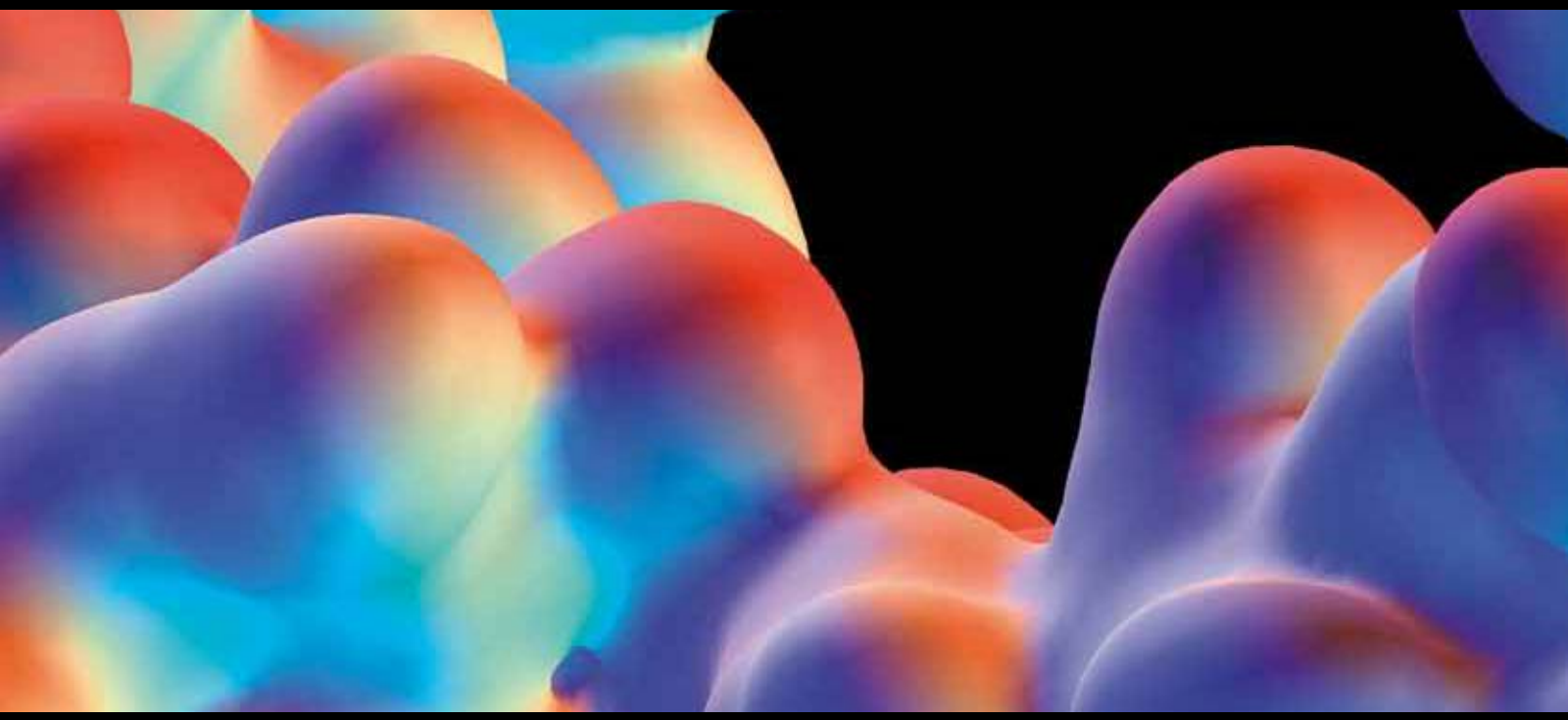
Enzyme Research

Skin Diseases-Related Enzymes: Mechanisms and Clinical Applications

Guest Editors: Yong-Doo Park, Jun-Mo Yang, and Zhi-Rong Lü



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Editorial Board

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Editorial

Skin Diseases-Related Enzymes: Mechanisms and Clinical Applications

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There are higher numbers of known diseases of the skin than any other organ system due to the fact that skin is the first defense system against the external environment and it primarily responses to the various outer challenges. In this regard, studies on the skin diseases-associated enzymes are, therefore, important to understand the complex nature of defense mechanisms and various pathogeneses.

In the present special issue, we focused on the several enzymes from the broad category of skin diseases-related enzymes that the authors mainly contributed the review and the research articles. We invited investigators to contribute to the several potential topics that expected to provide insights into the roles of enzymes in skin diseases, and, as the final outcome, six review articles that focused on matrix metalloproteinases, ADAMs family proteinases, autotaxin, AKT signaling, and manganese superoxide dismutase and four original research articles that are associated with pigmentation, psoriasis, and inflammation are published in the current special issue.

The review articles include the following:

(i) bifacial roles of matrix metalloproteinases (MMPs) was described: treatments for aging and cancer via inhibiting MMPs are paid attentions while stimulating MMPs is also applied to prevent wound scars and epidermal hyperproliferative diseases,

(ii) the roles of a disintegrin and metalloproteinases (ADAMs) belonging to the zinc protease superfamily in a wide variety of skin diseases,

(iii) mechanisms of autotoxin and its metabolite lysophosphatidic acid (LPA) in melanoma and potential treating strategy,

(iv) therapeutic ideas for melanoma by regulating Akt3 and the related molecules in the pathway of AKT signaling,

(v) the roles of manganese superoxide dismutase in skin cancer and in inflammation,

and the original research articles describe the following:

(i) inhibition kinetics for tyrosinase, a core enzyme of pigmentation, accompanying with the computational docking simulation,

(ii) enzymatic functional and structural studies for manganese superoxide dismutase that is well-known to associate with inflammation,

(iii) bioinformatic analyses of probing therapeutic candidates for psoriasis by using PPI mappings and DB analyses,

(iv) computational simulations and interactomics for the novel binding partners of creatine kinase.

In this special issue, with the efforts of the authors, several important diseases-associated enzymes have been newly understood, and the integrating studies between enzymology and computational simulations successfully revealed the factors associated with complex cutaneous diseases. This special

issue also handles the several enzymes that are important for the clinical applications.

Publication of this special issue marks the part of the long journey of *Enzyme Research*, and I hope that this special issue serves a vehicle for the high-quality research of enzyme researchers in some respects.

Acknowledgment

The author thank all the authors who contributed their papers to this issue and specially appreciate the invited guest editors and the reviewers for their endeavors and contributions.

Yong-Doo Park
Jun-Mo Yang
Zhi-Rong Lü

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Review Article

Beneficial Regulation of Matrix Metalloproteinases for Skin Health

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Matrix metalloproteinases (MMPs) are essential to the remodeling of the extracellular matrix. While their upregulation facilitates aging and cancer, they are essential to epidermal differentiation and the prevention of wound scars. The pharmaceutical industry is active in identifying products that inhibit MMPs to prevent or treat aging and cancer and products that stimulate MMPs to prevent epidermal hyperproliferative diseases and wound scars.

1. Introduction

Matrix metalloproteinases (MMPs) are essential to the remodeling of the extracellular matrix. While their upregulation facilitates aging and cancer, they are essential to epidermal differentiation and the prevention of wound scars. The pharmaceutical industry is active in identifying products that inhibit MMPs to prevent or treat aging and cancer and products that stimulate MMPs to prevent epidermal hyperproliferative diseases and wound scars.

2. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc-dependent extracellular proteinases, also called matrixins or collagenases, which remodel the extracellular matrix (ECM) [1–10]. The ECM gives tissue its structural integrity and predominantly comprises of the fibrillar collagens, basement membrane, and elastin fibers composed of elastin and fibrillin [11–13]. There are three predominant groups of MMPs: collagenases, gelatinases, and stromelysins [1–13]. The collagenases (MMP-1, -8, -13, and -18) cleave interstitial (structural) collagens, with MMP-1 as the predominant one. Gelatinases, primarily MMP-2 and MMP-9, degrade basement membrane collagens and degrade denatured structural collagens. The stromelysins (MMP-3, -10, -11, and -19) degrade basement membrane collagens as well as proteoglycans and matrix glycoproteins. The other MMP classes include membrane-type MMPs (MT-MMP: MMP-14, -15, -16, -17, -24, and -25) that activate MMPs, matrilysins that degrade the basement membrane (MMP-7 and -26), elastase that degrades primarily elastin (MMP-12), and others (MMP-20, -21, -22, -23, -27, and -28). MMPs are regulated in expression or activity at several different levels: gene expression, activation, and cellular inhibition of activity by tissue inhibitors of matrix metalloproteinases (TIMPs), especially TIMP-1 and TIMP-2 [12]. Epithelial cells, fibroblasts, neutrophils, and mast cells are some of the cell types that produce MMPs [1–13].

Transforming growth factor-β (TGF-β) is a predominant regulator of the expression of MMPs and the ECM [1, 2, 12]. It is secreted extracellularly in a latent form and activated by proteases to form the mature TGF-β that binds to its receptor complexes to activate Smads and thereby the regulation of various genes, including MMPs, collagen, and elastin [1, 2, 12]. TGF-β has differential effects in different cell types [1, 2, 12]. It inhibits MMP-1 and stimulates collagen, MMP-2, and TIMPs in fibroblasts, whereas in keratinocytes it stimulates the expression of MMPs and inhibits cell growth [1, 2, 12].

3. Skin Aging and Carcinogenic Effects of Matrix Metalloproteinases

3.1. Skin Aging and Its Prevention. Skin aging is the result the intrinsic chronological aging process superimposed by environmental factors, predominantly exposure to ultraviolet (UV) radiation (photoaging) [1, 2, 11–17]. UV radiation shifts the cellular balance to oxidative stress, inflammation, immunosuppression, and inhibition of TGF-β [1, 2, 12]. In addition, UV radiation stimulates proinflammatory and proangiogenic cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) [1, 2]. Cellular damage in photoaged skin includes cell loss, DNA damage, lipid peroxidation, and compromise of skin barrier function [13].

Alterations in collagen and elastin of the ECM are primarily responsible for the clinical manifestations of skin aging such as wrinkles, sagging, and laxity [1, 2, 11–13, 17, 18]. The atrophy of collagen and elastin fibers in skin aging is predominantly from the increased expression of their degradative enzymes, collagenases (MMP-1), gelatinases (MMP-2 and -9), and elastases [1–13]. Collagen fibers are degraded by MMP-1 and MMP-2 and the elastin fibers by elastases, MMP-2, and MMP-9 [12].

Natural products that have been identified in our laboratory to inhibit MMPs and elastase and simultaneously stimulate collagen and elastin are *Polypodium leucotomos* extract, lutein, and xanthohumol [12–18]. These products consist of polyphenols, carotenoids, or flavonoids with antioxidant, anti-inflammatory, photoprotective, or anticarcinogenic properties [12–18].

P. leucotomos is rich in polyphenols [1]. It directly inhibits activities of MMPs [12]. *P. leucotomos* inhibits expression of MMPs in epidermal keratinocytes and fibroblasts and stimulates fibrillar collagens, elastin, and TGF-β in dermal fibroblasts [12, 13]. In addition, it inhibits cellular oxidative stress and thereby membrane damage and lipid peroxidation [13]. Furthermore, it functions as a sunscreen [14, 15].

Lutein is a non-provitamin A carotenoid that inhibits epidermal hyperproliferation, expansion of mutated keratinocytes, and the infiltration of mast cells in response to solar radiation, and thereby photoaging [16]. The mechanism to lutein's antiaging and antiphotoaging effects includes the inhibition of MMP-to-TIMP ratio in dermal fibroblasts and the inhibition of cell loss and membrane damage in ultraviolet radiation-exposed fibroblasts [17].

Xanthohumol, a flavonoid, directly inhibits MMPs (-1, -3, and -9) and elastase activities while dramatically increasing the expression of types I, III, and V collagens, elastin, fibrillin-1, and fibrillin-2 in dermal fibroblasts [18].

3.2. Skin Cancer and Its Prevention. Carcinogenesis is characterized by development of tumors from genetic alterations and immunosuppression. The mechanisms also include oxidative stress from reactive oxygen species, inflammation and DNA damage [19]. Malignant tumors metastasize, with MMPs playing a central role [19]. Cancer invasion and metastasis of various cancer types parallelly increased expression of MMPs [19]. MMPs activate growth factors such as TGF-β and VEGF to induce angiogenesis [19]. Furthermore, MMPs contribute to cancer progression by degrading the ECM, basement membrane, and E-cadherin molecules that hold cells together [19–22]. In our laboratory we have investigated two categories of agents that may prevent or facilitate carcinogenesis through the regulation of expression of MMPs: (a) plant extracts or vitamins and (b) hormones.

The plant extracts or vitamins examined in our laboratory for MMP regulation in cancer cells are *P. leucotomos,* lutein and ascorbic acid [12, 17, 23, 24]. *P. leucotomos* inhibits MMP-1 expression transcriptionally, through AP-1 promoter sequence, and stimulates the expression of TIMP-2 in melanoma cells [4]. *P. leucotomos* in addition inhibits TGF-β, known to stimulate MMPs in cancer cells [12]. In photo-carcinogenesis experiments with rats, lutein inhibits tumor multiplicity, volume, and tumor-free survival time [16]. Furthermore, lutein inhibits MMP-1 and stimulates TIMPs (-1 and -2), to reduce MMP/TIMP ratio and thereby carcinogenesis [17]. Ascorbate has dose dependent differential effects on cancer cell growth versus expression of MMPs/metastasis potential [23, 24]. At lower concentrations, ascorbate inhibits cell growth with dramatic increases in the expression of MMPs, which are inhibited by cotreatment with *P. leucotomos* or gene silencing with MMP siRNA [23, 24].

4. Beneficial Effects of Matrix Metalloproteinases

4.1. Epidermal Differentiation and Wound Repair. Hyperproliferative diseases of the skin are associated with reduced MMPs [25]. The expression of MMP-9 is reduced in psoriatic keratinocytes, with hyperproliferative keratinocytes [26]. Hyperproliferative skin diseases are also associated with reduced generation of ceramides (the major lipids of the stratum corneum) or increased ceramidase activity [27–29]. Ceramides are intracellular messengers of the sphingomyelin cycle that activate protein kinase C-alpha and stress-activated protein kinases to induce apoptosis and epidermal differentiation [30]. The experiments in our laboratory indicate that ceramide induces MMP-1 expression in keratinocytes through the activator protein-1 (AP-1) sequence and simultaneously inhibits keratinocyte cell viability by apoptosis [25]. The mechanism of MMP-1 gene regulation by ceramide may be through the stimulation of TGF-β, known to inhibit epidermal cell viability [25].

MMPs are essential to wound healing. They remove wound debris, facilitate epithelization, and prevent wound scars from excess collagen [31–34]. In our laboratory, copper is effective in inducing wound healing whereas the antibodies to TGF-β associated with wound scars are ineffective [31–34].

The biologically active concentrations of copper range from 1 to 200 μM in tissue engineering for wound care, without toxicity [35]. The lower copper concentrations (0.3–3 μM) stimulate activity of MMPs whereas the higher

concentrations (1–100 μM) stimulate the expression of MMPs in fibroblasts [31]. Adult wound scars are attributed to increased TGF-β expression and subsequent collagen deposition [33]. However, TGF-β antibodies cause feedback stimulation of TGF-β [32, 33]. The feedback stimulation of TGF-β in turn inhibits MMPs and stimulates TIMPs in fibroblasts, with further scarring potential [32, 33].

5. Summary

MMPs are potent proteases that remodel the ECM. It is central to the aging and cancer process. *Polypodium leucotomos* extract, lutein, and xanthohumol are effective in inhibiting expression and/or activities of MMPs, and thereby aging and cancer. Conversely, MMPs can prevent psoriasis and wound scars. Ceramide stimulates expression of MMP-1 and simultaneously inhibits cell viability. Copper stimulates MMPs, which may be its mechanism to improve wound healing. However, the antibodies to TGF-β cause feedback stimulation of TGF-β, and further scarring potential.

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Review Article

The Roles of ADAMs Family Proteinases in Skin Diseases

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A disintegrin and metalloproteinases (ADAMs) are members of a new gene family of transmembrane and secreted proteins, which belong to the zinc proteinase superfamily. These molecules are involved in various biological events such as cell adhesion, cell fusion, cell migration, membrane protein shedding, and proteolysis. Growing evidence now attests to the potential involvement of ADAMs proteinases in diverse processes such as skin wound healing, inflammation, pigmentation, tumor development, cell proliferation, and metastasis. This paper focuses on the roles of ADAMs proteinases in a wide variety of skin diseases.

1. Introduction

A disintegrin and metalloproteinases (ADAMs) are members of a new gene family of transmembrane and secreted proteins, which belong to the zinc protease superfamily. These molecules are involved in various biological processes such as cell adhesion, cell fusion, cell migration, membrane protein shedding, and proteolysis. It has become clear in recent years that ADAMs proteinases have important roles in skin homeostasis and in skin diseases, and the signaling cascades involved are gradually being identified [1, 2].

Many transmembrane proteins are processed by one or several proteolytic steps to their biologically active forms [1, 2]. Examples include growth factors such as epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α), and cytokines such as tumor necrosis factor-α (TNF-α), all of which are synthesized as precursor proteins that need to be cleaved to gain their functional activity. In addition, there are a number of cell surface receptors that undergo cleavage near the transmembrane domain, a process called ectodomain shedding [1, 2]. These include the TNF-α receptor, CD44, L-selectin and KIT. The soluble, released ectodomains of those receptors may be part of their downmodulation in response to ligand activation and/or those fragments may have functions of their own.

In this paper, we discuss the implications of ADAMs proteinases in several physiological and pathological functions in skin tissue and their roles in various skin diseases.

2. Structure and Function of ADAMs Proteinases

There are two distinct groups in the ADAMs family: the membrane-anchored ADAMs and the secreted-type ADAMTS (ADAM with thrombospondin motifs), which are not discussed in this paper. Each member of the ADAMs family contains common domains, including the propeptide, metalloproteinase, disintegrin, cysteine-rich, EGF-like (though this is absent in ADAM10 and ADAM17), transmembrane and cytoplasmic domains (Figure 1). Although the structure of ADAMs and ADAMTS proteinases is closely related, ADAMTS molecules are characterized by various numbers of thrombospondin type one (TSP-1) motifs at their C-terminal ends and the absence of transmembrane and cytoplasmic domains.

The ADAMs group is comprised of more than 30 members (reviewed in [1, 2]). Proteinase activities have

Figure 1: Schematic of domain structure of ADAM proteinases. P: propeptide domain, M: metalloproteinase domain, D: disintegrin domain, CR: cysteine-rich domain, EGF: EGF-like domain, T: transmembrane domain, C: cytoplasmic domain, SP: spacer domain, TS: thrombospondin type I motif.

been demonstrated for ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, and 33, and these molecules contain a common HEXGHXXGXXHD sequence. About 60% of the members of this group are nonproteolytic ADAMs molecules.

Several ADAMs are expressed in multiple spliced forms. For example, ADAM22, ADAM29, and ADAM30 have two to three forms that vary in the lengths of their cytoplasmic tails, although no functional differences in those isoforms have been reported. In contrast, ADAM12 has two splice forms, a membrane-bound form and a secreted form, which have markedly different activities.

ADAM17 is the most extensively investigated ADAM proteinase and is known to release soluble TNF-α from its membrane precursor, pro-TNF-α, which permits TNF-α paracrine signaling; thus, ADAM17 is also called TACE (TNF-α-converting enzyme) [3]. ADAM17 plays a critical role in the ectodomain shedding of many soluble proteins, including TNF-α, TNF receptors I and II, TGF-α, HB-EGF, amphiregulin (AR), and interleukin-6 receptor (IL-6R). The development of pharmaceutical inhibitors for ADAM17 has focused on its role as a modulator of TNF-α in rheumatoid arthritis [4]. As TNF-α is a potent proinflammatory cytokine, ADAM17 inhibitors may be of particular utility in respiratory disease, ulcerative colitis, and other diseases. ADAM9 cleaves and releases EGF, HB-EGF, and fibroblast growth factor receptor 2 IIIb. ADAM10 is involved in the ectodomain shedding of various substrates, including adhesion molecules such as L1 cell adhesion molecule (L1-CAM) and CD44, E-cadherin, and N-cadherin, IL-6R, CD30. ADAM12 is known to participate in the ectodomain shedding of several potential substrates, including HB-EGF and EGF [5]. Moreover, ADAM12 regulates transforming growth factor-β (TGF-β) receptor trafficking. ADAM12 interacts with TGF-β receptor and enhances TGF-β signaling by controlling the localization of TGF-β receptors to early endosomes [6].

The ectodomain shedding of proHB-EGF actually produces two fragments, an extracellular fragment (HB-EGF), and a remnant fragment (HB-EGF-C). Recently, promyelocytic leukemia zinc finger (PLZF) was identified as a binding protein of the cytoplasmic tail of proHB-EGF [7]. PLZF is a transcriptional repressor of cyclin A and suppresses cell growth by inhibiting entry or progression into the S-phase of the cell cycle. Subsequent to proteolytic cleavage of proHB-EGF, HB-EGF-C translocates from the plasma membrane into the nucleus, which triggers nuclear export of the transcriptional repressor PLZF. Suppression of cyclin A and delayed entry into S-phase of cells expressing PLZF can be reversed by the production of HB-EGF-C. These results indicate that released HB-EGF-C functions as an intracellular signal and coordinates cell cycle progression with HB-EGF.

3. Involvement of ADAMs Proteinases in Skin Diseases

3.1. Skin Cancer. Since ADAMs can mediate the shedding of growth factors and regulate the adhesion and motility of cells, ADAMs family proteinases are involved in signaling events that are dysregulated in cancers and during tumor progression [8]. In many types of cancers, ADAMs are upregulated and several recent studies have highlighted the potential of targeting ADAMs family members as a new approach for antitumor therapy [9]. A schematic summarizing the functions of ADAMs proteins is shown in Figure 2.

3.1.1. Squamous Cell Carcinoma (SCC). G protein-coupled receptors (GPCRs) have been shown to activate some ADAMs proteinases and to transactivate epidermal growth factor receptor (EGFR) [10]. Ultraviolet (UV) radiation of skin cancer cells activates ADAMs and induces EGFR ligand shedding and EGFR transactivation [11]. It is likely that UV irradiation induces reactive oxygen species (ROS) generation, and that those ROS activate ADAM9 and ADAM17, which then cleave EGFR ligands, particularly AR. The soluble form of AR subsequently binds to EGFR and can induce skin cancer proliferation.

