistry and Biophysics*, vol. 471, no. 1, pp. 85–93, 2008.

[3] A. A. Tandara, O. Kloeters, J. E. Mogford, and T. A. Mustoe, "Hydrated keratinocytes reduce collagen synthesis by fibroblasts via paracrine mechanisms," *Wound Repair and Regeneration*, vol. 15, no. 4, pp. 497–504, 2007.

[4] S. Braun, C. Mauch, P. Boukamp, and S. Werner, "Novel roles of NM23 proteins in skin homeostasis, repair and disease," *Oncogene*, vol. 26, no. 4, pp. 532–542, 2007.

[5] G. P. Marti, P. Mohebi, L. Liu, J. Wang, T. Miyashita, and J. W. Harmon, "KGF-1 for wound healing in animal models," *Methods in Molecular Biology*, vol. 423, pp. 383–391, 2008.

[6] S. M. Tsai and W. P. Wang, "Expression and function of fibroblast growth factor (FGF)7 during liver regeneration," *Cellular Physiology and Biochemistry*, vol. 27, no. 6, pp. 641–652, 2011.

[7] B. Berent-Maoz, E. Montecino-Rodriguez, R. A. Signer, and K. Dorshkind, "Fibroblast growth factor-7 partially reverses murine thymocyte progenitor aging by repression of Ink4a," *Blood*, vol. 119, no. 24, pp. 5715–5721, 2012.

[8] P. L. Santa Maria, S. L. Redmond, M. D. Atlas, and R. Ghassemifar, "Keratinocyte growth factor 1, fibroblast growth factor 2 and 10 in the healing tympanic membrane following perforation in rats," *Journal of Molecular Histology*, vol. 42, no. 1, pp. 47–58, 2011.

[9] S. Raffa, L. Leone, C. Scrofani, S. Monini, M. R. Torrisi, and M. Barbara, "Cholesteatoma-associated fibroblasts modulate epithelial growth and differentiation through KGF/FGF7 secretion," *Histochemistry and Cell Biology*, vol. 138, no. 2, pp. 251–269, 2012.

[10] S. Aguilar, C. J. Scotton, K. McNulty et al., "Bone marrow stem cells expressing keratinocyte growth factor via an inducible lentivirus protects against bleomycin-induced pulmonary fibrosis," *PLoS ONE*, vol. 4, no. 11, Article ID e8013, 2009.

[11] K. Mann and B. Meng, "The triple gene block movement proteins of a grape virus in the genus Foveavirus confer limited cell-to-cell spread of a mutant Potato virus X," *Virus Genes*, vol. 47, no. 1, pp. 93–104, 2013.

[12] N. N. Kakareka, Iu. G. Volkov, Z. N. Kozlovskaia, and T. I. Pleshakova, "Production of immunodiagnosticum preparations based on plant virus strains," *Mikrobiolohichnyĭ Zhurnal*, vol. 75, no. 1, pp. 69–78, 2013.

[13] M. R. Park and K. H. Kim, "Molecular characterization of the interaction between the N-terminal region of Potato virus X, (PVX) coat protein (CP) and Nicotiana benthamiana PVX CP-interacting protein, NbPCIP1," *Virus Genes*, vol. 46, no. 3, pp. 517–523, 2013.

[14] K. L. Hefferon, "Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins," *Virology*, vol. 433, no. 1, pp. 1–6, 2012.

[15] L. G. Tyulkina, E. V. Skurat, O. Y. Frolova, T. V. Komarova, E. M. Karger, and I. G. Atabekov, "New viral vector for superproduction of epitopes of vaccine proteins in plants," *Acta Naturae*, vol. 3, no. 4, pp. 73–82, 2011.

[16] B. Wagner, H. Fuchs, F. Adhami, Y. Ma, O. Scheiner, and H. Breiteneder, "Plant virus expression systems for transient production of recombinant allergens in Nicotiana benthamiana," *Methods*, vol. 32, no. 3, pp. 227–234, 2004.

[17] C. Lacorte, S. G. Ribeiro, D. Lohuis, R. Goldbach, and M. Prins, "Potato virus X and Tobacco mosaic virus-based vectors compatible with the Gateway cloning system," *Journal of Virological Methods*, vol. 164, no. 1-2, pp. 7–13, 2010.

[18] C. Peng, B. Chen, H.-K. Kao, G. Murphy, D. P. Orgill, and L. Guo, "Lack of FGF-7 further delays cutaneous wound healing in diabetic mice," *Plastic and Reconstructive Surgery*, vol. 128, no. 6, pp. 673e–684e, 2011.

[19] G. Marti, M. Ferguson, J. Wang et al., "Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model," *Gene Therapy*, vol. 11, no. 24, pp. 1780–1785, 2004.