Overexpression of protein kinase Cε (PKCε) in mouse epidermis results in the rapid development of papilloma-independent metastatic SCCs via the two-stage model of carcinogenesis [12]. PKCε transgenic mice have elevated serum TNF-α levels during skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Since TNF-α is linked to skin tumor promotion by TPA, this increase may be linked to the development of metastatic SCC. TPA-stimulated shedding of TNF-α could be completely prevented in PKCε transgenic mice and isolated keratinocytes by an ADAM17 inhibitor, TAPI-1. These results indicate that PKCε signal transduction pathways to TPA-stimulated TNF-α ectodomain shedding are mediated by ADAM17. Injection of a TNF-α synthesis inhibitor during skin tumor promotion completely prevented the development of metastatic SCC in PKCε transgenic mice. The sum of these results indicates that ADAM17 is involved in the development of SCC in PKCε transgenic mice.

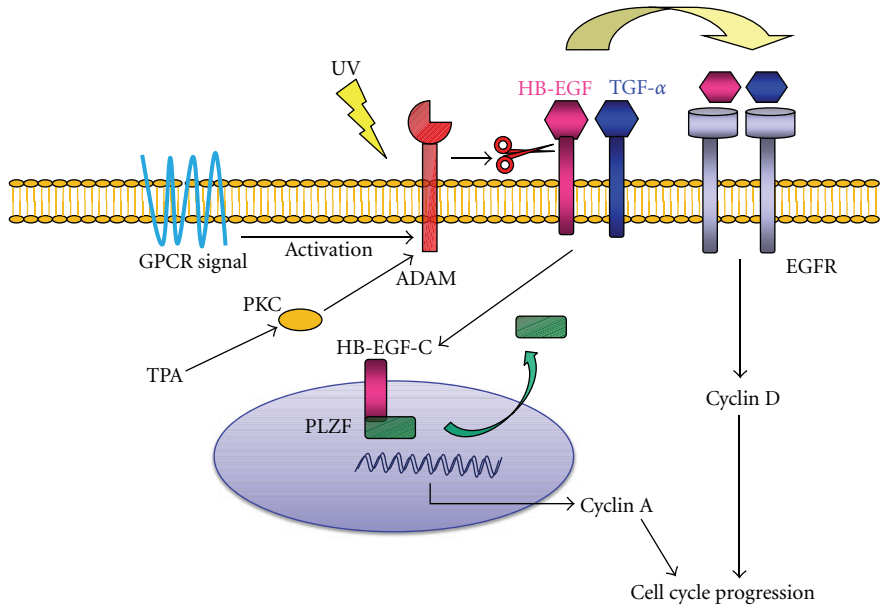

Figure 2: Physiological functions of ADAM proteinases in cancer.

3.1.2. Basal Cell Carcinoma (BCC). BCC is the most common type of skin tumor. BCC rarely metastasizes but is locally invasive and highly destructive. ADAM10, ADAM12, and ADAM17 are increased at the peripheral tumor margin compared with central areas of BCC tumor cell nests. Expression of ADAM10 and ADAM12 is increased in the deep margin of invading tumor cell nests. In contrast, ADAM17 is increased in superficial BCC. ADAM10, ADAM12, and ADAM17 show different expression patterns in BCC histologic subtypes, indicating their different roles in the pathogenesis of BCC [13].

3.1.3. Malignant Melanoma (MM). ADAM9, ADAM10, ADAM12, ADAM15, and ADAM17 are overexpressed in more than 10 MM cell lines, and HB-EGF and TGF-α are overexpressed in more than 10 MM cell lines [11].

ADAM10 expression was significantly elevated in melanoma metastases compared with primary melanomas [14]. Furthermore, expression of several components of the Notch pathway, which can be cleaved by ADAM10, were upregulated in MM compared with common melanocytic nevi [15]. Down-regulation of ADAM10 with specific siRNA resulted in the suppression of cell growth and the reduced migration of MM cells. In addition, overexpression of ADAM10 induced the migration of MM cells. Soluble L1-CAM can induce cell migration through binding to integrins. Elevated levels of L1-CAM in a metastatic variant of an MM cell line suggest a role for L1-CAM in tumor progression. Knockdown of L1-CAM reduced the migration of MM cells and abrogated their chemoresistance against cisplatin [14]. On the other hand, ADAM10 is the major protease responsible for constitutive CD44 cleavage from MM cells, and its expression can impair tumor cell proliferation [16]. CD44 proteins are cell surface receptors for hyaluronic acid (HA), a component of the extracellular matrix that has multiple effects on cell behavior. ADAM10, ADAM17, and matrix metalloproteinase (MMP) 14 have previously been implicated in the shedding of CD44 from various tumor cells. In MM cells, ADAM10 and ADAM17 but not MMP14 are significantly expressed in histological sections. However, only ADAM10 but not ADAM17 is involved in the constitutive shedding of native CD44 from MM cells. HA promotes the proliferation of MM cells [17], and soluble CD44 inhibits HA-stimulated proliferation of melanoma cells in vitro and in vivo [18].

ADAM9 is detected in MM cells and in peritumoral stromal fibroblasts, while it is absent in fibroblasts distal to the tumor site. In contrast, in nevi, ADAM9 expression is absent both in nevus cells and in stromal cells close to nevus cell nests [19].

3.2. Wound Healing. Wound healing is a complex process involving multiple cellular events, including cell proliferation, migration, and tissue remodeling. Members of the EGF family, such as EGF, TGF-α, HB-EGF, AR, and their receptor EGFR, are the most important factors in skin wound healing [20–22]. TGF-α, AR, and HB-EGF are autocrine growth factors in normal keratinocytes [23, 24], and both TGF-α and HB-EGF accelerate keratinocyte migration [25–28]. TNF-α regulates keratinocyte migration in vivo and in vitro via a MMP9 dependent pathway [29]. Blocking TNF-α function inhibits keratinocyte migration. HB-EGF mRNA is rapidly induced after scrape wounding of keratinocytes, with a slight increase in TGF-α, AR, and blocking HB-EGF inhibits keratinocyte migration [27, 28]. Therefore HB-EGF is the predominant growth factor involved in the epithelialization of skin wound healing in vivo and it functions by accelerating keratinocyte migration. ADAM9, ADAM10, ADAM12, and ADAM17 are considered candidate HB-EGF sheddases [30–33], which are also expressed in keratinocytes [34–37].

However it has been reported that ADAM9, ADAM10, and ADAM17 reduce cell motility in cDNA-transfected HaCaT cells [38].

Gene array data and immunohistochemistry from human venous reflux ulcers demonstrated that ADAM12 is upregulated in the nonhealing edge of chronic skin ulcers [39]. In addition, skin explants from ADAM12-deficient mice revealed a significant increase in keratinocyte migration and proliferation compared to skin explants from wild-type mice. Based on these findings, expression of ADAM12 in chronic wounds impairs wound healing through the inhibition of keratinocyte migration and proliferation.

ADAM10 represents the major E-cadherin sheddase [36]. ADAM10-mediated shedding of E-cadherin affects epithelial cell-cell adhesion as well as cell migration. The shedding of E-cadherin by ADAM10 modulates the β-catenin subcellular localization and downstream signaling. ADAM10 overexpression in epithelial cells increases the expression of the β-catenin downstream gene cyclin D1 in a dose-dependent manner and enhances cell proliferation. ADAM10 is also involved in the cleavage of CXC-chemokine ligand 16 (CXCL16), which is expressed as a transmembrane adhesion molecule and can be released as a chemoattractant [40]. CXCL16 is expressed in epidermal keratinocytes and is released into the wound exudate upon injury.

ADAM9 expression is increased during the first 7 days after-wounding in a mouse wound model [41]. ADAM9 knockout mice show accelerated wound repair compared with control littermates. No changes in the infiltration of inflammatory cells into the wound areas were observed. Since no differences in proliferation are observed in vivo or in vitro, the increased migration of keratinocytes is responsible for this effect.

3.3. Inflammatory Skin Diseases

3.3.1. Psoriasis. Psoriasis is a common hyperproliferative and chronic inflammatory skin disease, and it has been proposed that TNF-α is involved in its pathogenesis [42]. Increased concentrations of TNF-α have been detected in psoriatic skin lesions [43, 44]. Previous reports have shown that TGF-α, AR, and HB-EGF are overexpressed in psoriatic epidermis [45–47]. Further, soluble p55 and p75 TNF receptors are upregulated in synovial fluid from patients with psoriatic arthritis [48]. EGFR and its ligands are considered to be the most important mechanism for the proliferation of keratinocytes. So far, overexpression of ADAM10, ADAM12, and ADAM17 has been demonstrated in psoriatic skin [35, 49]. ADAM17 cleaves TNF-α and TNF receptors. ADAM10, ADAM12, and ADAM17 are critically involved in EGFR-ligand shedding including HB-EGF. By this mechanism, these sheddases may play a role in the regulation of keratinocyte proliferation and inflammation. Levels of ADAM17 are elevated in peripheral blood mononuclear cells of patients with active psoriasis (compared with healthy subjects), are correlated with the plasma concentration of soluble TNF receptor, with the severity of the disease, and are decreased after treatment [50].

ADAM33 has been identified as a novel psoriasis susceptibility gene [51]. This gene has been previously reported to be linked to asthma [52], which indicates that ADAM33 controls general effects on dermal inflammation and immunity.

3.3.2. Eczema. Acute eczema is an inflammatory skin disease characterized by scaling, redness, and itching. In acute eczema, small intraepidermal blisters may occur, which is characterized by reduced cohesion of keratinocytes. E-cadherin is a prime mediator of epithelial cell-to-cell interactions [53]. In several inflammatory skin diseases, such as psoriasis and pemphigus vulgaris, increased serum levels of soluble E-cadherin have been described [54]. Many cytokines, growth factors, and chemokines contribute to the pathogenesis of inflammatory epithelial skin diseases. Proinflammatory cytokines significantly increased levels of soluble E-cadherin in keratinocytes, which was abrogated in the presence of an ADAM10 inhibitor. ADAM10 levels increase in areas of blisters [55]. These findings indicate that inflammatory responses are able to activate ADAM10-mediated proteolysis of E-cadherin in keratinocytes.

3.4. Pigmentary Disorders. The signaling activated by the Kit ligand (Kitl), also referred to as stem cell factor (SCF), and its receptor KIT (membrane-bound KIT; m-KIT) plays an important role in melanocyte development, survival, proliferation, and melanogenesis [56]. Kitl is synthesized as a transmembrane protein and is processed to produce the soluble secreted form Kitl. Alternative splicing generates two Kitl RNA transcripts that encode two cell-associated Kitl proteins, Kitl1, and Kitl2. Membrane-bound KIT protein (m-KIT) is expressed as a transmembrane protein and has a tyrosine kinase domain [57]. A cleaved, soluble product of KIT, the soluble form of KIT (s-KIT), exists and has an ability to bind SCF, and functions as a decoy receptor to inhibit SCF/m-KIT signaling [58]. Mutations in the human gene encoding KIT results in piebaldism, a congenic disorder that is characterized by amelanotic patches on acral and/or ventral skin surfaces [59]. Intradermal injection of SCF enhances the number, size, and dendricity of melanocytes in normal human skin xenografts, whereas interruption of SCF binding to its receptor m-KIT decreases these parameters [60]. UVB radiation augments the expression of membrane-bound SCF in epidermal keratinocytes, and injection of a KIT-inhibitory antibody abolishes the UVB-induced increase in pigmentation in guinea pig skin [61]. SCF expression is increased in epidermal lentigo seniles [62]. SCF secreted by dermal fibroblasts stimulates melanocytes located in the epidermis overlying dermatofibroma [63]. s-KIT has been detected in vivo in human serum and plasma, and levels of s-KIT are increased in the sera of patients with mastocytosis and correlates with disease severity [64]. Additionally, the amount of s-KIT in the serum correlates with graft-versus-host disease following bone marrow transplantation [65]. Both ADAM17 and ADAM19 affect Kitl1 shedding in different ways: ADAM17 is the major sheddase for Kitl1 and Kitl2, while ADAM19 reduces ADAM17-dependent phorbol-ester-stimulated Kitl1

Table 1: Principal ADAMs proteinases involved in skin diseases.

ADAM	substrate	Pathology association
ADAM9	HB-EGF, EGF, Collagen XVII	Cancer, Wound healing
ADAM10	E-cadherin, N-cadherin, CD44, EGF, HB-EGF, L1-CAM, CXCL16 Collagen XVII	Cancer, Wound healing, Psoriasis, Eczema
ADAM12	EGF, HB-EGF	Cancer, Wound healing, Psoriasis
ADAM17	TNF-α, TNFR, TGF-α, HB-EGF, AR, Collagen XVII, KIT, SCF	Cancer, Migration, Psoriasis, Melanogenesis, SSc
ADAM33		Psoriasis

ectodomain shedding [66]. The production of s-KIT is enhanced by an ADAM17 activator in melanocytes, and the ADAM17 activator-induced production of s-KIT abolishes SCF-induced melanogenesis [67]. Moreover, melanogenesis is significantly suppressed by the addition of an ADAM17 activator, whereas TAPI-1, an inhibitor of ADAM17, is found to significantly stimulate melanin synthesis in a 3D skin model [67].

The melanocortin-1 receptor (MC1R) is a key regulator of pigmentation in mammals and is tightly linked to an increased risk of skin cancers, including melanoma, in humans [68, 69]. Agouti signal protein (ASP) antagonizes MC1R function, and is also associated with increased risk of skin cancer [70]. Many genes that are upregulated by ASP are involved in morphogenesis, cell adhesion, and extracellular matrix-receptor interactions. Microarray analysis indicates that ASP up-regulates several ADAM family genes such as ADAM11, ADAM12, ADAM19, and ADAM23 in melanocytes [71]. However the precise roles of these ADAM proteinases on melanocytes remain unclear.

Color loci in mammals are genetic loci in which mutations can affect pigmentation of the hair, skin, and/or eyes. In mice, over 800 phenotypic alleles are now known, at more than 200 identified color loci [72]. In the ADAMs family, ADAM17 and ADAMTS20 are known to be involved in regulating pigmentation. ADAM17 knockout mice reveal a disorganized distribution and structure of hair follicles, which contain hairs with irregular pigment deposition [73]. A defect of ADAMTS20 is the cause of the belted (*bt*) white-spotting mutation. It presents as a mostly pigmented mouse except for a region proximal to the hindlimbs that appears as a white belt [74]. ADAMTS20 is a secreted metalloprotease which shows a highly dynamic pattern of expression in the developing embryo that generally precedes the appearance of melanoblasts, and is not expressed in the migrating cells. It has been suggested that the ADAMTS20 proteinase plays a role in the regulation of cell migration [74]. In humans, ADAM17 and ADAMTS20 are also known to be candidate genes that regulate pigmentation in East Asians [75].

3.5. Others

3.5.1. UV Radiation. UV radiation is clearly an important environmental factor in human skin carcinogenesis. ADAMs family proteinases are involved in regulating these signaling pathways. UVA and UVB up-regulate ADAM17 mRNA expression in immortalized keratinocyte HaCaT cells [76]. A low, nonlethal dose of UVA (1–4 J/cm^2) induces a dose-dependent EGFR activation, cyclin D1 accumulation, and cell cycle progression in HaCaT cells [77]. Knockdown of ADAM17 blocks UVA-induced EGFR activation and cell cycle progression, which demonstrates that ADAM17 mediates the EGFR/cyclin D1 pathway and cell cycle progression to the S phase induced by UVA radiation [77]. UVC also induces EGFR phosphorylation in melanocytes and keratinocytes, which is inhibited by a broad spectrum metalloproteinase inhibitor, BB94 [11]. AR is required for UV-induced EGFR activation in SCC-9 cells, which depends on ADAM9 and ADAM17.

3.5.2. Systemic Sclerosis. Systemic sclerosis (SSc) is a disease characterized by progressive fibrosis of multiple systems including the skin. Elevated serum concentrations of the soluble TNF receptor p55 and TNF-α are known to correlate with the severity of SSc [78–80]. Up-regulation of ADAM17 was observed in peripheral monocytes of patients with early SSc [81]. Furthermore, the ADAM17 inhibitor, TAPI-1, significantly suppressed skin sclerosis induced by bleomycin and reduced fibrogenic cytokines [82]. These findings indicate that ADAM17 could be a new target for the therapy of SSc.

3.5.3. Collagen XVII. Collagen XVII (also called BP180 or BPAG2) is a hemidesmosomal adhesion component in the skin and mucosa [83]. Mutations in the *COL17A1* gene are associated with junctional epidermolysis bullosa, a genetic skin blistering disease [84]. Patients with bullous pemphigoid and related autoimmune bullous dermatoses have tissue-bound and circulating autoantibodies targeting collagen XVII. Collagen XVII is an epithelial adhesion molecule and is proteolytically released from the membrane via the action of several ADAM proteases. ADAM9, ADAM10, and ADAM17 are known to be collagen XVII sheddases [38, 85].

4. Concluding Remarks

The current status and future potential of ADAMs proteinase activities in the fields of cutaneous biology and skin disorders were discussed in this paper, and the functions of relevant ADAMs proteinases are summarized in Table 1. A better understanding of the regulatory mechanisms and physiological functions of ADAMs proteinases in human skin may generate novel targets for the diagnosis and/or therapeutics of skin diseases.

Conflict of Interests

The authors has no conflict of interests to declare.

Abbreviations

ADAMs: A disintegrin and metalloproteinases
ADAMTS: ADAM with thrombospondin motifs
AR: Amphiregulin
ASP: Agouti signal protein
BCC: Basal cell carcinoma
CXCL16: CXC-chemokine ligand 16
EGF: Epidermal growth factor
EGFR: EGF receptor
GPCR: G protein-coupled receptor
HA: Hyaluronic acid
HB-EGF: Heparin-binding EGF-like growth factor
IL-6R: Interleukin-6 receptor
Kitl: Kit ligand
L1-CAM: L1 cell adhesion molecule
m-KIT: Membrane-bound KIT
s-KIT: Soluble form of KIT
MC1R: Melanocortin-1 receptor
MM: Malignant melanoma
MMP: Matrix metalloproteinase
PKC: Protein kinase C
PLZF: Promyelocytic leukemia zinc finger
ROS: Reactive oxygen species
SCC: Squamous cell carcinoma
SCF: Stem cell factor
SSc: Systemic sclerosis
TACE: TNF-α-converting enzyme
TGF-α: Transforming growth factor-α
TGF-β: Transforming growth factor-β
TNF-α: Tumor necrosis factor-α
TPA: 12-*O*-tetradecanoylphorbol-13-acetate
TSP-1: Thrombospondin type one
UV: Ultraviolet.

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Review Article

Autotaxin: Its Role in Biology of Melanoma Cells and as a Pharmacological Target

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Autotaxin (ATX) is an extracellular lysophospholipase D (lysoPLD) released from normal cells and cancer cells. Activity of ATX is detected in various biological fluids. The lysophosphatidic acid (LPA) is the main product of ATX. LPA acting through specific G protein-coupled receptors (LPA_1-LPA_6) affects immunological response, normal development, and malignant tumors' formation and progression. In this review, the impact of autotoxin on biology of melanoma cells and potential treatment is discussed.

1. Biochemistry and Structure of Autotoxin

Autotaxin, ATX (E.C. 3.1.4.39), is a member of the family of nucleotide pyrophosphatases/phosphodiesterase (NPP1-7) and is also referred, as NPP2 [1]. It is to a glycoprotein with four possible N-glycosylation sites, synthesized as a pre-proenzyme and is secreted to extracellular space following two N-terminal cleavages (27 and 8 amino acids) [2, 3]. ATX is a constitutively active enzyme possessing activity of phospholipase D. It hydrolyzes the head groups of lysophospholipids to lysophosphatidic acid (1 or 2-acyl-*sn*-glycerol-3-phosphate, LPA) and also acts on sphingosylphosphorylcholine to produce sphingosine 1-phosphate (S1P) [4, 5]. Both, LPA and S1P are strong inhibitors of ATX with affinity to enzyme approximately 1000-fold higher than reported for ATX substrates [6]. mRNA for ATX has been detected in brain, ovary, lung intestine, and kidney but enzyme activity has been detected in blood, cerebrospinal and seminal fluid, urine, and saliva [7–11]. It is not filtered in the glomerulus because of high molecular weight (~125 kDa) but is cleared from the circulation by the scavenger receptors of liver sinusoidal endothelial cells [12]. Moreover, ATX is the main source of blood LPA (~0.1 μM plasma and ~1 μM serum), however, not for S1P [13, 14]. There is evidence that S1P is produced intracellularly *via* sphingosine kinases and transported through ATP-binding cassette transporter [15, 16]. ATX hydrolyzes also ATP; however, affinity to ATP is at least 50-fold lower then for lysophospholipids [17, 18]. LPA acts on target cells through specific G-protein-coupled receptors: LPA_1/Edg2, LPA_2/Edg4, LPA_3/Edg7, LPA_4/GPR23/$P2Y_9$, LPA_5/GPR92, LPA_6/$P2Y_5$ broadly expressed in normal and cancer cells [19, 20]. The LPA-binding proteins for example, albumin, determine the activation of the specific LPA receptors; LPA_3, in contrast to LPA_1 and LPA_2, is not activated by complex LPA-albumin [21].

The structure of autotoxin is presented in Figure 1. At the N-terminus, ATX possesses hydrophobic signal sequence (SS) followed by two somatomedin B-like domains (SBLD) with RGD tripeptide motif suggesting that this domain may play a role in cell-extracellular matrix interactions. At catalytic domain (CD), Thr^{210} and N542-linked glycan are suggested to be responsible for hydrolytic activity of ATX, but motif G/FXGXXG is responsible for metal binding. ATX activity is stimulated by divalent cations for example, Ca^{2+}, Mg^{2+}, and Co^{2+}. C-terminally to CD is the nuclease-like domain (NLD). It contains EF-hand-like motif and is structurally similar to DNA and RNA-nonspecific endonucleases; however, it lacks the catalytic activity. In humans, NLD is covalently linked with catalytic domain *via* disulfide

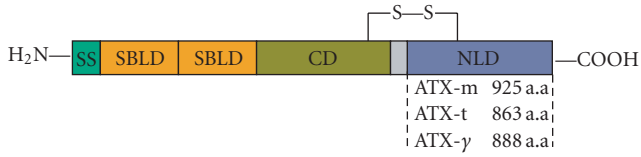

Figure 1: Scheme of the structure domains of autotaxin isoforms. SS: signal sequence, SBLD: somatomedin B-like domains, CD: catalytic domain, NLD: nuclease-like domain.

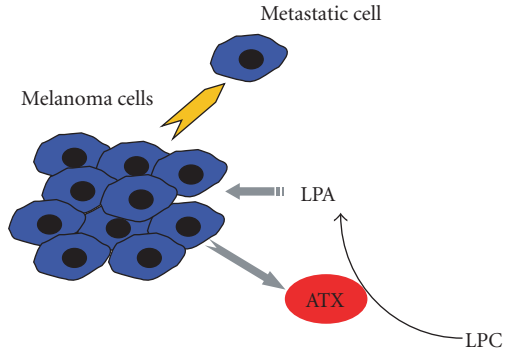

Figure 2: Role of ATX-LPA axis in motility of melanoma cells. ATX: autotaxin, LPA: lysophosphatidic acid, and LPC: lysophospholipids.

bridge (C413-C805). The ATX gene is located on chromosome 8 at position 8q24.1 and ATX has three alternative splicing isoforms in humans: ATX teratocarcinoma-derived ATX-t (925 a.a); melanoma-derived ATX-m (863 a.a), and brain specific, ATX-γ (888 a.a) [22–25].

2. Role of Autotoxin during Normal Development

ATX has a critical role in formation of vasculature by vasculogenesis and angiogenesis. ATX knockout mice ($atx^{-/-}$) are lethal around embryonic day 10.5. Admittedly, ATX is a major producing enzyme for LPA, nevertheless the phenotypes of LPA receptors knockout mice is less severe, suggesting that ATX-induced cellular signal may involve others pathways. This speculation is supported by results of experiments where modification of LPA level in blood (2-fold increase) by driving ATX expression is not sufficient to induce tumorigenesis [13, 26–28].

3. Role of Autotoxin in Biology of Melanoma Cells

ATX was identified in the cultured cell supernatant of human melanoma cells (A2058) as a cell motility-stimulating factor acting at pM-nM concentrations in pertussis toxin-sensitive manner [29]. Further studies have provided evidence that LPA, product of ATX, mediates chemotaxis and proliferation of melanoma cells [30]. The recent experiments suggest that ATX expression is one of the factors involved in metastasis of melanoma cells (Figure 2). Inhibition of ATX production blocks LPA-induced migration of melanoma cells [31]. It has been detected that melanoma metastatic specimens have increased ATX level, and ATX expression in primary melanoma is higher than in melanoma *in situ* [32]. Moreover, reduced expression of ATX predicts survival in uveal melanoma [33].

It has been shown that ATX-stimulated motility is suppressed by an LPA_1-selective antagonist, Ki16425, in melanoma cells [34]. Accumulating evidence suggest the various intracellular signaling pathways may be involved in ATX-induced motility of melanoma cell. It has been shown that this action is mediated through G-protein coupled isoform of phosphatidylinositol 3-kinase γ (PI3Kγ) suggesting involvement of proteins located downstream of PI3Kγ, for example, small G proteins [35]. Accordingly, there is evidence that ATX induces Cdc42/Rac1/p21-activated kinase (PAK1) complex formation [36]. The experimental data suggest that this complex is required for LPA-induced activation of focal adhesion kinase (FAK) [37]. The changes of PAK1 and FAK activity affect cytoskeleton proteins and structural integrity of melanoma cells. Moreover, ATX in melanoma cells induces the expression and activity of urokinase-type plasminogen activator (UPA) in a dose-dependent manner. This action is mediated by G_i proteins and PI3K/Akt signaling involving translocation of p65 into the nucleus and DNA binding of necrosis factor kappa B [38]. In general, action of LPA on melanoma cells enhances their metastaic potential (Figure 3). Recent experiments have provided evidence about the role of LPA receptors in biology of melanoma cells [32]. Downregulation of LPA_3 and using sequence-specific small interfering RNA (siRNA) reduces melanoma cells viability and proliferation. The effects of LPA receptors activation in melanoma cells are presented in Figure 4.

4. Autotoxin as a Pharmacological Target

The approved melanoma therapy lacks significant efficiency, hence, new therapeutic targets are under investigation. The current research focuses on the autotoxin-LPA axis [39]. Because LPA acts on multiple receptors with overlapping activities, currently the LPA receptors are not attractive as a pharmacological target, and the main stream of investigation concerns ATX activity.

4.1. L-Histidine. L-histidine inhibits activities of ATX in a non-competitive manner with $IC_{50} \sim 4$ mM and ATX-stimulated migration of human melanoma cells; 10 mM L-histidine induces 90%–95% reduction in stimulated motility. The proposed mechanism of L-histidine action is based on inhibition of a process that is required for the hydrolysis of both nucleotides and phospholipids [40].

4.2. Analogs of Bioactive Lipids. ATX is negatively regulated by LPA. The effect is dependent on the length of the acyl chain; maximal inhibition is induced by 1-oleoyl-LPA ($IC_{50} \sim 0.1$–$2\,\mu$M), 1-palmitoyl-LPA, and 1-myristoyl-LPA whereas short-chain LPA (6 : 0) has no measurable effect. The inhibition of ATX activity results from a combination

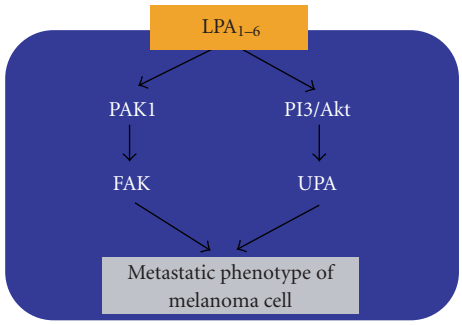

Figure 3: A proposal role for LPA receptors during melanoma progression. LPA receptors activation in melanoma cells leads to p21-activated kinase (PAK) and focal adhesion kinase (FAK) and to the activation of phosphoinositide 3-kinase/Akt-kinase and urokinase-type plasminogen activator (UPA). Finally, the melanoma cells express the metastatic phenotype.

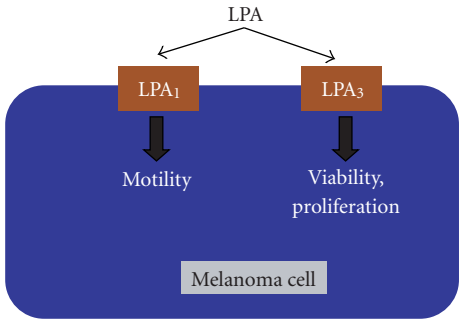

Figure 4: The LPA_1 and LPA_3 receptors as a potential target for pharmacological intervention.

of a decreased turnover number and decreased affinity of the active site for its substrates. Of note, LPA both inhibits ATX and activates LPA receptors (EC_{50} for LPA 18 : 1 at LPA_{1-4} ~ 0.007–0.5 μM), therefore it may induce the opposite of the intended effect [6, 41].

The other group of ATX inhibitors are analogues of cyclic phosphatic acid (1-acyl-*sn*-glycero-2,3-cyclic-phosphate, cPA) [42, 43]. The naturally occurring cPA, 1-oleoyl-*sn*-glycero-2,3-cyclic-phosphate (cPA 18 : 1) possesses unique properties having two targets: enzyme and receptor. cPA 18 : 1 inhibits ATX activity with a maximum of 22% at 1 μM and inhibits signaling pathway mediated through LPA_1 and LPA_3 receptors. Because of this properties, cPA 18 : 1 is described as having a "one-two punch" [44]. Another naturally occurring cPA, cPA 16 : 1 (1-palmitoleoyl-*sn*-glycero-2,3-cyclic-phosphate) possesses similar activity against ATX with maximum of 45% at 1 μM.

Interestingly, replacement of either the *sn*-2 or *sn*-3 oxygen by methylene (carba group) increases the inhibitory properties of these analogues (carba analogues, ccPA) [45]. 2ccPA 16 : 1 and 2cPA 18 : 1 (replacement of *sn*-2 oxygen with a methylene group) inhibit ATX activity about 90% with IC_{50} ~ 140 nM and ~370 nM, respectively. Furthermore, 3ccPA 16 : 1 and 3ccPA 18 : 1 (replacement of *sn*-3 oxygen with methylene group) inhibit ATX activity about 70% with IC_{50} ~ 300 nM and ~60 nM, respectively. Moreover, 3ccPA does not interact with LPA receptors. The invasion assay using melanoma cells (A2058) has provided evidence that ccPA-induced inhibition of ATX activity results in inhibition of cell migration. Furthermore, cPA and ccPA exert an inhibitory effect on experimental pulmonary metastasis in mice. Recently, a new generation of ccPA with potential therapeutic modality has been developed. Thio-ccPA 18 : 1 possesses multitarget properties. It inhibits ATX activity (~90% at 10 μM) and blocks LPA_1 (IC_{50} ~ 800 nM) and LPA_3 (IC_{50} ~ 440 nM) receptors without effect on LPA_2 receptors. Thio-ccPA influences metastatic melanoma tumors *in vivo,* reducing the number of pulmonary metastases and metastatic lesions to kidney, liver, pancreas, and intestines [32].

4.3. Nonlipid Small Molecule. It has been recently shown that thiazolidinediones compounds with incorporated boric acid moiety into catalic T210 residue (HA 130) inhibit ATX-mediated LPA production with IC_{50} ~ 30 nM [46]. Intravenous injection of HA 130 decreases 3.8-fold plasma LPA level in mice at 10 min. Furthermore, HA 130 inhibits ATX-mediated melanoma cells migration without affecting LPA receptor signaling pathways.

A report has been recently published describing the pharmacokinetic and pharmacodynamic properties of PF-8380 [47]. It inhibits activity of isolated ATX or ATX activity in blood with IC_{50} ~ 3 and 100 nM, respectively. There are no data about influence on melanoma cells, hoverer, PF-8380 (30 mg/kg) taken orally decreases the plasma LPA level about 95%, suggesting its potential usage in melanoma treatment.

There are several small-molecule, nonlipid ATX inhibitors including hexachlorophene, merbromin, bithionol, and others under investigation [48, 49]. Their mechanism of action differ (competitive, noncompetitive or mixed inhibition) and the most potent compounds inhibit ATX activity with IC_{50} at micromolar range. Their biological action was confirmed in experiments *in vivo* measuring effects on melanoma cell motility and invasion. A recently developed new TX autotaxin inhibitor pipemidic acid inhibits ATX with IC_{50} ~ 900 nM [50]. The natural phenolic antioxidants, including flavonols, possess inhibitory properties against ATX; however, the effect on ATX activity is about 2-fold lower than LPA 16 : 1 (1-palmitoleoyl-sn-glycerol-3-phosphate). Moreover, it has been estimated that it would be difficult to affect ATX activity *in vivo* by flavonoids supplementation in diet because plasma concentration of flavonoids in plasma may reach 10 μM [51].

The recently published crystallography results are used in ligand-based computational approaches for optimization of the current ATX inhibitors and development of new ones [52, 53].

Taken together, the increasing incidence of melanoma and poor average survival of metastatic melanoma are the main reason for the development of the new chemical compounds used in melanoma treatment. Autotaxin, melanoma cell motility-stimulating factor, and their receptors seem to be promising targets for pharmacological treatment

of melanoma. Much more research is needed for synthesis and pharmacological characterization of new specific ATX or LPA receptors inhibitors.

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Review Article

Therapeutic Implications of Targeting AKT Signaling in Melanoma

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Identification of key enzymes regulating melanoma progression and drug resistance has the potential to lead to the development of novel, more effective targeted agents for inhibiting this deadly form of skin cancer. The Akt3, also known as protein kinase B gamma, pathway enzymes regulate diverse cellular processes including proliferation, survival, and invasion thereby promoting the development of melanoma. Accumulating preclinical evidence demonstrates that therapeutic agents targeting these kinases alone or in combination with other pathway members could be effective for the long-term treatment of advanced-stage disease. However, currently, no selective and effective therapeutic agent targeting these kinases has been identified for clinical use. This paper provides an overview of the key enzymes of the PI3K pathway with emphasis placed on Akt3 and the negative regulator of this kinase called PTEN (phosphatase and tensin homolog deleted on chromosome 10). Mechanisms regulating these enzymes, their substrates and therapeutic implications of targeting these proteins to treat melanoma are also discussed. Finally, key issues that remain to be answered and future directions for interested researchers pertaining to this signaling cascade are highlighted.

1. Introduction

Kinases and phosphatases are the key components of signaling cascades regulating metabolic processes such as cell survival, proliferation, apoptosis, differentiation, and cell motility [1–4]. Aberrant expression and activities of these enzymes have been reported to lead to the development of several cancers including melanoma [4–10]. Due to genetic and epigenetic modifications, deregulating oncogenic kinases and tumor inhibitory proteins, melanocytes acquire transformed characteristics leading to malignant melanoma [4–10]. Members of the PI3K and Akt3 signaling cascades have been implicated in initiation, progression, invasive, and drug resistance phenotypes of melanomas [1–4]. Enzymes in this signaling cascade are therefore attractive targets for treating or preventing melanoma development [11–13]. This paper provides an overview of enzymes involved in PI3K-Akt signaling pathway focusing specifically on the tumor suppressor phosphatase PTEN, lipid kinase PI3K, and the oncogenic survival kinase Akt3. Key structural features, mechanisms regulating the expressionm and activities of these proteins as well as therapeutic implications of targeting this pathway to treat melanoma are reviewed.

1.1. Tumor Suppressor PTEN Is a Key Phosphatase Regulating PI3K-Akt3 Signaling and Thereby Melanoma Development. The PTEN (phosphatase and tensin homolog deleted on chromosome 10) gene, which is also known as MMAC1 (mutated in multiple advanced cancers), and TEP1 (TGF-β regulated and epithelial cell-enriched phosphatase) is a unique 55 kDa dual specificity phosphatase located on

the long arm of chromosome 10 at 10q23 [14, 15]. PTEN dephosphorylates proteins [16–18] and hydrolyzes the secondary messenger inositol trisphosphates (PIP_3s) [17, 19] thereby inhibiting the activities of several proteins or pathways, which regulate cell proliferation, survival, and apoptosis [14, 20].

Preclinical and clinical evidence has demonstrated the inactivation of PTEN in 29 to 43% of melanoma cell lines or tumors from patients. For example, a recent study showed decreased PTEN expression in 43% melanoma cases and demonstrated a significant correlation between alterations in PTEN expression and primary tumor ulceration [20]. However, only a few studies, although controversial, demonstrated the clinical implications of PTEN as a prognostic marker in melanoma. Addressing this aspect, a recent study concluded that the utility of PTEN status as a prognostic marker is limited despite decreased PTEN expression observed commonly in primary melanomas associated with aggressive tumor behavior [20]. This clinical study analyzed 127 primary melanomas and found no significant association between PTEN expression and patient survival.

Key experiments demonstrating the tumor suppressor function of PTEN in melanomas include (a) triggering and sensitization of melanoma cells to apoptosis following introduction of PTEN (Figure 1) [8, 22] as demonstrated by increased cleaved caspase-3 only when wild-type PTEN is expressed compared to inactive G129R mutant in UACC 903 cells [8]; (b) enhanced phosphorylation of Akt3 in melanocytes and early melanoma WM35 cells expressing wild-type PTEN protein only upon PTEN inhibition [23]; (c) loss of cell viability, decreased activity of Akt3, and downstream pPRAS40 following the introduction of PTEN in melanoma cells lacking functional protein (Figure 2) [8, 9, 21]; (d) appearance of spontaneous melanoma tumors in mice lacking $PTEN^{-/-}$ and expressing conditionally induced ^{V600E}B-Raf. B-Raf, one of the key regulators of proliferation, has been shown to be mutated in ~60% melanomas [4, 24]. The most common mutation T1799A, which results in the substitution of valine to glutamic acid, has been identified in >90% of melanoma patient tumors harboring a mutant B-Raf protein [4, 24]. Furthermore, targeted deletion of PTEN in melanomas induced BCl_2, which resulted in enhanced resistance to growth factor receptor inhibitors and chemotherapeutic agents [8, 9, 20, 21].

PTEN expression has been shown to inhibit melanoma cell proliferation and survival. Mechanistically, decreased PTEN activity alters cell cycle progression, migration, and adhesion of melanoma cells [14]. Recently, PTEN loss and ^{V600E}B-Raf have been shown to cooperate to promote metastatic melanoma development [24]. Furthermore, PTEN loss increased melanoma cell and nontransformed melanocytes invasion and migration by increasing Akt2 activity and by E-cadherin downregulation [25]. It is interesting that targeting PTEN differentially regulates Akt3-mediated cell survival and Akt2-mediated metastasis in melanomas.

In addition to PI3K-Akt pathway regulation, PTEN also regulates the synthesis of proteins in melanoma cells. Mechanistically, PTEN controls phosphorylation of eukaryotic initiation factor-2α (eIF2α), independent of its phosphatase activity, thereby inducing the antiproliferation and apoptotic signals [26]. Isogenic melanoma cells lacking PTEN had low phosphorylated eIF2 compared to cells expressing wild-type PTEN. Furthermore, reconstitution of wild-type or phosphatase-defective PTEN in PTEN-null human glioblastoma cells enhanced phosphorylation of eIF2α via binding to PTEN's PDZ domain [26]. Phosphorylated eIF2α inhibits eIF2B and blocks the initiation of translation and overall protein synthesis [26].

PTEN also regulates several key processes such as inhibition of cell proliferation by altering cell cycle progression through G1 to S phase and control of apoptosis by modulating Akt activity [20, 27–29]. Furthermore, PTEN expression using a variety of vectors or from an introduced chromosome in melanoma cell lines lacking PTEN induced apoptosis and inhibited tumor development [8, 20, 22]. Cells containing functionally active PTEN protein exhibited elevated p27 expression, decreased cyclin-D1 and cyclin-D2 protein levels [14, 29, 30].

1.2. Mechanisms Regulating the Expression and Activity of PTEN in Melanomas. PTEN expression and activity are regulated at transcriptional and posttranslational levels [15, 19, 31] by positive regulators EGR-1 (early growth regulated transcription factor), PPARγ (peroxisome proliferator-activated receptors), and p53 [19] as well as negative regulators MKK-4 (mitogen-activated protein kinase kinase 4), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), TGF-β (transforming growth factor beta), and c-JUN [14, 19, 32]. At a posttranslational level, phosphorylation, oxidation, acetylation, and ubiquitination are key factors regulating PTEN activity [19, 33, 34]. For example, oxidation of cysteine residues C71 and C124 by reactive oxygen radicals inhibits PTEN phosphatase activity. Similarly, acetylation of PTEN catalytic domain lysine residues 125 and 128 by histone acetyltransferase PCAF (p300/CBP-associated factor) decreases affinity towards PIP_3's thereby regulating melanoma development [35]. Other posttranslational modifications such as phosphorylation of the C-terminal tail serine and threonine residues (S362, T366, S370, S380, T382, T383, S385) by CK2 (casein kinase 2), LKB1, Src, GSK3β have been shown to play critical roles regulating PTEN activity by altering protein stability [19, 33]. Phosphorylated PTEN is stable but less active compared to unphosphorylated PTEN [19, 36]. For example, PTEN proteins either lacking the C-tail or harboring mutations in C-tail are short lived [33, 36, 37]. However, it is not known whether such posttranslational modifications contribute to human melanoma development.

The subcellular localization of PTEN also plays a key role in melanoma development [14, 19] as PTEN has intrinsic membrane translocation signals in the phosphatase and C2 domains. Mutations in these regions and interactions with other proteins impair ability of PTEN to translocate to the plasma membrane. For example, ubiquitination of PTEN catalyzed by NEDD4-1 (neural precursor cell expressed, developmentally downregulated 4) influences

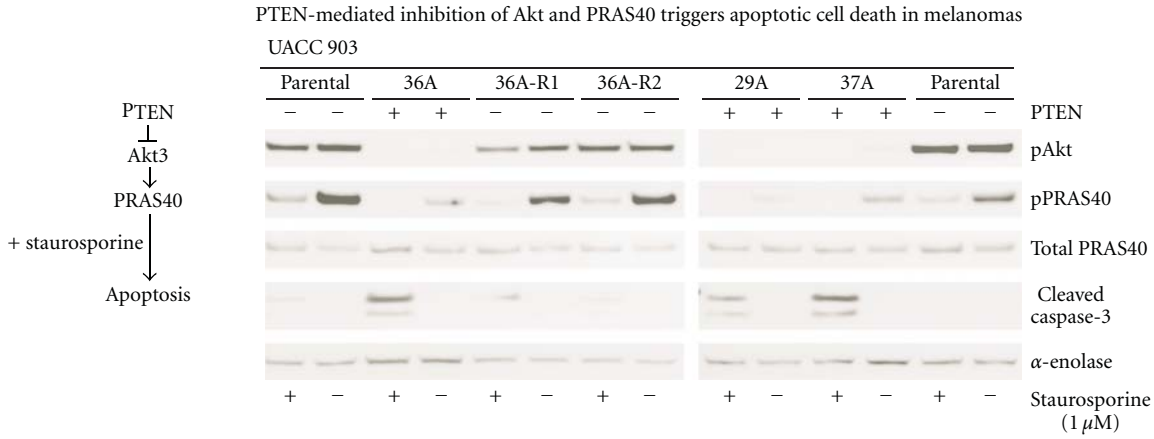

Figure 1: PTEN (EC number: 3.1.3.67) regulates Akt signaling in melanomas. Isogenic UACC 903 cell lines expressing (29A, 36A, 37A) or not expressing (parental UACC 903, revertant 36A-R1, 36A-R2) PTEN were treated with 1 μM staurosporine for 4 h and cell lysates analyzed for the expression of pAkt, pPRAS40, and cleaved caspase-3. The data demonstrates that PTEN null cell lines express high pAkt, pPRAS40, and decreased cleaved caspase-3 fragments compared to cells harboring PTEN, indicating that PTEN sensitizes melanoma cells to chemotherapeutic agents [21].

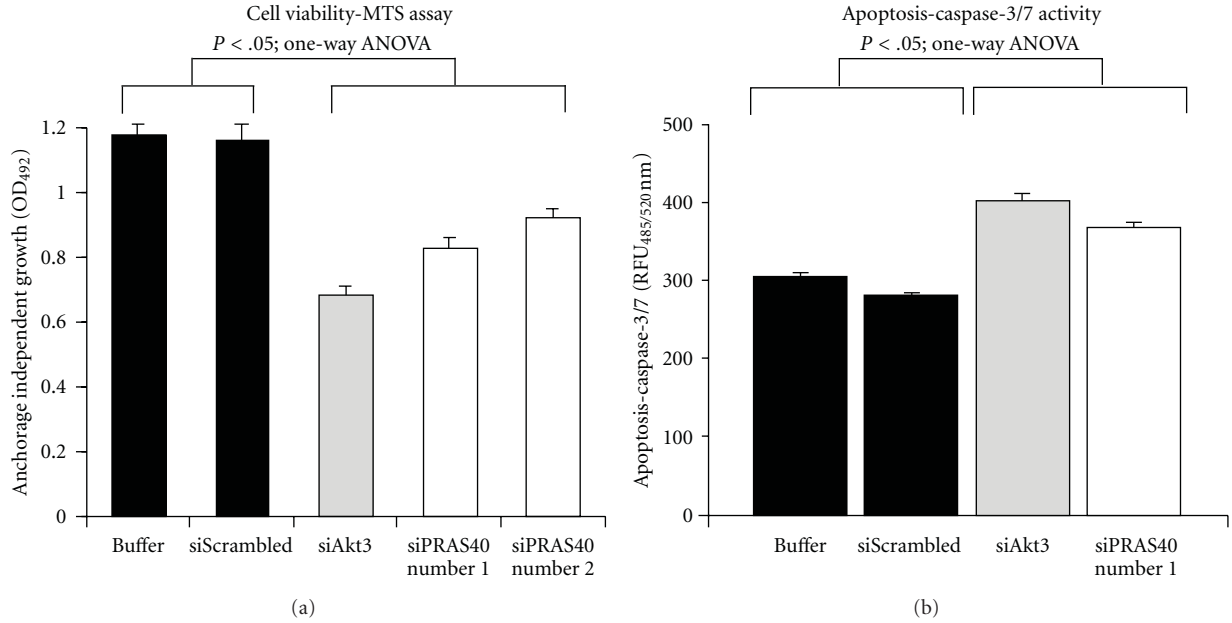

Figure 2: Targeting Akt3 signaling inhibits melanoma cell survival. SiRNA-mediated targeting of Akt3 or downstream PRAS40 inhibited anchorage independent growth and induces caspase-3/7 activity in melanomas compared to control buffer or scrambled siRNA transfected cells [21].

its cellular localization, as monoubiquitination promotes nuclear localization while polyubiquitination causes PTEN protein to remain in the cytosol [38, 39]. It would be interesting to determine whether similar ubiquitination patterns occur in melanomas.

Genetic and epigenetic mechanisms such as loss of a whole chromosome, mutations, and epigenetic miRNAs also modulate the expression as well as activity of PTEN [18, 19]. For example, loss of chromosome 10 (the site of PTEN gene), occurs in 30%–60% of noninherited melanomas [8]. Similarly, screening of melanoma cell lines and paired uncultured metastatic melanoma to peripheral blood specimens for PTEN loss and/or alterations, found homozygous deletions in ~20% melanoma cell lines, while 9% had nonsense, frame shift, or intronic mutations. However, only ~12% of uncultured melanoma specimens contained nonsense mutations or homozygous deletions [40, 41]. Intragenic polymorphisms in introns have also been reported to regulate PTEN expression in melanomas. Furthermore, ~12% of melanomas have mutations in the PTEN gene [40, 41].

Early melanocytic lesions frequently have undergone loss of one allele of PTEN or haploinsufficiency occurring due to loss of the entire chromosome 10 [14, 42–45]. Allelic

loss or mutations of PTEN have been reported in 5%–15% of uncultured melanoma specimens and in 30%–40% of established melanoma cell lines [40, 41]. Decreased PTEN levels due to haploinsufficiency plays an important role in early melanomas by specifically increasing Akt3 activity in these cells, thereby protecting these cells from apoptosis as well as releasing quiescent melanocytes from ^{V600E}B-Raf induced cell senescence [25, 46–48]. Loss or inactivation of PTEN also induces phosphorylation and monoubiquitination of DNA damage checkpoint kinase, Chk1, which then causes genomic instability, double-stranded DNA breaks, culminating in cancer development [41]. Amino acid altering mutations (P95S, F154L, L325F) induced by UV radiation have also been shown to impair PTEN function and thereby promote the development of early melanomas [49].

Epigenetic modifications such as methylation of CpG islands or microRNAs also regulate PTEN activity. For example, CpG islands methylation has been reported in melanomas [14, 50, 51]. Recently, PTEN promoter methylation leading to inactivation of transcription was found to occur in ~62% of metastatic melanoma patients [52–60]. However, contradictory to this observation, the most recent studies by Liu et al., 2008, and Bonazzi et al., in 2009, found no DNA methylation of the PTEN promoter [61, 62]. Therefore, although regulation of PTEN expression by DNA methylation appears to be important, further studies are warranted to measure its contribution to melanoma development. If mutational and epigenetic silencing studies of PTEN are combined together, functional inactivation might occur in ~77% of nonfamilial melanomas [54, 63–65].

1.3. PI3K Is a Key Regulator of Melanoma Development. Phosphatidyl inositol 3 kinases (PI3Ks) are a family of intracellular lipid kinases that phosphorylate the 3′ hydroxyl group of phosphatidylinositols (Pis) and phosphoinositides [66, 67]. Activation of PI3K affects cell growth, proliferation and survival thereby influencing the tumorigenic potential of melanoma cells [66, 67]. PI3Ks are classified based on substrate specificity and structure, into class-I, class-II, and class-III kinases [66, 68] (Figure 3). Whereas class-I PI3Ks convert PIP2 into PIP3, class-II, and class-III PI3Ks use PIs to generate PI-3-P. Class-I PI3Ks are further subdivided into class-Ia and class-Ib [66, 67]. Activity of class-Ia PI3K is triggered by growth factor receptor tyrosine kinases, whereas class-Ib is activated by G protein-coupled receptors. Class-Ia PI3K is a heterodimer comprising of p85 regulatory and p110 catalytic subunits [66, 68]. At the plasma membrane class-Ia PI3Ks phosphorylate PIP2 at the 3′ position and convert it into PIP3 upon growth factor stimulation. PIP3 binds to the PH domain containing PDK1 and Akt proteins leading to recruitment to the plasma membrane. In addition to lipid kinase activity, class-I PI3Ks also exhibit protein kinase activity. The physiological relevance of this activity differs between the members of class I PI3Ks. For example, class-Ia PI3K phosphorylates insulin receptor substrate-1 (IRS-1), whereas class-Ib PI3K activates the MAPK signaling cascade [66, 68].

PI3K-mediated activation of Akt occurs as a result of ligand-dependent activation of tyrosine kinase receptors, G-protein-coupled receptors, or integrins [69]. Many of theses ligands are overexpressed in cancers, making this a route for Akt activation in melanomas [70, 71]. Receptor-independent activation of PI3K also occurs in 10%–20% melanomas expressing constitutively active Ras proteins [72–74]. Elevated PI3K activity itself can also cause Akt activation. Chromosome 3q26 containing the p110 catalytic subunit of PI3K, which is frequently amplified in cancer of the ovary [75] and cervix [76], leads to increased PI3K catalytic activity. The importance of PI3K signaling in melanoma was demonstrated by overexpressing a deleted subunit of PI3K (Deltap85) to reduce PI3K signaling [77]. In this manner, Delta p85 functioned as a dominant-negative disrupting p85/p110 subunit interaction, consequently inducing apoptosis in the melanoma cell line G361.

PI3K is a critical regulator of melanoma progression. Targeting this kinase using siRNAs or pharmacological agents such as ZSTK474, which binds to ATP-binding pocket of PI3K, reduced melanoma tumor development [66, 68]. In addition, preclinical studies have shown that therapeutic agents inhibiting PI3K activity synergizes with MAPK inhibitors. Although PI3K appears to be a critical regulator of melanoma development, to date, not many studies have shown its expression in patient tumor samples. Furthermore, results of publications are controversial, as some studies found no or low expression of PI3K in patient samples, while others have reported high level of expression [66, 68]. Addressing this aspect a recent study quantitatively assessed the expression of PI3K in 523-melanoma and 540-nevi samples, and showed that p85 and p110 subunit expression is high in melanomas compared to nevi [66, 68]. However, neither subunit served as prognostic marker in either primary or metastatic patients.

1.4. Akt3 Is Central to the Development of Melanomas. Akt, also known as protein kinase B, is a member of AGC family kinases and has three isoforms Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ) [71, 78] (Figure 4). These three isoforms share >80% homology and contain pleckstrin homology (PH), catalytic and regulatory domains [70, 71, 78–84]. The N-terminal PH domain spans amino acids 1 to 107, mediating protein-protein and protein-lipid interactions [85, 86], whereas the central catalytic domain (CD) contains a key phosphorylation residue T^{305} [87, 88]. The carboxy terminal regulatory domain (RD), also referred as hydrophobic motif (HM), contains a second phosphorylation site serine (S^{472}), whose phosphorylation is required for complete activation of this kinase. Other possible phosphorylation sites may also be important, and research in this area continues [89]. In one study it has been shown that the E40K mutation enhances the enzymatic activity of Akt3 in melanomas [21, 46]. Splice variants of Akt3 lacking serine 472 have been identified but the significance of this form of the protein remains unknown [90, 91].

Akt is a positive regulator of cell proliferation and survival, which are controlled by various growth factors and

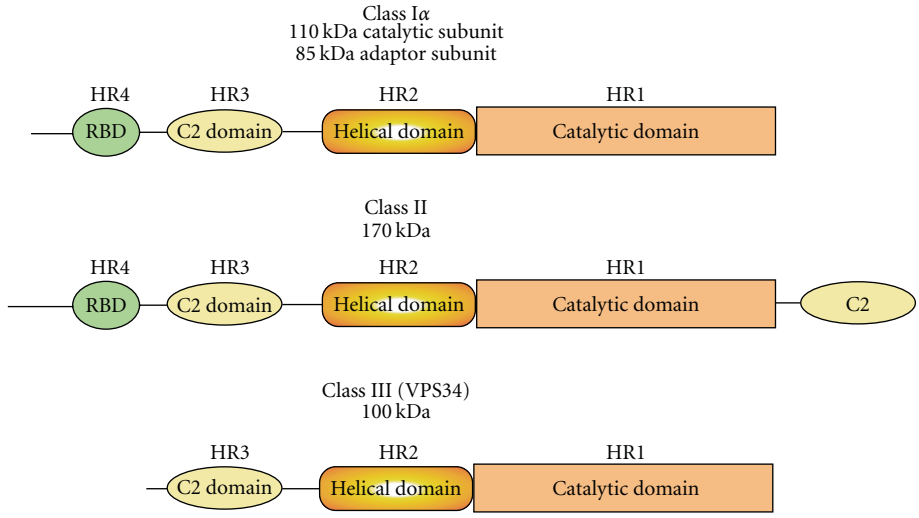

Figure 3: Schematic representation of PI3 kinase enzymes. PI3 kinases are a group of enzymes involved in regulating cell growth, proliferation, differentiation, motility, and survival. Based on structure and substrate specificity PI3 kinases have been divided into Class I (EC number: 2.7.1.153), Class II (EC number: 2.7.1.154), and Class III (2.7.1.137) enzymes. Class I and II PI3Ks are composed of regulatory and catalytic subunits, whereas Class III contains only catalytic subunit. Depending on the type of subunits, heteromeric Class I PI3Ks are further subdivided into Class IA and Class IB. Whereas Class IA PI3K consists of p85 regulatory subunit and p110 catalytic subunit, the Class IB PI3K is made up of p101 regulatory subunit and p110γ catalytic subunit. Class IA PI3K is the most common PI3Ks implicated in the development of cancers.

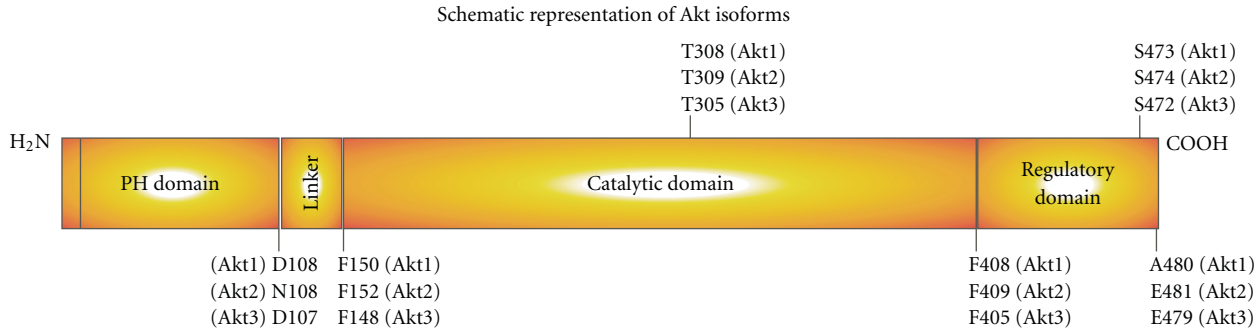

Figure 4: Schematic representation of Akt kinases (EC number: 2.7.11.1). Akt, also known as protein kinase B, is a serine, threonine protein kinase implicated in the development and chemotherapeutic resistance of cancers. Although the three isoforms, Akt1, Akt2, and Akt3, share high degree of structural homology in terms or domain (PH, catalytic, and regulatory) architecture, they exhibit variations in some of the key amino acid residues and cellular functions. Domain organization and key phosphorylation site are represented.

extracellular stimuli (Figure 5) [10]. Despite sharing high degree of homology and some cellular activities, Akt1, Akt2, and Akt3 also exhibit isoform-specific functions [92, 93]. Studies using isoform-specific knockout mice have demonstrated that Akt1 has a key role in maintaining cell survival [93]. Akt1 knockout mice are smaller compared to wild-type mice, and cells lacking Akt1 undergo apoptotic cell death [93, 94]. Likewise, Akt2 knockout mice developed type-2 diabetes, whereas mice lacking Akt3 displayed impaired brain growth [95, 96]. However, not much is known about how these isoform-specific functions are regulated. Some of the key factors regulating isoform-specific functions of Akt include (a) differential tissue distribution [93], (b) differences in the Akt responses to extracellular stimuli [97, 98], (c) structural variations in the key domains regulating translocation, substrate binding, and catalytic activity [10, 99], and finally (d) intracellular compartmentalization [93, 97].

Aberrant activation of Akt kinases has been reported in several malignancies including melanoma. Mechanisms leading to Akt activation can involve (a) mutations in the upstream regulators PI3K and PTEN; (b) overexpression of the gene due to increased copy number or activating point mutations in Akt itself, (c) deletion of negative regulators such as PTEN; (d) altered expression of interacting proteins such as TCL1, HSP90, APPL1, and RasGAP [5, 6, 8, 67]. Akt activity is also regulated by posttranslational modifications such as phosphorylation, ubiquitination and also by physical interactions with effector proteins such as Hsp90 and Pin1 [92, 100, 101]. Although physiological functions of Akt

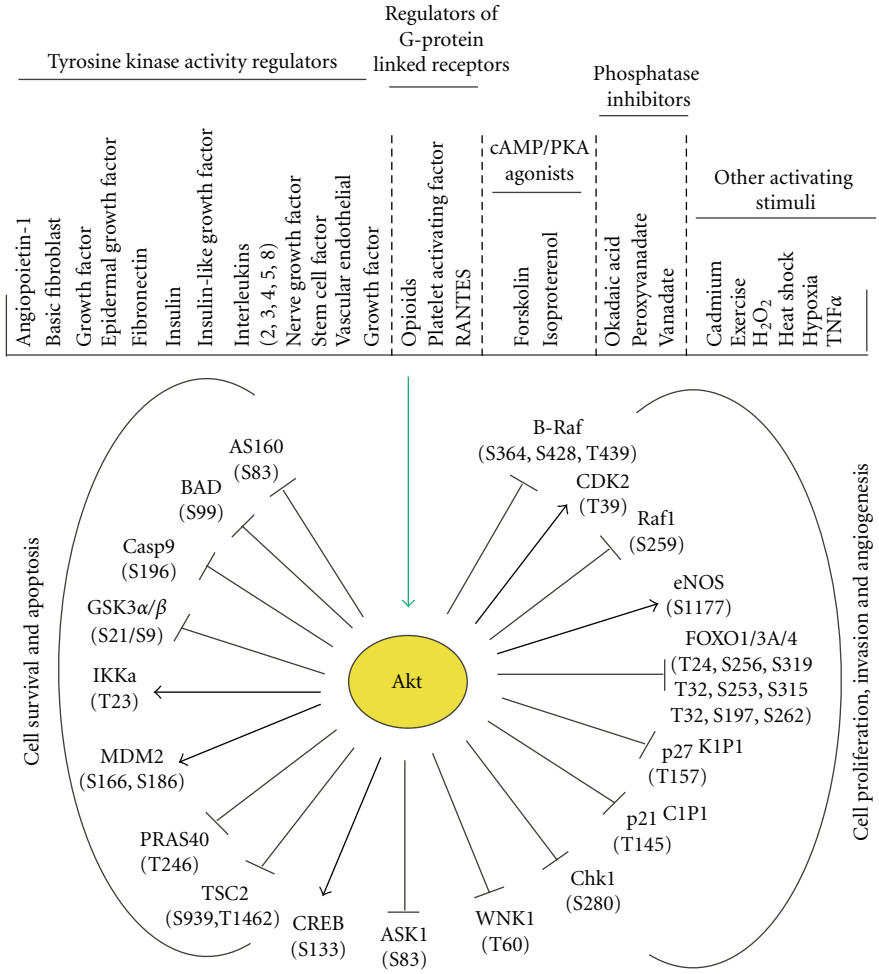

Figure 5: Akt is the key regulator of cell survival and proliferation. Growth factors and hormones trigger the Akt pathway by a series of phosphorylation and dephosphorylation events. Shown are some of the substrates of Akt implicated in cell survival, proliferation, and migration.

isoforms are regulated through similar mechanisms, the activity of a particular isoform is cell type dependent [70, 71, 78–84].

Translocation to the cell membrane followed by phosphorylation of key amino acid residues activates Akt signaling. In normal cells, Akt is cytoplasmic and inactive. Upon growth factors binding to a cell surface receptor or through G-protein-coupled receptors, PI3K gets activated, which, then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) on the 3-OH group, generating second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [102]. PIP_3 binds to the PH domain of Akt thereby facilitating translocation to the plasma membrane. Akt activation is initiated when the translocated Akt is phosphorylated on T308 residue by membrane-localized phosphoinositide-dependent kinase-1 (PDK-1). However, complete activation is achieved only when a PDK2 phosphorylates the second phosphorylation site S473. Recent studies have shown that serine 473 phosphorylation is mediated by rapamycin-insensitive mTORC2 [103, 104]. PDK2 activity of the mTOR complex has been recently established but in melanomas its role in maintaining Akt3 phosphorylation has not been studied [103, 104]. Akt autophosphorylation might also play a role in the activation process [70, 71, 78–84]. Furthermore, it has been shown that prior phosphorylation of tyrosine 315 (Y315) and 326 (Y326) residues is a prerequisite for the activation of Akt kinases (Figure 4).

Preferential activation of a specific Akt isoform has been reported in cancers of breast, ovarian, pancreas, and skin [93]. For example, Akt2 gene amplification has been observed in ovarian and pancreatic cancers. Similarly, Akt3 found to be selectively activated in melanomas [5, 6, 9]. Therefore, selective activation of a speficic Akt isoform appears to regulate the development of a particular cancer. But, to date, the mechanistic basis for this isoform-specific regulation is not known and slowly starting to emerge [93].

Akt activity is also regulated by dephosphorylation of phosphorylated Thr308 and Ser473. Phosphatases are the key enzymes controlling phosphorylation status of Akt. Whereas PTEN dephosphorylates secondary messenger PIP3 into PIP2, the protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP)

remove phosphate groups from T308 and S473, respectively, to deactivate Akt kinases [92, 105, 106]. Okadaic acid, a strong inhibitor of PP2A, promotes phosphorylation of Akt, whereas ceramide, an activator of PP2A, reduces Akt activity [92, 107, 108]. Although extensive studies have been reported on the critical role of PTEN phosphatase, not much is known about the status of PP2A and PHLPP in melanomas.

Another posttranslational modification that has been shown to regulate Akt activity is ubiquitination. Ubiquitination-dependent upregulation of Akt activity has been reported in various cancers [92, 100]. Studies have demonstrated that TRAF6 E3 ligase is required for Akt ubiquitination [109]. Cells overexpressing TRAF6 exhibit high Akt kinase activity, whereas TRAF6 null cells had negligible phosphorylated active Akt [109]. Experiments measuring the effect of TRAF6 expression on Akt activity before and after translocation revealed that TRAF6 is essential for membrane translocation of Akt, but once the Akt is bound to cell membranes, TRAF6 has no effect on regulating Akt activity [100, 109].

Primary structure of Akt also influences its activity. Since the constituent amino acid sequences, especially ubiquitination sites, are involved in regulating protein stability, mutations in these key sites might influence the protein stability thereby enzyme activity. For example, site-directed mutagenesis analysis showed that ubiquitination of Akt occurs at K8 and K14 residues located in the PH domain [100]. Growth-promoting somatic mutations in the PH domain of Akt have been reported in several cancers [92, 100, 110]. For example, E17K mutation in Akt is found in cancers of breast (8%), colon (6%), skin (1.5%), and ovaries (2%) [92, 99, 100, 110, 111]. Similarly, E49K mutation is identified in a subset of bladder cancer patients [112]. However, the abundance of E17K mutation is either too low, or, in some instances, no such mutations were found making it difficult to consider this as a biomarker of tumor progression. For example, in nonsmall cell lung cancers and acute myelogenous leukaemias no such mutations in Akt1 were identified [113, 114]. Moreover, no differences in the ability to translocate to the plasma membrane were observed in experiments where the PH domain of Akt1 was switched with Akt2 PH domain indicating that it is not just the PH domain mediating translocation of the protein but other factors such as posttranslational modification, which might also play a role [93]. Thus, basal Akt activity in a cell primarily depends upon several factors regulating subcellular localization, specifically a balance between positive and negative regulators controlling Akt phosphorylation status and proteins that interact with the kinase itself.

2. Functional Characterization of Akt3 Signaling in Human Melanomas

2.1. Akt3 Is a Key Protein Kinase Regulating Melanoma Development and Chemotherapeutic Resistance. Increased Akt3 expression/activity occur in 60%–70% of sporadic melanomas demonstrating a key role in melanoma development [23, 115]. Activated Akt3 phosphorylates several substrate proteins containing Arg-X-Arg-X-X-[Ser/Thr]-Hyd (where X is any amino acid and Hyd is a bulky hydrophobic amino acid) consensus sequences thereby regulating cellular survival and chemotherapeutic resistance (Figure 5). For example, phosphorylation of (a) GSK3β inhibits its activity thereby promoting cell cycle progression through increased cyclin D levels [116, 117]; (b) PRAS40 at threonine 246 (T^{246}) inhibits the interactions with mTORC1 thereby increasing the nutrient status of the cells [21, 118, 119]; (c) ^{V600E}B-Raf decreases its activity to levels that promote rather than inhibit cell proliferation [46, 120]; (d) osteopontin, a glycophosphoprotein, promotes melanoma progression levels to a highly metastatic state [121]. Furthermore, Akt3 inhibits cellular apoptosis by decreasing caspase-3/7 activity and increasing the expression of cleaved caspase-3 and cleaved PARP levels [9, 122]. Decreased apoptosis makes melanoma cells less sensitive to chemotherapeutic agents functioning through this mechanism.

Increased Akt3 activity also plays a significant role in progression to more advanced aggressive tumors [6, 21, 23]. For example, expression of Akt3 has been elevated in cell lines derived from primary melanoma tumors at the radial and vertical stages of cell growth compared to normal human melanocytes (Figure 6). No significant changes were observed in the levels of Akt1 and Akt2 in the same lysates, indicating that Akt3 activation performs critical functions in melanoma development [6, 23]. Consistent with this observation, a recent report also showed elevated Akt3 expression in melanoma cell lines having high pAkt [123]. Analysis of a panel of 58 melanoma cell lines and 96 melanoma metastases showed elevated pAkt in cells harboring a mutant ^{V600E}B-Raf compared to cells containing N-Ras [123]. However, it is unknown why Akt3 and not the other isoforms is activated in melanomas. Various explanations with experimental evidence have provided some clues by demonstrating (a) increased copy numbers of the Akt3 gene compared to other isoforms in melanomas, which might contribute to some extent for the preferential Akt3 activation [14, 23] and (b) selective activation of Akt3 by preferentially interacting with PIP_3 and/or accessory proteins that bind to PH domain of Akt3 but not to Akt2 or Akt1. Structurally Akt3 has different phosphorylation sites within the PH domain compared to other Akt isoforms [70, 71, 78–84]. For example, TCL1 selectively binds to the Akt3 PH domain, thereby promoting hetero-oligomerization of Akt1 with Akt3, causing transphosphorylation of Akt in leukemias [124–126]. Thus, factors interacting preferentially with Akt3 may lead to selective activation in melanomas.

Recent studies have identified another mechanism for isoform-specific regulation of Akt signaling. These studies have shown that the phosphatidylinositol 3-phosphate binding FYVE domain-containing protein WDFY2, localizes to a distinct subset of early endosomes that are close to the plasma membrane thereby serves as a molecular scaffold to regulate the phosphorylation of Akt kinases in an isoform-specific manner [127]. For example, WDFY2-depleted cells expressed very low levels of Akt2 and pAkt compared to

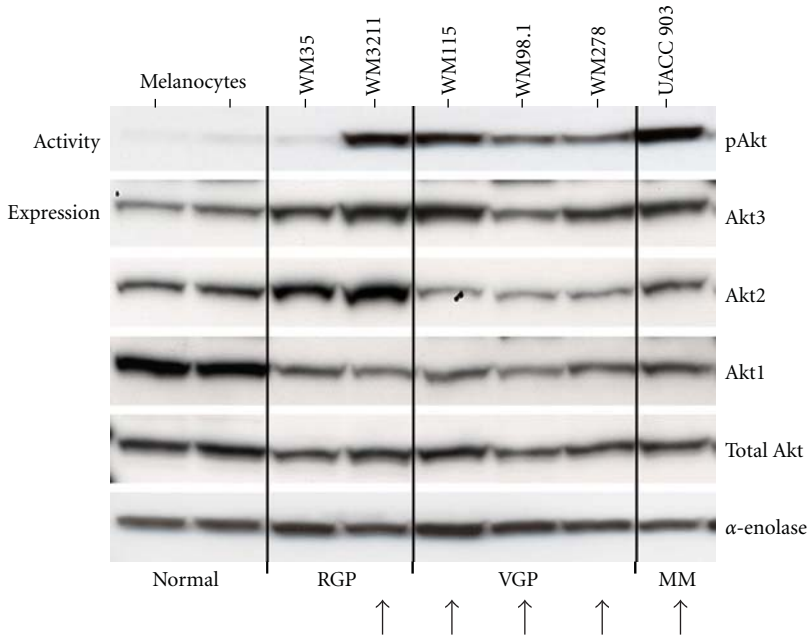

Figure 6: Akt3 but not Akt2 or Akt1 is upregulated in the majority of melanoma cells. Protein lysates were harvested from melanocytes and melanoma cell lines representing radial (WM35, WM3211), vertical (WM115, WM98.1, and WM278), and metastatic (UACC 903) stages and analyzed by western blotting. Arrows indicate the cell lines expressing high Akt3 and pAkt. *Note.* Expression of Akt1 and Akt2 is not changed in majority of advanced melanomas, indicating that Akt3 is the key regulator of cell survival in melanomas [9].

control cells containing WDFY2. Similarly another study also demonstrated the endosome mediated, isoform-specific regulation of Akt activity and substrate selectivity [127]. Rab5 effector endosomal protein Appl1 interacts with transmembrane receptors and Akt thereby influencing Akt activity and substrate selectivity [128]. Human adaptor protein containing PH domain, PTB domain, and leucine zipper (Appl1) is an Akt-interacting protein involved in the regulation of cell survival and proliferation [128]. Targeted depletion of Appl1 decreased phosphorylation of GSK3 but not TSC2 indicating the substrate selectivity induced by this endosomal protein [128]. However, it is not known whether similar regulatory mechanisms also occur in melanoma. We now know that GSK3 regulates cell survival and proliferation rates of melanoma cells but whether Appl1 regulates GSK3 activity in melanoma is not known. Addressing this might open up new avenues for understanding the mechanistic basis of Akt-mediated cell survival regulation and help designing potent therapeutic agents to inhibit this signaling cascade in melanomas.

A central role of the Akt3 isoform in melanoma development is well established; however, a recent report using human melanoma biopsy samples found Akt2 as a predominantly activated isoform in melanomas [129]. In addition, a different study demonstrated that loss of PTEN promoted melanoma cell metastasis via activating Akt2 but not Akt1 or Akt3 [25]. Interestingly, this study also demonstrated that expression of myrAkt3 inhibited invasion of melanoma cells without influencing the expression levels of pFAK and pSTAT3 [25]. Mechanistically, Akt2 induces the expression of miR-200 microRNAs thereby decreasing the expression of E-Cadherin [25], which in turn increases cell invasion. Therefore, it might be interesting to study whether different isoforms of Akt have different functions in regulating melanoma tumor development and metastasis, and if so, how this preferential regulation is occurring. These studies will help addressing some of the key aspects of Akt signaling in melanomas and provide new insights for understanding the mechanistic basis of melanoma tumor development.

2.2. Mechanism Promoting Akt3 Deregulation in Melanomas. A key mechanism for increased Akt activity in cancer cells involves gene copy number increases or mutations leading to constitutive activation. Recently a low-frequency activating mutation (E17K) in the PH domain of Akt3 has been identified in melanoma cell lines and ~4% of patient tumors [111]. This mutation enables Akt3 to get recruited to cell membranes independent of PI3K, which leads to cellular transformation. Genetic amplifications increasing Akt1 or Akt2 expression occur in carcinomas of the stomach, ovary, pancreas, and breast [130–137]. Specifically, Akt2 amplification occurs as part of the 19q13.1–q13.2 amplicon in high-grade aggressive ovarian tumors [138]. Even though no amplifications of Akt genes have been reported in melanomas [139, 140], Akt3 protein is frequently overexpressed [23], as a result of copy number increases of the long arm of chromosome 1 containing the gene [43–45]. No increases in the long arms of chromosome 14 or 19 containing the Akt1 and Akt2 genes, respectively, have been reported [43–45]. Thus, increase in Akt3 copy number is one mechanism contributing to increased expression and

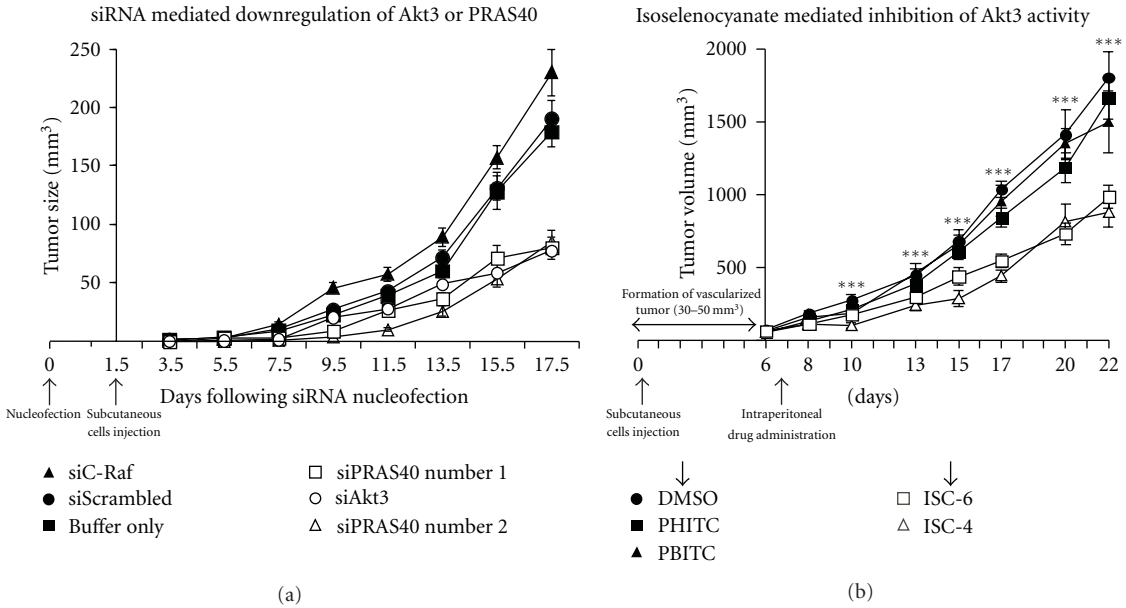

Figure 7: Targeting Akt3 inhibits melanoma tumor development. SiRNA targeting Akt3 and downstream PRAS40 is introduced into UACC 903 melanoma cells by Amaxa transfection and effect on xenografted subcutaneous tumors measured. The data shows ~50%–60% decreased tumor volume when the expression of Akt3 and PRAS40 is inhibited compared to control cells transfected with a scrambled siRNA, siRNA to C-Raf, or buffer [21]. Inhibiting Akt activity using pharmacological agent ISC-4 that contains selenium also reduced melanoma tumor growth as effectively as siRNA mediated downregulation of Akt3 expression, indicating the potential utility of developing pharmacological agents targeting this key protein in melanomas [122].

activity in melanoma. Since expression of Akt3 protein might not represent the activation status of the protein, it is thought that other processes must also contribute, leading to preferential activation of Akt3 versus the other isoforms in melanomas.

Phosphatases that directly dephosphorylate Akt may also play a role in the preferential activation of Akt3 in melanomas. While the identity of these phosphatases remains uncertain, PP2A phosphatase may be involved in this process [141]. Proteins binding to Akt also have the potential to modulate cellular activity as is observed with the protooncogene TCL1, which enhances oligomerization of Akt1 with Akt3, thereby facilitating activation of Akt3 in leukemia [124–126]. Another example is the heat-shock-protein 90 (Hsp90) that can complex with Akt. Inhibition of Akt-Hsp90 complex formation can inactivate Akt by PP2A-mediated dephosphorylation [142, 143].

Deregulated Akt3 expression and activity could also be controlled by noncoding microRNAs. For example, miR-149 has been shown to inhibit Akt1 activity thereby inducing apoptosis in neuroblastoma and cervival cancers [144]. Ectopic expression of miR-149 induced apoptosis by inhibiting Akt1 expression and activity [144]. However, miRNAs specifically regulating Akt3 expression have not been identified. Only recently, miRNAs 15a and 16 have been shown to inhibit the expression of Akt3 in multiple myelomas [145]. However, these miRNAs also inhibited ribosomal protein-S6, MAP-kinases, and NF-κB activator MAP3KIP3 [145]. Discovering miRNAs specifically regulating Akt3 is important to assess the clinical progression of the disease and for developing novel tools for preventing or treating melanomas.

3. Cellular Processes Regulated by Akt3 Signaling in Melanomas

3.1. Akt3 Signaling Regulates Cell Survival, Migration, Metastasis, and Chemoresistance. Akt3 has been shown to be a prosurvival kinase in melanomas [23]. SiRNA or pharmacological agents targeting Akt3 protein levels or expression of PTEN selectively lowers Akt3 activity, thereby reducing the tumorigenic potential of melanoma cells by altering apoptotic sensitivity of the cells (Figure 7) [21, 23]. Furthermore, increased activity correlates with tumor progression, providing cells with a selective advantage in the tumor environment [21, 23]. Inhibiting Akt signaling in tumor cells by adenoviral transfer of an Akt kinase-dead mutant, in which the two regulatory phosphorylation sites have been mutated to alanines thereby converting it to a dominant negative, led to selective induction of apoptosis in tumor cells expressing activated Akt [146]. In contrast, a minimal effect was observed in normal or tumor cells expressing low levels of active Akt [146]. In addition, a recent study showed that Akt could act as a molecular switch by increasing angiogenesis and producing superoxides [147]. Experimentally, overexpression of Akt in radial growth phase WM35 cells transformed them into malignant phenotypes by stabilizing

cells with extensive mitochondrial DNA mutations, which can generate ROS, and by inducing expression of NOX4, a ROS generating enzyme [147].

The vital role of Akt in melanoma tumor progression and development is further confirmed by showing that hyperactivated Akt signaling upregulated Notch1 via NFκB activity thereby inducing the transformation of melanocytes under prevailing hypoxic conditions in tumors [148]. Upregulated Akt activity in melanoma tumors appears to protect cells from low oxygen pressures and apoptotic cell death induced by various chemotherapeutic agents [48]. For example, a recent study demonstrated that Akt3 mediates the resistance to apoptosis in B-Raf-targeted melanomas [48]. Targeting ^{V600E}B-Raf using PLX-4072 rendered invasive melanoma cells susceptible to anoikis, a form of apoptosis generally induced by loss of adhesion and is mediated by BH3-only proteins, Bim-EL and Bcl2-modifying factor (Bmf) [48, 149]. However when expression of Akt3 is induced by adhesion to fibronectin in these cells, the cells exhibited resistance to apoptosis caused by B-Raf inhibition [48]. Therefore, Akt3 is a key regulator of melanoma cell survival and helps protect cells from apoptosis induced by chemotherapeutic agents [48]. Another recent study also demonstrated the induction of chemotherapeutic resistance by Akt activation. Human melanoma cells under endoplasmic reticulum stress showed more resistance to apoptosis induced by microtubule-targeting chemotherapeutic agents such as docetaxel and vincristine, which is mediated by Akt activation [150].

Processes such as metastasis, cell-cell adhesion, cell migration, and development of chemoresistant tumors under hypoxic conditions are also regulated by PI3 kinase and Akt signaling pathways [6, 147, 152]. Activated Akt in metastatic melanoma cells have been shown to regulate Notch1 expression via NFκB thereby promoting tumor development under hypoxic tumor conditions [148]. A different study has shown that Akt inhibits RhoB, a GTPase, in melanomas and thereby induces tumor cell survival, transformation, invasion, and metastasis [153].

3.2. Substrates of Akt3 Kinase Involved in Melanoma Development. Akt substrates can be cytoplasmic or nuclear proteins, and numbers of proteins regulated by Akt continue to increase as studies such as those using the minimal consensus peptide sequence Arg-X-Arg-X-X-[Ser/Thr]-Hyd (where X is any amino acid and Hyd is a bulky hydrophobic amino acid) search for putative substrates [154, 155]. The functions of many of these substrates in cellular processes have been identified, demonstrating that Akt regulates multiple processes in cells including apoptosis and proliferation (Figure 5).

Substrates for Akt3 do not appear to be specific but rather seem to be identical to those acted on by all three Akt isoforms. Substrates of Akt3 involved in melanoma development include (a) Bad-whose inactivation promotes cell survival [156]; (b) NFκB-inhibition of Akt activity led to increased apoptosis and decreased NFκB promoter activity in melanoma cells [115]; (c) hTERT-inhibition of Akt reduced hTERT peptide phosphorylation and telomerase activity [157]; and (d) Rac1-targeting Akt decreased phosphorylated serine 71 of Rac1 thereby modulating the Rac1 signal transduction pathway in SK-MEL-28 melanoma cells [158] (Figure 5).

Recently, Akt3 has been reported to phosphorylate ^{V600E}B-Raf on S364 and/or S428 to reduce its activity to levels that promote rather than inhibit melanoma development from melanocytes [46]. Ectopic expression of ^{V600E}B-Raf in primary cell lines such as melanocytes has been shown to induce cellular senescence not only by elevating the levels of MAPK activity for unusually longer periods but also by upregulating the expression of cyclin-dependent kinase (cdk) inhibitors [159, 160]. Therefore, genetic changes such as loss of tumor suppressor genes (PTEN, p53 or p16INK4A) or upregulation of cooperating oncogenes is necessary to progress into advanced metastatic stages [161]. For example, activation of Akt3 has been demonstrated to facilitate the progression of quiescent melanocytic nevi into aggressive vertical and metastatic stages by inhibiting ^{V600E}B-Raf activity thereby releasing cells from a senescence block [46, 162]. This demonstrates that in melanomas, Akt3 kinase not only increases cell survival but also aids early melanoma development [46]. Likewise, a recent study demonstrated the induction of melanomas only when PTEN is silenced [47]. Using a conditional mouse model it has been demonstrated that ^{V600E}B-Raf induction resulted in the development of melanocytic hyperplasias, which failed to progress into melanomas [47]. However, when the PTEN gene was silenced while maintaining the expression of ^{V600E}B-Raf, melanocytic hyperplasias progressed into melanomas with 100% penetrance, short latency, and metastatic ability [47].

PRAS40 is another substrate regulated by Akt3. PRAS40 is a cytosolic protein found ubiquitously in all eukaryotes [118] and is phosphorylated by Akt at T246 [118]. Phosphorylated PRAS40^{T246} protects neuronal cells from ischemic injury by inhibiting caspase-3-mediated apoptotic cell death [163]. Expression of pPRAS40 levels are also associated with tumor malignancy [164]. Compared to paired control normal cells, cancer cell lines (MCF10A/MCF7, BEAS/H1170) exhibited high pPRAS40 expression [164]. However, the detailed functional characterization, in vitro and in vivo, was not reported. Compared to normal melanocyte controls, 43 to 60% of flash frozen tumors collected from melanoma patients express high pPRAS40 and pAkt [21]. Furthermore, targeted inhibition of PRAS40 using siRNA reduced melanoma tumor growth in xenografted melanoma studies (Figure 8) [21]. Mechanistically, PRAS40 inhibition induced caspase-3/7-mediated apoptosis in melanoma cells and increased sensitivity to apoptosis inducers such as staurosporine [21]. However, the molecular basis for caspase-3/7 activity inhibition by pPRAS40 in melanomas remains unknown.

Recent reports have demonstrated that PRAS40 is a physiological target of mTORC1 kinase and regulates its activity by functioning as a direct inhibitor of substrate binding [119, 165, 166]. PRAS40 has two additional phosphorylation sites S183 and S221 other than the one phosphorylated by Akt [167]. A recent study demonstrated that phosphorylation

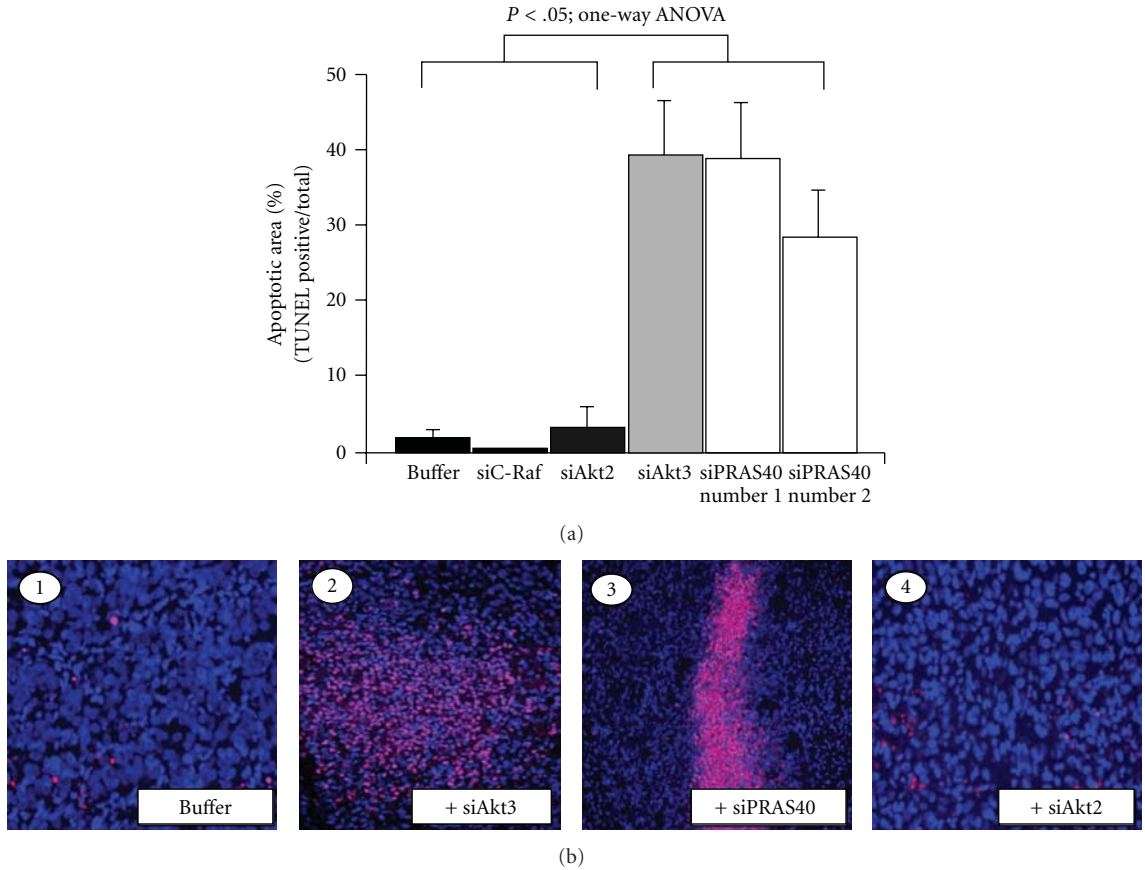

Figure 8: Akt3 and downstream PRAS40 are the key regulators of melanoma cell survival. SiRNA-mediated downregulation of Akt3 and PRAS40 induced apoptosis in subcutaneous melanoma tumors. Shown are the representative pictures of TUNEL assay performed on size- and time-matched tumors. The bar graph shows the percentage TUNEL positive cells in the tumors [21].

of T246 by Akt facilitates the phosphorylation of S183 and S221 by mTORC1 [167]. 14-3-3 proteins via physical interactions sequester phosphorylated PRAS40 thereby release mTOR. Therefore, increased $pPRAS40^{T246}$ may releasing its inhibitory effects on mTORC1 kinase and downregulate caspase-3/7 activity. Although phosphorylation of PRAS40 at threonine 246 by Akt is primarily involved in inhibiting cellular apoptosis and increasing survival, the role of unphosphorylated PRAS40 in the cancer cells needs further investigation.

Another well-known substrate of Akt is GSK3 [6, 117], which is present in cells as two structurally similar GSK3α and GSK3β isoforms [168]. Akt has been shown to phosphorylate S9 of GSK3β and S21 of GSK3α to inhibit their activity thereby promoting cell proliferation by stabilizing cyclin-D1 protein [29, 117]. Therefore, unphosphorylated active GSK3 is considered as an apoptosis inducing tumor suppressor. However, recent studies have shown that GSK3 expression is elevated in advanced cancers and deregulation of GSK3 activity increases malignant transformation of cells [168–171]. Recently it has been observed that inhibition of GSK3β enhances sorafenib-induced apoptosis in melanoma cells [172]. Furthermore, targeting GSK3β using organometallic inhibitors (DW1/2) decreased Mdm2 activity thereby elevating p53-mediated apoptosis in melanoma cell lines expressing wild-type p53 protein [173]. Finally, GSK3α and not GSK3β has been shown to regulate pancreatic cell survival [174]. However, similar studies have not been reported in melanomas. Therefore, identifying the actual isoform regulating melanoma development by selectively inhibiting these isoforms using siRNA is warranted. Furthermore, studies are also warranted to demonstrate which isoform is expressed in melanoma patient tumors in order to establish its clinical relevance.

4. Clinical Implications of Targeting the PI3k-Akt3 Signaling Cascade in Melanomas

Targeting PI3K-Akt3 signaling has significant clinical potential for inhibiting melanoma tumor development [23]. For example, introduction of PTEN into melanoma cells or inhibiting Akt3 using siRNA or small molecule inhibitors such as ISC-4 inhibited melanoma tumors growth in vitro and in animals [175, 176]. Furthermore, expression of PTEN or reduction of Akt3 activity has been found to increase melanoma cell sensitivity to apoptotic stimuli that occurs with most chemotherapeutic agents [9, 21, 176].

4.1. PTEN Reexpression Prevents Melanoma Development. Tumor suppressor PTEN has been demonstrated to inhibit melanoma development in cultured cells and in spontaneous melanoma models [9, 21, 47]. For example, introduction of PTEN into advanced-stage melanoma cells via a chromosomal transfer or ectopic expression using a plasmid-based vector inhibited tumor development in xenografted melanoma models [8, 15, 177]. Thus, targeted delivery of PTEN into melanoma cells has potential to be an effective therapeutic agent. Restoration of functional PTEN in these cells would increase the therapeutic efficacy of antimelanoma agents [8, 9, 21]. For example, isogenic cell lines expressing PTEN containing low pAkt are sensitive to staurosporine-induced apoptotic cell death, whereas cell lines that lost PTEN are resistant to therapeutic agents [8, 9, 21] (Figures 1 and 2). Furthermore, if PTEN expression was regulated by endogenous microRNAs, inhibiting using antimers could significantly upregulate PTEN activity thereby restoring the chemosensitivity of the melanoma cells. However, PTEN regulation by microRNAs has not yet been reported in melanomas.

4.2. Therapeutic Agents Targeting PI3K and Akt3 Signaling Cascades in Melanoma. Targeting Akt3 alone or in combination inhibits growth by inducing apoptosis, decreasing survival, and inhibiting proliferation of melanoma cells [5, 9, 21]. Several synthetic and naturally occurring small molecule inhibitors specifically inhibiting Akt activity have been developed and efficacy tested for inhibiting melanoma development in cultured cells and xenografted animal models [6, 9, 21, 46, 122, 151, 176, 178]. For example, isoselenocyanate ISC-4 has been demonstrated to inhibit early melanocytic lesions and advanced xenografted melanoma tumors in animals [122]. Compared to known inhibitors of Akt signaling such as API-2, the selenium containing ISC-4 appears to be more effective for melanoma treatment [122]. BI-69A11 is another Akt inhibitor, demonstrated to effectively inhibit melanoma tumors [179]. However, clinical utility of these inhibitors for decreasing melanoma development has not been evaluated yet. Similarly, safety and efficacy of other inhibitors of PI3K-Akt signaling such as GSK690693 (developed by GlaxoSmithKline), a novel ATP-competitive inhibitor of Akt kinases [180] and SR13668 (developed by SRI international), an NCI approved Akt inhibitor [181, 182], also need to be tested in clinic.

Since the efficacy of a particular inhibitor in vitro or in preclinical animal models may not exactly reflect the potency of the compound in clinical trials, testing these new inhibitors in clinic is highly recommended. For example, perifosine, the alkylsphophocholine analogue, is found to be very effective (inhibiting growth of cancer cells at 0.2 to 0.3 μM concentration) in vitro and in animal models. However, a Phase-II study using perifosine in 18 previously untreated patients with metastatic melanoma demonstrated no objective response [183].

4.3. Targeting Multiple Pathways to Synergistically Treat Melanoma. Several lines of evidence now suggest that it might be required to target multiple signaling pathways to inhibit melanoma development. For example, a recent study shows that inhibiting ^{V600E}B-Raf using PLX-4720 induced resistance to apoptosis by activating Akt signaling [48, 149]. Furthermore, it is also now well established that targeting ^{V600E}B-Raf not only induces resistance to various therapeutic agents but also stimulates the activity of C-Raf and wt B-Raf signaling thereby triggering the formation of keratoacanthomas and basal cell carcinomas [7, 184]. Therefore, it is important to target multiple signaling cascades to inhibit melanoma development. However, it is not completely known which targets should be inhibited. Studies have provided some directions in this regard by showing synergistically acting melanoma tumor inhibition by simultaneously targeting PI3K/Akt3 and MAP kinase signaling pathways [32, 46, 151, 185–187]. For example (a) delivering siRNAs inhibiting Akt3 and ^{V600E}B-Raf synergistically inhibited melanoma tumor cells growth in culture or in xenografted melanoma tumors [46, 151] (Figures 9 and 10); (b) combining nanoliposomal ceramide with sorafenib synergistically reduced melanoma cell growth [188]; (c) pharmacological agents inhibiting MAPK (U0126, PD98059 and PD325901) and mTORC1 (using rapamycin) more effectively reduced melanoma cells growth compared to either of the agents tested singly [24, 189, 190]; (d) topical application of LY-294002 and U0126 in combination effectively decreased melanoma tumor incidence in the transgenic TPRas mouse model when compared to either of these agents alone [191]; (e) targeting PI3K and mTOR using dual inhibitors NVP-BBD130 and NVP-BEZ235 effectively reduced the size of primary melanoma tumors and inhibited cervical lymph node metastasis in a syngenic mouse melanoma model. Although these studies demonstrate the potential therapeutic efficacy of combined target inhibition, no complete tumor reduction occurred in any of these studies, warranting the identification of other candidates and target combinations for treating this disease.

Other key targets regulating melanoma development have been recently identified. For example, Yang et al. showed the effect conditional ablation of Ikkb on melanoma tumor development using an established HRasV12 mouse model of spontaneous melanoma [192]. Ink4a/Arf$^{-/-}$ mice with melanocyte-specific deletion of Ikkb were protected from HRasV12-initiated melanoma only when p53 was expressed. Ikkb ablation was found to decrease Aurora-A kinase and IL-6 in melanomas [192]. Therefore Ikkb and Aurora-A could be potential targets in melanomas [192]. However, it is not known whether targeting these pathways would synergize with Akt inhibition. In support of this direction of investigation, a recent study reported that Aurora-A regulates cancer cell survival by inducing Akt activity and inhibiting Aurora kinase with VX-680 synergizes with pharmacological inhibition of Akt (wortmannin) activity [193]. However, experimental evidence pertaining to which Aurora kinase isoform needs to be targeted for the greater synergistic effect is currently unknown.

It is fairly certain that both MAP and PI3 kinase pathways will need to be targeted for inhibiting melanoma. For

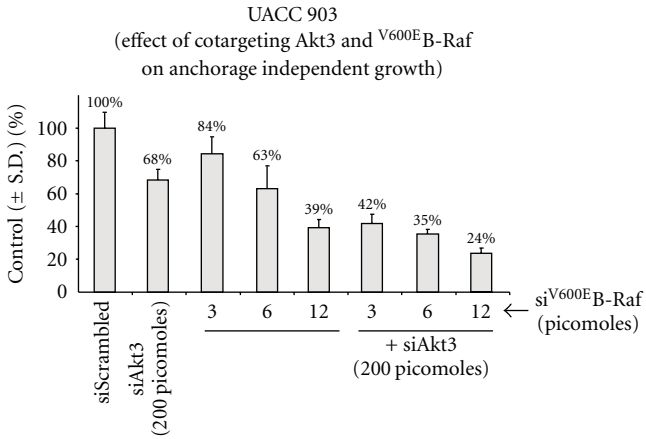

Figure 9: Cotargeting Akt3 and ^{V600E}B-Raf inhibits melanoma cell proliferation in vitro. Two hundred picomoles of siRNA targeting Akt3 and increasing (3, 6, and 12 picomoles) amounts of siRNA inhibiting ^{V600E}B-Raf were introduced alone or in combination into UACC 903 melanoma cell line by Amaxa transfection and effect on anchorage independent growth ability measured using MTS assay. The data shows a dose-dependent inhibition of cell viability when Akt3 and ^{V600E}B-Raf were inhibited. However, maximal effect was observed only when Akt3 and ^{V600E}B-Raf targeted together, indicating the necessity of inhibiting multiple signaling cascades [46, 151].

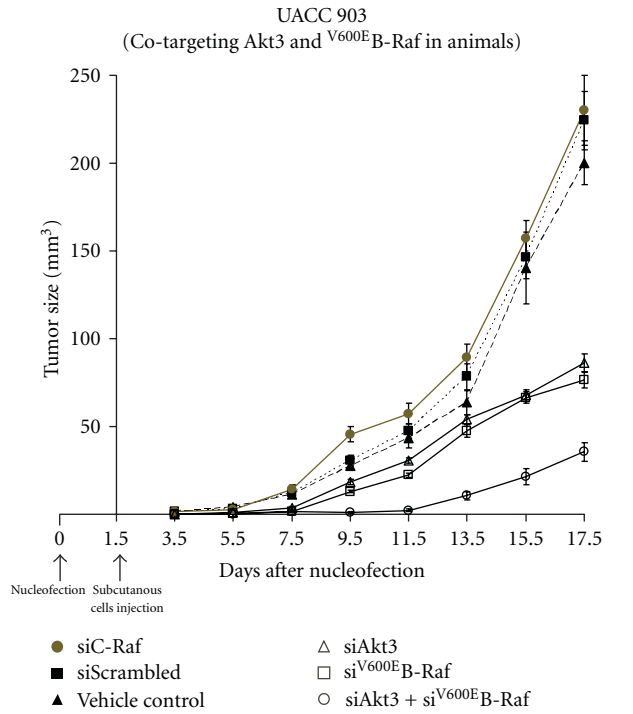

Figure 10: Akt3 synergizes with ^{V600E}B-Raf in melanomas. UACC 903 cells transfected with siRNA targeting Akt3 and ^{V600E}B-Raf alone and in combination were subcutaneously injected into mice and effect on tumor development measured. Cotargeting these two key kinases inhibited melanoma development more effectively than inhibiting each of these kinases alone [46, 151].

example, hybrid compounds that inhibit these pathways such as HMBA (hexamethylene bisacetamide—HMBA), which simultaneously inhibits Akt and MAPK pathways and represses NFκB activity in breast cancer cell lines, needs to be tested in melanomas [194]. Similarly, recent studies have demonstrated the utility of a novel selenium containing iNOS inhibitor, called PBISe, for inhibiting melanoma cell growth in vitro and in vivo [195]. Intraperitoneally administered or topically applied PBISe inhibited iNOS and PI3K/Akt3 signaling thereby inducing significant apoptosis in melanoma cells. Furthermore, PBISe-mediated inhibition of Akt3 signaling induced cell senescence by upregulating pErk1/2 to inhibitory levels which triggered the induction of cell cycle inhibitors p21, p16, and p27 [196, 197]. Inhibitors targeting PI3K also have potential to inhibit melanoma growth [67]. For example, SF1126, a conjugate of LY294002 and a pan-PI3K inhibitor, developed by Semafore Pharmaceuticals, is currently in Phase-I clinical trial for solid tumors [198]. Other PI3K inhibitors that are under clinical investigation include GDC-0941, an inhibitor of p110α subunit developed by Genentech, Inc; and XL-147 and XL-765, developed by Exelixis [198]. However, it is not known whether these inhibitors could synergistically inhibit melanoma development when combined with agents inhibiting other key signaling cascades.

5. Conclusions

Activation of Akt3 is a key event regulating the development of melanomas. Enzymes involved in this signaling pathway regulate cell survival, proliferation, metastasis and are implicated in development of resistance to a variety of chemotherapeutic agents. Mechanisms regulating Akt3 phosphorylation such as loss of PTEN, and activation of PI3K signaling need to be further unraveled to aid in the design of novel therapeutic strategies for treating melanoma. Therefore, PI3K-Akt3 signaling remains an attractive target in melanomas. Although this review provides an overview of the PI3K-Akt signaling in melanomas, several aspects need further investigation. Several unanswered questions also remain pertaining to this key signaling cascade. For example, it is not known (a) which microRNAs are regulating Akt signaling in melanomas, (b) whether endogenous Akt3 is also regulating metastasis development in human melanomas, and (c) how Akt2 is regulating melanoma metastasis and whether targeting Akt2 alone could inhibit metastasis development? Addressing these questions might provide a better understanding of the role of Akt signaling in melanoma and help design more potent selective agents for targeting this disease.

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Review Article

The Role of Manganese Superoxide Dismutase in Skin Cancer

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Recent studies have shown that antioxidant enzyme expression and activity are drastically reduced in most human skin diseases, leading to propagation of oxidative stress and continuous disease progression. However, antioxidants, an endogenous defense system against reactive oxygen species (ROS), can be induced by exogenous sources, resulting in protective effects against associated oxidative injury. Many studies have shown that the induction of antioxidants is an effective strategy to combat various disease states. In one approach, a SOD mimetic was applied topically to mouse skin in the two-stage skin carcinogenesis model. This method effectively reduced oxidative injury and proliferation without interfering with apoptosis. In another approach, Protandim, a combination of 5 well-studied medicinal plants, was given via dietary administration and significantly decreased tumor incidence and multiplicity by 33% and 57%, respectively. These studies suggest that alterations in antioxidant response may be a novel approach to chemoprevention. This paper focuses on how regulation of antioxidant expression and activity can be modulated in skin disease and the potential clinical implications of antioxidant-based therapies.

1. Introduction

Antioxidant enzyme expression is known to decrease with aging, which has been theorized to contribute to age-related diseases. One of the main contributors to disease progression is reactive oxygen species (ROS) generation. ROS is the result of incomplete reduction of oxygen within the electron transport chain. The reactive oxidants of ROS include superoxide anion ($O_2^{\bullet -}$), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH). Although these molecules can act as signaling molecules, they can also participate in cellular damage, such as lipid peroxidation and DNA damage that trigger altered downstream signaling and apoptotic pathways.

With skin being the largest, most readily exposed organ to the environment, it is imperative that mechanisms of protection against oxidative injury are in place within the skin. The skin consists of various antioxidant enzymes such as glutathione reductase, catalase, and superoxide dismutase. These enzymes are often activated to maintain homeostasis and to minimize the damaging effects of ROS. However, alterations in the expression/activity of these antioxidants increase the susceptibility of skin to ROS-mediated injury that contributes to skin disease. Many studies have shown that antioxidant activity, mainly manganese superoxide dismutase (MnSOD), is reduced in various skin cancers. For example, epidermal SOD activity is decreased in hyperproliferative keratinocytes in squamous cell carcinoma, basal cell epithelioma, as well as benign hyperproliferative keratinocytes in the psoriatic epidermis [1–3]. This paper focuses on the therapeutic potential of exogenous antioxidant inducers, the use of SOD mimetics as a chemopreventive agent, and dietary mechanisms of antioxidant induction in skin carcinogenesis.

2. MnSOD in Skin Disease

Psoriasis is a skin disease generally characterized by the incomplete differentiation of epidermal keratinocytes, and infiltration of leukocytes. Once localized within skin tissues, these inflammatory cells release various cytokines and ROS resulting in a high incidence of lipid peroxidation. Interestingly, malondialdehyde, a key marker of lipid peroxidation,

has been found at increased levels in the plasma and red blood cells of patients with psoriatic skin. In addition, plasma levels of b-carotene and a-tocopherol levels were decreased, along with decreased catalase and glutathione-peroxidase activities in RBC, contributing to the pathogenesis of the disease. Lontz et al. examined the mRNA expression of MnSOD in psoriatic skin tissue and found that MnSOD mRNA was significantly higher in lesional psoriatic skin compared to nonlesional skin tissues [4]. Many postulated that associated induction of MnSOD expression may serve as a protective mechanism of increased survival; however, it was not found to be directly correlated to disease pathogenesis. It is known that cytokines such as TNF-a can induce MnSOD expression, suggesting a direct correlation of increased MnSOD expression and the inflammatory mechanism of psoriasis. On the other hand, the oxidative stress-mediated mechanism of disease pathogenesis has been observed in other skin disease, as well, such as contact dermatitis [5], acne, and vitiligo, suggesting the need for further investigation of antioxidant-based interventions in various skin diseases. In this quest, MnSOD becomes a potential target, being that it is the first line of defense within skin tissues and when compared to other SODs such as Cu, Zn-SOD, it is affected differently in various skin abnormalities [6]. Therefore, we will continue to further observe the induction, the role of overexpression and chemopreventive potential of MnSOD in other skin diseases, particularly skin carcinogenesis.

3. Induction of MnSOD Expression

MnSOD can be induced by a variety of stimuli including cytokines [7, 8], radiation [9, 10], and chemical carcinogens such as 12-*O*-tetradecanoylphorbol-3-acetate (TPA). Several studies have shown that TPA can induce MnSOD expression; by direct activation of protein kinase C (PKC) or through the production reactive oxygen species that can act as cell signaling molecules activating redox-sensitive transcription factors such as AP-1 and NF-κB [11–13]. However, several studies have shown that MnSOD expression is reduced in most human cancers. It has been found that the reduction in MnSOD expression is not due to defects in the primary structure of the MnSOD protein, but rather changes in gene expression [14, 15]. Several transcription factors have been implicated in the induction of MnSOD expression; however, the most widely studied is specificity protein 1 (Sp1). The transcription factor Sp1 contains three zinc finger motifs in the DNA-binding domains that recognize GC-rich sequences of GGGCGG [16]. The GC-rich characteristics of the MnSOD promoter are conserved among mouse [17], bovine [18], rat [19], and human [20]. The Sp1 protein is capable of inducing gene expression by forming homotypic, Sp1-Sp1 interactions [21–23]. However, SP-1 binding affinity and transcription properties can be altered by interactions with other cofactors. Sp1 forms heterotypic interactions with different classes of nuclear proteins such as TATA box-binding protein (TBP) [24], C/EBP [25], and YY1 [26, 27]. SP-1 recognition sequences are often found to be near binding sites for othertranscription factors such as AP-1 [28], AP-2 [29], and even NF-κB [30] suggesting that SP-1 may work in conjunction with other transcription factors to modulate MnSOD gene expression. On the other hand, studies have shown that subcellular organelles, such as the mitochondria can regulate the induction of antioxidant genes such as *sod2*. Kim et al. showed that in an effort to maintain an optimal mitochondrial redox state, increased MnSOD expression led to endogenous *sod2* transcripts, and increased *sod2* mRNA levels were a result of increased transcriptional activation of the *sod2* gene in mice [31]. In addition, it is known that homozygous *sod2*−/− knockout mice exhibit a neonatal lethality phenotype that is not reversed or delayed by copper/zinc superoxide dismutase or sod1 overexpression [32]. Therefore, maintenance of the cellular redox state and induction of MnSOD expression is important to cell viability.

4. Mechanisms of Action of MnSOD

Initially, it was postulated that flavones and antioxidants inhibit skin carcinogenesis by interfering with the metabolism of carcinogens into the ultimate carcinogen form [33, 34]. Several studies have found that overexpression as well as deficiencies in MnSOD expression can have significant effects on tumor formation using this two-stage skin carcinogenesis model. Skin carcinogenesis is known to occur in a two-stage process. The two-stage skin carcinogenesis model is a well-established model utilized to study the multiple stages of skin carcinogenesis: tumor initiation, promotion, and metastasis. A single application of the polycyclic aromatic hydrocarbon, 7, 12-dimethylbenz[a]anthracene, is applied at a subthreshold dose. Chemical mutagens, such as DMBA are known to induce carcinogen-specific mutations in the H-*ras* gene at codon 61 [35, 36]. Mutations in the H-*ras* gene confer a selective advantage within H-*ras* initiated cells, which can develop into benign tumors after treatment with tumor-promoting agents such as TPA [37]. Following this subcarcinogenic dose, multiple applications of TPA are applied to induce epigenetic changes. The tumor promotion stage is essentially reversible; however, later in the tumorigenesis process, this stage becomes irreversible. Overall, tumor promotion inhibitors have common mechanisms of action: (1) altered metabolism of the carcinogen, (2) scavenging abilities of active molecular species of carcinogens, and lastly (3) competitive inhibition [38]. Several studies have shown the inverse relationship of ROS and MnSOD expression in the pathogenesis of hyperproliferative and inflammatory diseases. It is known that DMBA/TPA treatment induces cell proliferation and apoptosis; both believed to be modulated by oxidative stress propagation [39]. For example, it was found that overexpression of MnSOD, in the two-stage skin carcinogenesis mouse model, reduced the number and incidence of papillomas providing direct evidence of free radical involvement in skin carcinogenesis [40]. Zhao et al. showed that apoptosis preceded cell proliferation [39]. It was found that apoptosis peaked at the 6-hour time point, prior to the peak in cell proliferation at 24 h [39]. Providing a therapeutic window for antioxidant intervention,

MnTE-2-PyP^{5+}, a small molecule catalytic antioxidant (SOD mimetic), was applied following the peak in apoptosis. It was found that papilloma formation decreased 6-fold compared to their control counterparts, without effecting apoptosis. These results suggest that antioxidant therapy is an effective mode of tumor suppression and can potential be used in conjunction with traditional chemotherapeutics without interfering with drug-induced cell death. Consistent with that, it was found that in the presence of MnTE-2-Pyp^{5+}, the level of oxidative injury was significantly reduced. Therefore, these results suggest the oxidative stress-mediated tumor promotion of TPA, as well as, the antioxidant capabilities of MnSOD in tumor suppression. Furthermore, in a study using MnSOD transgenic mice, it was found that only 50% of transgenic mice developed papillomas, compared to 78% of their nontransgenic counterparts [40]. These results, again, suggest the antioxidant capabilities of MnSOD as a tumor suppressor.

Moreover, superoxide anions, one of the major constituents of ROS, act as signaling molecules that can regulate oncoproteins and downstream gene expression. As a key cellular redox regulator, MnSOD has been shown to affect the binding activities of transcription factors to transcriptional control elements, therefore modulating gene expression. The mechanism behind MnSOD mediated tumor suppression has been shown to involve suppression of activator protein-1 (AP-1) activity. AP-1 is a key mediator of oncogenic signaling [37]. There are many posttranslational modifications that can regulate AP-1 activity such as modulation of the phosphorylation states of the Jun or Fos protein [41] and redox regulation of the Jun protein. High levels of phosphorylated c-Jun, Fra-1, Fra-2, and ATF-2 proteins have been shown to positively correlate with malignant phenotypes in the multistage mouse skin carcinogenesis model [37]. In addition, the increased expression and posttranslational modifications of these oncoproteins account for a high percentage of the increased AP-1 activity. In malignant cell lines, the DNA binding and transactivation properties of AP-1 have been found to be elevated, peaking in fully metastatic cell lines [37]. The transcription factor, AP-1, is known to play a role in cellular differentiation, proliferation, and transformation. The AP-1 complex is known to consist of the homo- or heterodimer of the Fos, Jun, and Fra family members. Many of the subunits of AP-1 are redox sensitive and can be regulated by posttranslational modifications induced by TPA-mediated ROS signaling. It is known that AP-1 activation can be detected as soon as 6 hours post-TPA treatment. Zhao et al. showed that by overexpressing MnSOD in human MnSOD transgenic mice, the initial activation of AP-1 was delayed and resulted in a significant reduction in papilloma formation [40]. When both nontransgenic and MnSOD transgenic mice were treated with DMBA/TPA, it was shown that JunD was the only family member whose expression was increased within 24 h of TPA treatment [40]. Another kinase found to be involved in AP-1 activity is c-Jun N-terminal kinase (JNK). JNK activity has been shown to increase more than threefold in malignant cell lines [37]. It was found that the increased phosphorylated form of JNK seen at 6 h post-TPA treatment in nontransgenic mice was delayed and reduced in MnSOD transgenic mice 24 h post TPA treatment. These results therefore suggest that MnSOD overexpression can affect TPA-induced AP-1 activation by modulating JNK kinase activity. Nevertheless, we have shown that the induction of endogenous antioxidant enzymes, particularly MnSOD, is efficient in reducing tumor incidence, as well as, mediators of proliferation [40].

5. Overexpression of MnSOD

As mentioned previously, overexpression of MnSOD has been shown to be anticarcinogenic in the two-stage skin carcinogenesis model. Overexpression of MnSOD not only reduced tumor multiplicity and incidence, but also modulated cell proliferative pathways such as AP-1signaling and DNA binding activity. In a large number of *in vitro* and *in vivo* models, and in even in gene-radiotherapy, MnSOD overexpression has been shown to suppress the malignant phenotype and metastatic ability of tumor cells. MnSOD is an attractive therapeutic target because of its high inducibility and subcellular mitochondrial localization. While various physiological stimuli have been shown to induce MnSOD expression such as cytokines, oxidative stress, and growth factors, the main function of MnSOD is to protect mitochondrial DNA from oxidative injury. Mitochondria are known as the powerhouse of the cell. Not only is mitochondria one of the main energy-generating organelles of the cell, but it is also considered one of the main generators of ROS. Oxygen radicals cannot only act as signaling molecules, but can also promote cell death, which is mainly mediated through the mitochondria. It is known that ROS can amplify the apoptotic cascade by expediting the release of mitochondrial cytochrome *c* via mitochondrial oxidative damage [42]. With MnSOD being an effective regulator of cellular redox status, this endogenous antioxidant enzyme can also provide cytoprotection from ROS-mediated apoptosis. However, the complexity of MnSOD expression and its involvement cancer progression still remains elusive. Zhao et al., in 2002, showed that a deficiency in MnSOD expression in MnSOD heterozygous knockout (MnSOD KO) mice enhanced cell proliferative signaling [42]. As previously mentioned, AP-1 signaling was suppressed via MnSOD overexpression. Surprisingly, the number of apoptotic cells increased as well, suggesting that MnSOD expression may not only play a role in tumor suppression, but may contribute to cell survival. MnSOD expression has been shown to be increased in various malignancies including human cervical carcinoma [43], brain malignant tumors [44], lung [45], gastric and colon cancers [46]. Consistent with that, *in vitro* experiments have shown that overexpression of MnSOD protects cells from ionizing radiation and in some cases induces resistance to chemotherapeutic drugs such as adriamycin [47]. Many investigators suggest that the survival mechanisms of MnSOD are mediated by hydrogen peroxide (H_2O_2) generation that overwhelms the cell capacity to regulate H_2O_2 accumulation, promoting cell survival and proliferative signaling. Nevertheless, further studies are needed to elucidate the H_2O_2-mediated mechanism.

6. MnSOD in Disease: Skin Cancer

Enzymatic inactivation is known to be associated with most pathological states of disease. With various mechanisms of inactivation, determining the mechanism of inactivation can be complex. Consistent with that, determining the benefits/damaging contributions of MnSOD is controversial, particularly in diseases such as skin carcinogenesis. Therefore, further investigation of activity/expression modulation in various disease states is needed to identify potential therapeutic targets. Previous studies from our lab have shown that ROS generation is increased in the early stages of skin carcinogenesis. It was found that NADPH oxidase was a key contributor to oxidative stress propagation in the DMBA/TPA two-stage skin carcinogenesis model. However, further studies showed that oxidative stress propagation induced p53 mitochondrial translocation. In our *in vitro* studies, skin epidermal JB6 P+ cells were treated with TPA, 10 minutes post-TPA treatment, the tumor suppressor p53, monitored by immunofluorescence staining, rapidly translocated to the mitochondria. Utilizing immunogold labeling, p53 was found localized on the outer membrane, and surprisingly in the mitochondrial matrix. With both MnSOD and p53 mitochondrial localization being key elements in cell fate, it was found via immunoprecipitation that mitochondrial p53 interacts with MnSOD within the mitochondria. Currently, there is growing controversy surrounding MnSOD's involvement in disease and most importantly the state of MnSOD expression, as well as its activity. Interestingly, following the p53-MnSOD interaction, MnSOD protein levels increased by 60%, whereas its activity decreased 11%, suggesting that MnSOD activity levels may play a more significant role in disease rather than expression. Subsequently following the reduction in MnSOD activity, the transcriptional activity of nuclear p53 was increased 1-hour post-TPA treatment, represented by an increase in the proapoptotic protein Bax, a transcriptional target of p53. In addition, the increase in p53 activation was associated with an increase in DNA fragmentation and apoptotic cell death. However, when treated with the MnSOD mimetic, MnTE-PyP^{5+}, mitochondrial p53 levels were slightly reduced, and p53 nuclear translocation and transactivation was completely blocked. Previous studies showed that TPA induced both cell proliferation and p53-mediated apoptosis. However, the involvement of MnSOD modulation in this process remained unsolved. The results from this study provide a link between mitochondrial redox status and nuclear regulation of apoptotic signaling and cell survival.

7. MnSOD As a Chemopreventive Agent

MnSOD is a highly inducible enzyme important to cell viability, but can also modulate cell proliferation and apoptotic signaling. Our lab utilized these mechanisms of action as a chemopreventive modality in skin carcinogenesis. Protandim, a dietary supplement consisting of 5 well-established medicinal plant extracts, has received increasing attention for itstherapeutic effects in various disease pathologies [47–49]. Protandim consists of *B. monnieri* (45% bacosides), 150 mg; *S. marianum* (70–80% silymarin), 225 mg; *W. somnifera* (1.5% withanolides), 150 mg; *C. sinensis* (98% polyphenols and 45% (−)-epigallocatechin-3-gallate), 75 mg. [50]. Antioxidants have known anticancer effects. However, several large clinical trials using small molecule antioxidants have failed, which poses several questions: Do antioxidant-based therapies contribute to disease progression by becoming prooxidants themselves? Are antioxidant-based therapies potent enough to overcome the ROS-generating load of various disease states? In general, cancer cells have a higher ROS generation status than normal cells. As a result, a number of antioxidant enzymes are significantly reduced in expression levels and activity. In addition, polymorphisms in MnSOD have been shown to be associated with a higher risk of prostate, breast, and other various cancers. However, MnSOD is the only antioxidant enzyme shown, when overexpressed, to reduce multicancer cell growth both *in vitro* and *in vivo* [51]. In other studies, small molecule catalytic antioxidant enzymes have been shown to be a more potent and practical approach for cancer chemoprevention. Sporn and Suh define chemoprevention as a pharmacological approach to intervention in order to arrest or reverse the process of carcinogenesis [52]. In our study, the two-stage mouse skin carcinogenesis model was used to investigate the mechanisms of action of Protandim during the early stages of skin carcinogenesis. Overall, the process of carcinogenesis is mediated by ROS generation and the ability of oxygen radicals to act as signaling molecules to modulate downstream carcinogenic events. We have found that the positive feedback loop that is formed by oxidative stress, cell proliferation and p53-mediated apoptosis plays a major role in contributing to carcinogenesis. Thus, it was postulated that the induction of MnSOD via Protandim could break this positive feedback cycle leading to cancer prevention. Mice utilized in this study were fed the Protandim diet during the tumor promotion stage (i.e., 2 weeks following DMBA initiation and 2 weeks prior to TPA treatment and for the duration of the study). Overall, no tumors were formed in the vehicle control/basal diet groups. However, both tumor incidence and multiplicity were reduced by 33% and 57%, respectively, in the Protandim diet group [53]. These results suggest that modulation of oxidative stress through the induction of antioxidant enzymes via dietary administration is sufficient in reducing tumor formation.

Nonetheless, oxidative stress alters both gene expression and cancer biology. Another key component of tumor progression is inflammation. Within the tumor microenvironment, various inflammatory cells release ROS and other inflammatory mediators. Tumors often utilize these pro-inflammatory mediators to foster cell proliferation, angiogenesis and metastasis [54]. Utilizing the two-stage model, we found that dietary administration of Protandim significantly decreased TPA-mediated macrophage infiltration, as well as, pro-inflammatory signaling pathways. For example, nuclear factor kappa B (NF-κB), a central regulator of immunity and inflammation, is a transcription factor of biological interest because of its sensitivity to the intracellular redox status. NF-κB regulates the expression

of numerous genes that encode selectins, cytokines, and cellular adhesion molecules. Oxidative stress generation is known to induce NF-κB nuclear translocation. Intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are transcriptionally regulated by NF-κB. Skin tissues from mice fed the Protandim diet exhibited reduced NF-κB DNA binding activity, resulting in a reduction in the protein expression levels of both cellular adhesion molecules. Therefore, these results suggest that Protandim not only suppresses tumor formation, but also mechanistically modulates pro-inflammatory signaling and the immune response via gene transcription.

As mentioned previously, p53 interacts with MnSOD following TPA-mediated oxidative stress generation. Therefore, is it possible for a dietary-mediated induction of MnSOD expression/activity to modulate p53 mitochondrial translocation and accompanying apoptosis? Skin tissues from DMBA/TPA treated mice were analyzed to assess the effects of the Protandim diet on p53 mitochondrial translocation [55]. Interestingly, skin tissues from Protandim-fed mice showed a significant decrease in mitochondrial p53 protein expression. Consistent with that, the number of apoptotic cells was also significantly decreased. Thus, the induction of antioxidant enzymes via dietary administration of Protandim modulates both TPA-mediated cell proliferation and p53-mediated apoptotic signaling. Therefore, it can be concluded that oxidative stress forms a mechanistic linkage between cell proliferation, inflammation, and apoptosis, suggesting that potent multimodal antioxidant inducers may potentially be utilized with conventional chemotherapeutics.

8. Conclusion

For many decades, ROS generation has been known to not only cause oxidative injury, but also act as signaling molecules that regulate cell proliferation and downstream gene expression. However, the induction of MnSOD is gaining interest as an effective novel mechanism of chemoprevention, being that it is the only antioxidant enzyme that when overexpressed suppresses tumor formation. MnSOD also has the ability to modulate multiple pathways contributing to skin carcinogenesis. Continuous efforts are currently being made to develop compounds that effectively induce MnSOD in hopes to incorporate antioxidant-based therapies into current clinical practice. Therefore, the development of various MnSOD inducers to be used during the early-onset of tumorigenesis may be a plausible modality utilized to suppress underlying mechanisms involved in carcinogenesis.

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Review Article

The Role of Manganese Superoxide Dismutase in Inflammation Defense

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Antioxidant enzymes maintain cellular redox homeostasis. Manganese superoxide dismutase (MnSOD), an enzyme located in mitochondria, is the key enzyme that protects the energy-generating mitochondria from oxidative damage. Levels of MnSOD are reduced in many diseases, including cancer, neurodegenerative diseases, and psoriasis. Overexpression of MnSOD in tumor cells can significantly attenuate the malignant phenotype. Past studies have reported that this enzyme has the potential to be used as an anti-inflammatory agent because of its superoxide anion scavenging ability. Superoxide anions have a proinflammatory role in many diseases. Treatment of a rat model of lung pleurisy with the MnSOD mimetic MnTBAP suppressed the inflammatory response in a dose-dependent manner. In this paper, the mechanisms underlying the suppressive effects of MnSOD in inflammatory diseases are studied, and the potential applications of this enzyme and its mimetics as anti-inflammatory agents are discussed.

1. Introduction

Aerobic organisms utilize molecular oxygen (dioxygen; O_2) as the final electron receptor in the oxidative phosphorylation electron transport chain. Normally, O_2 is reduced to H_2O after receiving four electrons; however, partial reduction of O_2 leads to the formation of highly reactive oxygen species (ROS), including the superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$). ROS can damage lipids, proteins, and DNA, leading to aberrant downstream signaling or stimulation of apoptosis [1, 2]. Oxidative stress has been implicated in neurodegenerative diseases, aging, cancer, pulmonary fibrosis, and vascular diseases [3–6]; elimination of unwanted ROS is, therefore, very important for organismal survival. To confront oxidative stress caused by ROS, organisms have evolved a variety of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. However, ROS can also act as cell signaling molecules and cause damage to foreign bodies [2]. ROS are, therefore, double-edged swords with respect to biological processes.

Inflammation is a host defense response to infectious agents, injury, and tissue ischemia. Inflammation occurs because of lymphocyte and macrophage invasion and the secretion of mediators of inflammation such as cytokines, cyclooxygenase products, and kinins [7]. Inappropriate inflammation is a hallmark of various diseases. A large body of evidence suggests that antioxidant enzymes are key regulators of inflammation. Manganese superoxide dismutase (MnSOD) is an enzyme present in mitochondria that is one of the first in a chain of enzymes to mediate the ROS generated by the partial reduction of O_2. MnSOD has been implicated in a number of oxidative stress-related diseases. In this paper, we will discuss the role of MnSOD in various inflammation-associated diseases and explore the therapeutic potentials of agents that regulate its expression.

2. Regulation of MnSOD

MnSOD mRNA levels can be upregulated by several factors: LPS [8], cytokines such as TNF [9], IL-1 [10], and

VEGF [11], UVB irradiation, ROS [12], and thioredoxin [13]. The human MnSOD gene (*sod2*) has a housekeeping promoter with multiple copies of Sp-1- and AP-2- binding sequences. The promoter region also contains a GC-rich region and NF-κB transcription regulation elements [14]. Several enhancers are also present in the promoter region and in the second intron [15]. TNF and IL-1 inductions of *sod2* mRNA require a 238-bp TNF response element (TNFRE), which is located in intron 2. Both C/EBP and NF-κB bind to the TNFRE enhancer to interact with the *sod2* promoter, resulting in the upregulation of MnSOD transcription [9]. TPA-induced MnSOD expression is due to the transcription factor specificity protein 1- (SP1-) mediated PKC signaling [16]. Dimeric SP1 can bind to GC-rich sequences of GGGCGG, but the binding affinity and transcription properties vary according to the interacting cofactors [17–19].

The downregulation of mRNA levels is as important in biological processes as is upregulation. Because ROS can act as intracellular secondary messengers, maintaining proper levels of these molecules is important for normal cellular function. This suggests that antioxidant enzymes are likely maintained at low levels in cells.

Many studies have reported the downregulation of MnSOD mRNA levels in disease states. Many tumor cell lines have mutations in the promoter region of the MnSOD gene that increase the number of AP-2-binding sites. AP-2 can interact with SP-1 within the promoter region and decrease promoter activity, thus downregulating transcription [17]. VEGF can upregulate MnSOD mRNA levels through the ROS-sensitive PKC-NF-κB and PI3K-Akt-Forkhead signaling pathways [11]. FOXO3a is a member of the Forkhead family of transcription factors. Phosphorylation of Ser253 of FOXO3a decreases DNA binding and consequently gene expression, which results in the age-related activation of Akt [18].

Aging-related disorders are often associated with oxidative stress. Epigenetic silencing of the MnSOD gene has also been observed in human breast cancers. Both DNA methylation and histone modification contribute to this regulation [19]. Epigenetic modification influences the abilities of SP1, AP-1, and NF-κB to bind to cis-elements in the promoter region of the MnSOD gene, resulting in silencing of this gene.

MnSOD mRNA upregulation always results in increased levels of MnSOD protein [20]. MnSOD is located in mitochondria; therefore, its major role appears to be controlling the levels of $O_2^{-\cdot}$ in mitochondria. H_2O_2 is a product of MnSOD-catalyzed reactions; increased MnSOD activity results in H_2O_2 accumulation. H_2O_2 can act as a second messenger or as a Fenton reaction agent, thereby causing damage to cells. To elucidate the significance of MnSOD regulation, the function of MnSOD must be considered.

3. The Function of MnSOD

In cancer cells, MnSOD is almost always suppressed by certain transcription factors or through epigenetic modification of cis-elements or chromatin. Overexpression of MnSOD in cancer cells can alter the phenotype in culture; the cells lose the ability to form colonies, a trait characteristic of malignant cells [21]. A large number of studies have reported that ROS play an important role in tumor metastasis [22, 23]. ROS can activate cell signaling pathways and/or mutate DNA, thereby promoting tumor proliferation and metastasis. This may explain why tumor cells almost always express MnSOD at low levels. Exogenous MnSOD can block ROS signaling to inhibit tumorigenesis, suggesting that MnSOD may be a potential antitumor therapeutic target. Overexpression of MnSOD can enhance the activity of the superoxide-sensitive enzyme aconitase and inhibit pyruvate carboxylase activity, thereby altering the metabolic ability of the cell and inhibiting cell growth [24].

A mouse knockout model of manganese superoxide dismutase has proven to be a useful model for elucidating the function of MnSOD. As stated previously, the major function of MnSOD is to protect mitochondria from ROS damage. However, although ROS can damage organisms, they are also mediators of cell signaling. Developing mice fetuses lacking manganese superoxide dismutase do not survive to birth; overexpressions of other types of SOD cannot attenuate this symptom [25]. Newlyborn MnSOD knockout mice have extensive mitochondrial injuries in multiple tissues. Disorders such as Leigh's disease and Canavan disease are characterized by mitochondrial abnormalities. Reductions in the levels of a variety of energy metabolism enzymes, especially those with a role in the TCA cycle, have also been noted in these disorders. Treatment of *Sod2*$^{\text{tm1cje}}$(−/−) mutant mice with the manganese superoxide dismutase mimetic manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) improved these mice and dramatically prolonged their survival times [26–28].

Miki et al. studied the cytological differences between wild-type mice and heterozygous *sod2* knockout (*sod2* −/+) mice after permanent focal cerebral ischemia (FCI). Cytochrome c accumulated at an early stage and was significantly more elevated in *sod2* −/+ mice than it was in wild-type mice. A remarkable increase in DNA laddering was also observed in the *sod2* −/+ mice but not in the wild-type mice, suggesting that MnSOD can block the release of cytosolic cytochrome c and prevent apoptosis [29]. Neurotoxins such as 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), 3-nitropropionic acid (3-NP), and malonate are commonly used in neurodegenerative functional models. Mice with a partial deficiency in MnSOD are more sensitive to these mitochondrial toxins than are normal mice [30], suggesting that MnSOD is an antitoxin agent that scavenges free radicals generated by environmental toxins that may cause neurodegeneration.

MCF-7 human carcinoma cells exposed to single-dose radiation and radioresistant variants isolated from MCF-7 cells following fractionated ionizing radiation (MCF and FIR cells) were found to possess elevated MnSOD mRNA levels, activity, and immunoreactive proteins. MnSOD-silenced cells were sensitive to radiation. The genes P21, Myc, 14-3-3 zeta, cyclin A, cyclin B1, and GADD153 were overexpressed

in both MCF + FIR and MCF + SOD cells (MCF-7 cells overexpressing MnSOD). These genes were suppressed in *Sod2* knockout mice (−/−) and in MnSOD-silenced cells [31]. These six genes are survival genes [32–34] that protect cell from radiation-induced apoptosis.

4. MnSOD in Diseases with Inflammation

Inflammation is a complex response to harmful stimuli, such as tissue injury, pathogens, autoimmune damage, ischemia and other irritants [35]. Numerous inflammation-associated molecules and cells remove injurious stimuli and repair damaged tissues. The healing process includes the destruction of "foreign objects" and the repair of injured self-tissues. If targeted destruction and associated repair are not correctly programmed, inflammatory disorders resulting in diseases such as psoriasis, inflammatory bowel disease, and neurodegenerative diseases develop [36, 37]. Superoxide anions have proinflammatory roles, causing lipid peroxidation and oxidation, DNA damage, peroxynitrite ion formation, and recruitment of neutrophils to sites of inflammation [38–40]. Elimination of superoxide anions by MnSOD and its isoenzymes can, therefore, be considered to be anti-inflammatory (Figure 1).

Inflammatory bowel disease (IBD) is accompanied by the excessive productions of reactive oxygen and nitrogen metabolites [41]. The concentration of malondialdehyde (MDA), which can serve as an index for lipid peroxidation, was found to be increased in inflamed mucosa cells [42]. Lipid peroxidation is associated with hydroxyl radicals and superoxide anions. In inflamed cells, levels of MnSOD are suppressed relative to those of normal cells, indicating that MnSOD may be a therapeutic target. NOD2 is a susceptibility gene for IBD; the NOD2 protein can activate the immune system by triggering NF-κB and can negatively regulate the Toll-like receptor-mediated T-helper type 1 response, thereby increasing susceptibility to infection [43, 44]. The pathology of IBD requires further investigation. Currently, drugs targeting NF-κB or ROS have been found to be somewhat effective.

The skin is the largest organ of the human body and acts as a physical boundary to protect the internal organs against the environment. Skin dysfunction could result in injury to deeper tissues. Skin injuries can activate the acute inflammatory response, and infection can heighten this response. Psoriasis is a chronic disease characterized by inflamed, scaly, and frequently disfiguring skin lesions. Epidermal keratinocytes in this disease show altered differentiation and hyperproliferation, and immune cells such as T-cells and neutrophils are present at lesion sites [45]. JunB is a component of the AP-1 transcription factor complex that regulates cell proliferation, differentiation, the stress response, and cytokine expression [46]. Both JunB and c-Jun are highly expressed in lesional skin, but levels of JunB have been shown to be low in severe psoriasis and intermediate in mild psoriasis, while c-Jun is expressed in the opposite manner [47].

Most components of the AP-1 transcription factor are redox-sensitive proteins regulated by ROS signaling. Exposure of keratinocytes to chemical irritants, allergens, or inflammatory stimuli triggers activation of several stress-sensitive protein kinases that are mediated by ROS. ROS enhance EGFR phosphorylation and activate ERKs and JNKs [48]. ROS also activate NF-κB during skin inflammation. These findings indicate that antioxidant enzymes may have potential as therapeutic agents.

MnSOD was found to be highly expressed in psoriasis, but this expression was not associated with the pathology of psoriasis [49]. A reasonable hypothesis is that lesional skin cells are induced to express MnSOD by cytokines released from inflammatory cells in order to counteract inflammation-induced oxidative stress. Although native MnSOD has shown promising anti-inflammatory properties against many diseases in both preclinical and clinical studies, there are several drawbacks to using native MnSOD as a therapeutic agent and pharmacological tool. Low molecular weight mimetics of SOD were, therefore, developed to address some of the drawbacks of native SOD use.

To date, frequently used SOD mimetics are MnTBAP, the Mn(III)-salen complex, and Mn II-pentaazamacrocyclic ligand-based SOD mimetics [50]. In a mouse model of lung pleurisy, treatment with MnTBAP before carrageenan administration was found to suppress inflammatory responses in a dose-dependent manner [51]. The mechanism of attenuation of inflammation by SOD mimetics is the reduction of peroxynitrite formation through the elimination of superoxide anions before they react with nitric oxide. Because peroxynitrites are numerous and have pro-inflammatory and cytotoxic effects, administration of SOD mimetics is clinically very important. M40403 (Figure 2) was derived from 1,4,7,10,13-pentaazacyclopentadecane containing added bis(cyclohexylpyridine) functionalities. It is the best products achieved high stability and catalytical activity based on the computer-aided design. M40403 gets a high specificity for scavenging superoxide anion, while other oxidants, such as hydrogen peroxide, peroxynitrite, and hypochlorite, are hardly oxidative to Mn-II packaged in the complex. The biological function of M40403 has been tested in several models [52]. The global mechanism seems that M40403 could block nitrosation of tyrosine in proteins, indicating that superoxide anion driven formation of peroxynitrite might be responsible for the nitrosation. While increasing evidences are suggesting that nitrosation plays important role in many inflammation-related diseases [50, 53], this low molecular mass synthetic is a potential therapeutic agent for curing inflammation.

5. Conclusions

Inflammation is a traditional but complex problem that still requires extensive investigation. ROS play a very important role in the triggering and promotion of inflammation. Thus, antioxidant enzymes that can function as ROS scavengers are ideal therapeutic agents. Data generated from mouse models have shown that native MnSOD has anti-inflammatory properties but also some practical disadvantages. MnSOD mimetics were, therefore, developed to address the shortcomings of native MnSOD. These low molecular weight

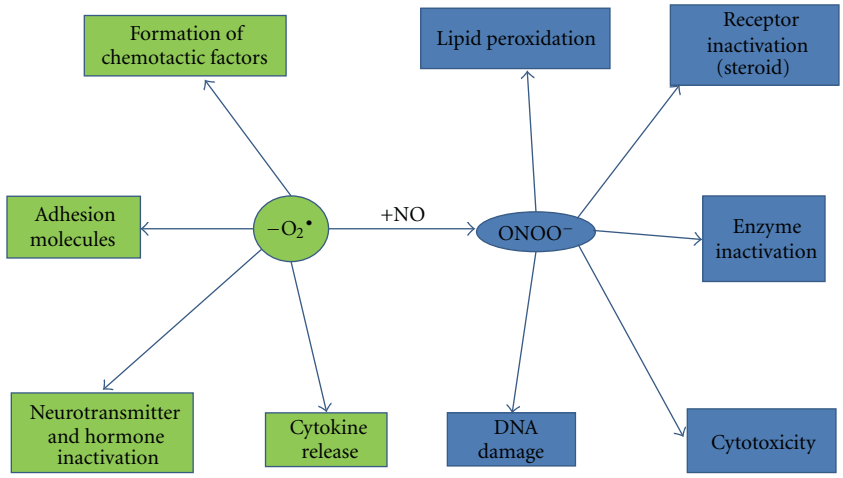

Figure 1: Biological basis and effects of superoxide generation. Excessive production of superoxide anions can lead to inflammation through many pathways, such as generation of peroxynitrite.

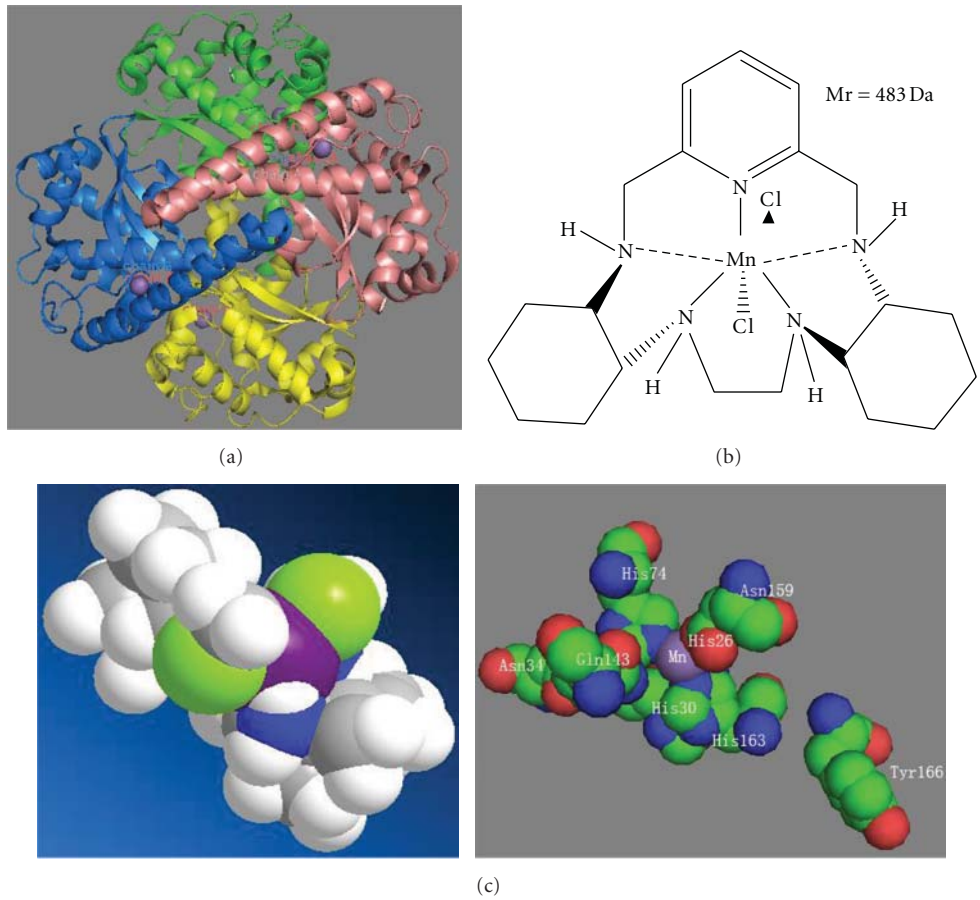

Figure 2: The three-dimensional (3D) structure of human manganese superoxide dismutase (a) and that of the synthetic superoxide dismutase mimetic M40403 (b). The 3D structure of M40403 and the active site of MnSOD (c). This manganese-containing biscyclohexylpyridine has superior catalytic activity compared to that of the native enzyme. Note that Mn^{2+} is purple and Cl^- is light green in the 3D structure of M40403.

molecules have been tested in several *in vivo* and *in vitro* models; they have all been shown to be effective mimics of SOD. Despite the great achievements made over the past few decades, however, there is still a need to develop even more efficient and compatible anti-inflammatory agents suitable for clinical pharmaceutical therapy.

Abbreviations

MnSOD: Manganese superoxide dismutase;
ROS: Reactive oxygen species;
MnTBAP: Manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Inhibitory Effect of Phthalic Acid on Tyrosinase: The Mixed-Type Inhibition and Docking Simulations

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Tyrosinase inhibition studies are needed due to the medicinal applications such as hyperpigmentation. For probing effective inhibitors of tyrosinase, a combination of computational prediction and enzymatic assay via kinetics was important. We predicted the 3D structure of tyrosinase, used a docking algorithm to simulate binding between tyrosinase and phthalic acid (PA), and studied the reversible inhibition of tyrosinase by PA. PA inhibited tyrosinase in a mixed-type manner with a $K_i = 65.84 \pm 1.10$ mM. Measurements of intrinsic and ANS-binding fluorescences showed that PA induced changes in the active site structure via indirect binding. Simulation was successful (binding energies for Dock6.3 $= -27.22$ and AutoDock4.2 $= -0.97$ kcal/mol), suggesting that PA interacts with LEU73 residue that is predicted commonly by both programs. The present study suggested that the strategy of predicting tyrosinase inhibition based on hydroxyl groups and orientation may prove useful for screening of potential tyrosinase inhibitors.

1. Introduction

Melanogenesis is a complex mechanism for melanin pigment production. Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1) plays a central role in melanin synthesis as a copper-binding metalloenzyme. It is ubiquitously distributed in organisms and has multi catalytic functions such as the hydroxylation of tyrosine to DOPA, the oxidation of DOPA to DOPAquinone, and the oxidation of 5,6-dihydroxyindole [1–3]. Besides the catalytic features, tyrosinase is distinctive from the other enzymes as it displays various inhibition patterns. Several previous studies suggest [4–6] that tyrosinase can induce neurotoxicity by activating apoptotic stress signaling pathways and is directly associated with a neurotoxic overproduction of cellular dopamine. It implies that tyrosinase gene expression results in oxidative metabolites and reactive oxygen species at the cellular level which are known to have cytotoxic effects.

Tyrosinase is directly related with severe skin diseases such as type 1 albinism (http://albinismdb.med.umn.edu/) and melanoma [7–9]. Mutations of tyrosinase gene can cause severe skin disease such as oculocutaneous albinism (OCA). Three types of OCA—tyrosinase-negative, yellow-mutant, and temperature-sensitive OCA—have been well known [10, 11], and new subtypes of albinism have been reported [12]. Tyrosinase is also involved in the skin hyperpigmentation and in this regard, tyrosinase inhibition study is directly associated with the treatment of melanin overproduction [13–15].

The tyrosinase mechanism is complex, in that this enzyme can catalyze multiple reactions. The crystallographic structure of tyrosinase has not been determined; thus, the overall 3D structure and architecture of the active site are not well understood regardless of several reports [16–18]. Studies of this enzyme mechanism must involve a variety of computational methods and kinetics to derive the structure-function relationships, for example, between substrates and ligands of the enzyme.

In the current study we determined the mechanism of tyrosinase inhibition by PA using computational simulation and kinetic analysis. We hypothesized that the two hydroxyl groups of PA may block L-DOPA oxidation by binding to tyrosinase. Previous findings have shown the importance of

hydroxyl groups in tyrosinase inhibition [19–22] in terms of molecular position, number, and specific interactions with the enzyme; these findings further support our hypothesis. PA has two hydroxyl groups, thus implying that PA might have an inhibitory effect on tyrosinase. Direct use of PA in medicinal or cosmetic applications is limited due to the toxicity; however, effective approaches derived from the combination of computational simulation and enzymatic kinetics as an example case are of interest for further screening tyrosinase inhibitor candidates. The computational simulation suggested that PA could be a potent inhibitor for tyrosinase where PA can directly interact with some residues locating near to the active site. Experimentally, PA exerts a mixed type of inhibition on tyrosinase. Kinetic parameters have consistently supported the result of docking simulation, and measurements of ANS-binding fluorescence have revealed changes in the regional structure. A combination of inhibition kinetics and computational modeling may facilitate testing of potential tyrosinase inhibitors, such as PA and prediction of the inhibitory mechanisms.

2. Materials and Methods

2.1. Materials. Tyrosinase (M.W. 128 kDa), L-DOPA, and PA were purchased from Sigma-Aldrich (Seoul, Korea). When L-DOPA was used as a substrate in our experiments, the purchased tyrosinase had a K_m of 0.30 ± 0.02 mM ($V_{\max} = 0.13 \pm 0.05$ mmol·min^{-1}) according to a Lineweaver-Burk plot. All kinetic reactions and measurements in this study were performed in 50 mM sodium phosphate buffer (pH 6.9).

2.2. Tyrosinase Assay and Kinetic Analysis for the Mixed-Type Inhibition. A spectrophotometric tyrosinase assay was performed as previously described [23, 24]. To begin the assay, a 10-μL sample of enzyme solution was added to 1 mL of reaction mix. Tyrosinase activity (v) was recorded as the change in absorbance per min at 492 nm using a Perkin Elmer Lambda Bio U/V spectrophotometer. To describe the mixed-type inhibition mechanism, the Lineweaver-Burk equation in double reciprocal form can be written as

$$\frac{1}{v} = \frac{K_m}{V_{\max}}\left(1 + \frac{[\mathrm{I}]}{K_i}\right)\frac{1}{[\mathrm{S}]} + \frac{1}{V_{\max}}\left(1 + \frac{[\mathrm{I}]}{\alpha K_i}\right). \quad (1)$$

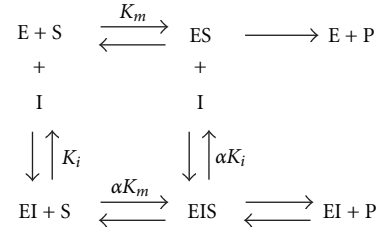
Secondary plots can be constructed from

$$\text{Slope} = \frac{K_m}{V_{\max}} + \frac{K_m[\mathrm{I}]}{V_{\max}K_i}, \quad (2)$$

$$Y\text{-intercept} = \frac{1}{V_{\max}^{\mathrm{app}}} = \frac{1}{V_{\max}} + \frac{1}{\alpha K_i V_{\max}}[\mathrm{I}]. \quad (3)$$

Then, the α, K_i, K_m, and $V_{\max}$ values can be derived from the above equations. The secondary replot of Slope or Y-intercept versus [I] is linearly fitted, assuming a single inhibition site or a single class of inhibition site, as shown in Scheme 1.

2.3. Intrinsic and ANS-Binding Fluorescence Measurements. Fluorescence emission spectra were measured with a Jasco FP750 spectrofluorometer using a cuvette with a 1-cm path length. Tryptophan fluorescence was measured following excitation at 280 nm, and the emission wavelength ranged between 300 and 410 nm. Changes in the ANS-binding fluorescence of tyrosinase were measured following excitation at 390 nm, and the emission wavelength ranged from 400 to 520 nm. The tyrosinase was labeled with 40 μM ANS for 30 min prior to measurements.



Scheme 1

2.4. Determination of the Binding Constant and the Number of Binding Sites. According to a previous report [25], when small molecules are bound to equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the following equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{n} + \frac{1}{K}\frac{1}{[Q]}, \quad (4)$$

where F_0 and F are the relative steady-state fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the quencher (PA) concentration. The values for the binding constant (K) and number of binding sites (n) can be derived from the intercept and slope of a plot based on (4).

2.5. Homology Modeling of Tyrosinase and Docking Simulations. The 3D structure of tyrosinase from *Agaricus bisporus* was modeled using the SWISS-MODEL [26] to assemble 556 amino acids (Protein CAA11562) selected with a homology-modeling protocol. This method differs from those described in previous reports [17, 18]. We retrieved the known homologues of tyrosinase (average, 23% sequence identity), as well as partial tyrosinase homologues, from the Protein Data Bank (PDB) (http://www.pdb.org/) and identified a PDB entry (2zmx chain A) to provide a suitable structural template. Based on the sequence alignment, the 3D structure of tyrosinase was constructed with a high level of confidence (final total energy, 38297.020 KJ/mol).

Among the many tools available for protein-ligand docking, Dock6.3 and Autodock4.2 programs were applied because of their automated capability [27]. The program uses a set of predefined 3D grids of the target protein with a systematic search technique [28]. The original structure of PA was derived from the PubChem database (Compound ID: 1017, http://pubchem.ncbi.nlm.nih.gov/). To prepare for the docking procedure, the following steps were taken: (1) conversion of 2D structures to 3D structures; (2)

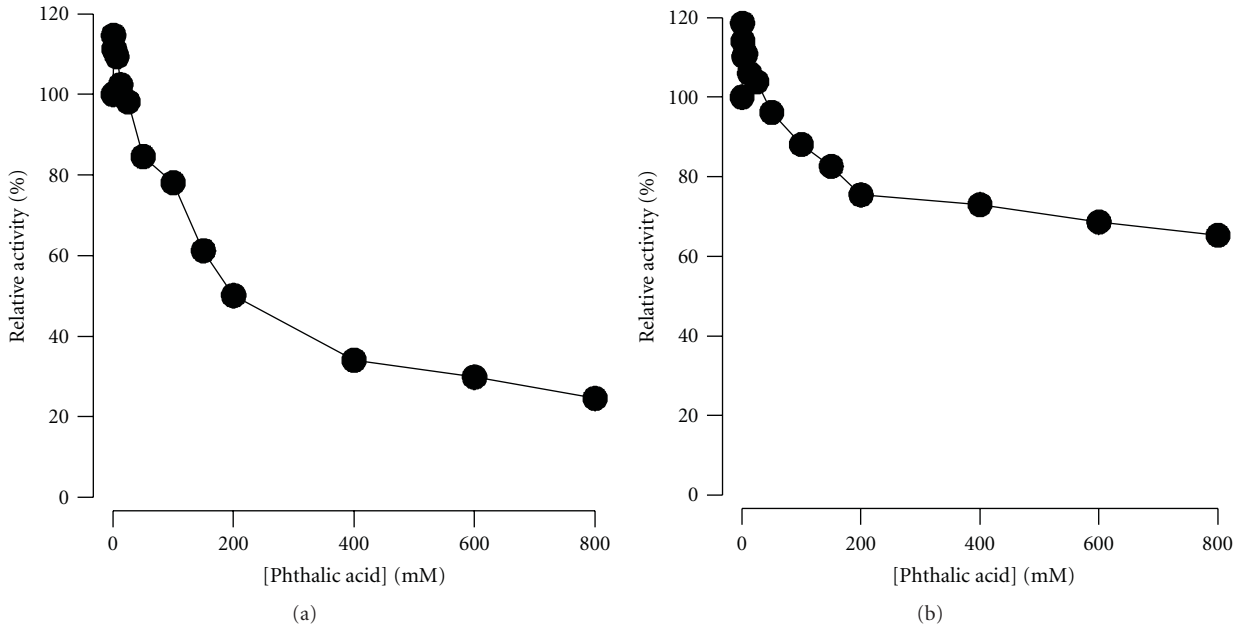


Figure 1: Inhibitory effect of PA on tyrosinase. Data are presented as the means ($n = 3$). Tyrosinase was incubated with PA at various concentrations for 3 h at 25°C and then added to the assay system at the corresponding PA concentrations (a) or in the absence of PA. (b) The final concentrations of L-DOPA and tyrosinase were 2 mM and 2.0 μg/mL, respectively.

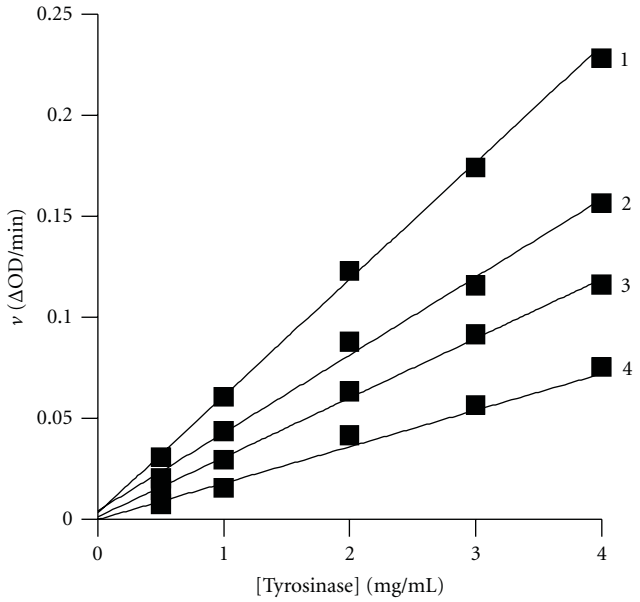


Figure 2: Plots of v versus [E]. The v value indicates the change in absorbance at 492 nm per minute at PA concentrations of 0, 100, 200, and 400 mM (labels 1 to 4, resp.). The final L-DOPA concentration was 2 mM.

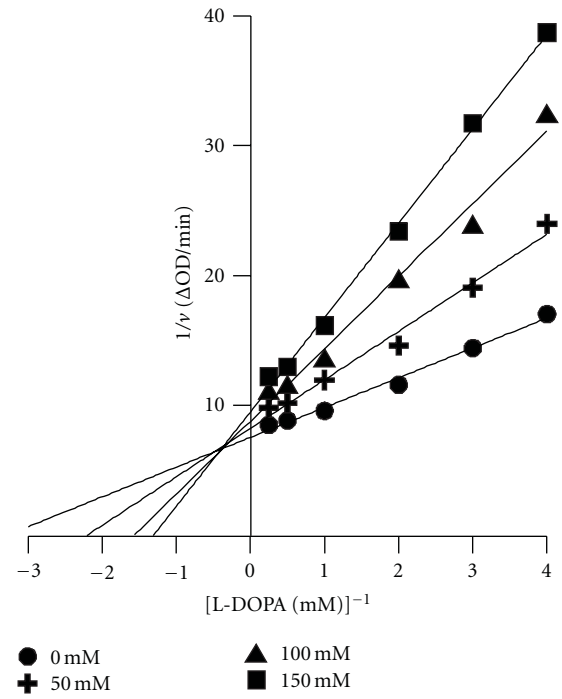


Figure 3: Lineweaver-Burk plot. (a) The PA concentrations were 0 (•), 50 (+), 100 (▲), and 150 mM (■). (b) The final enzyme concentration was 2.0 μg/mL.

calculation of charges; (3) addition of hydrogen atoms; (4) location of pockets. For these steps, we used OpenEye (http://www.eyesopen.com/).

3. Results

3.1. Effect of PA on Tyrosinase Activity. We assayed tyrosinase activity changes in the presence of PA. Tyrosinase activity was conspicuously inactivated by PA in a dose-dependent manner with an IC_{50} of 200 ± 5.0 mM ($n = 3$), where PA was present both in reaction and assay buffers (Figure 1(a)). At less than 50 mM PA, tyrosinase was slightly activated up to 18% and, then, gradually inactivated by increasing PA concentration (Figure 1(a)). When PA was removed from

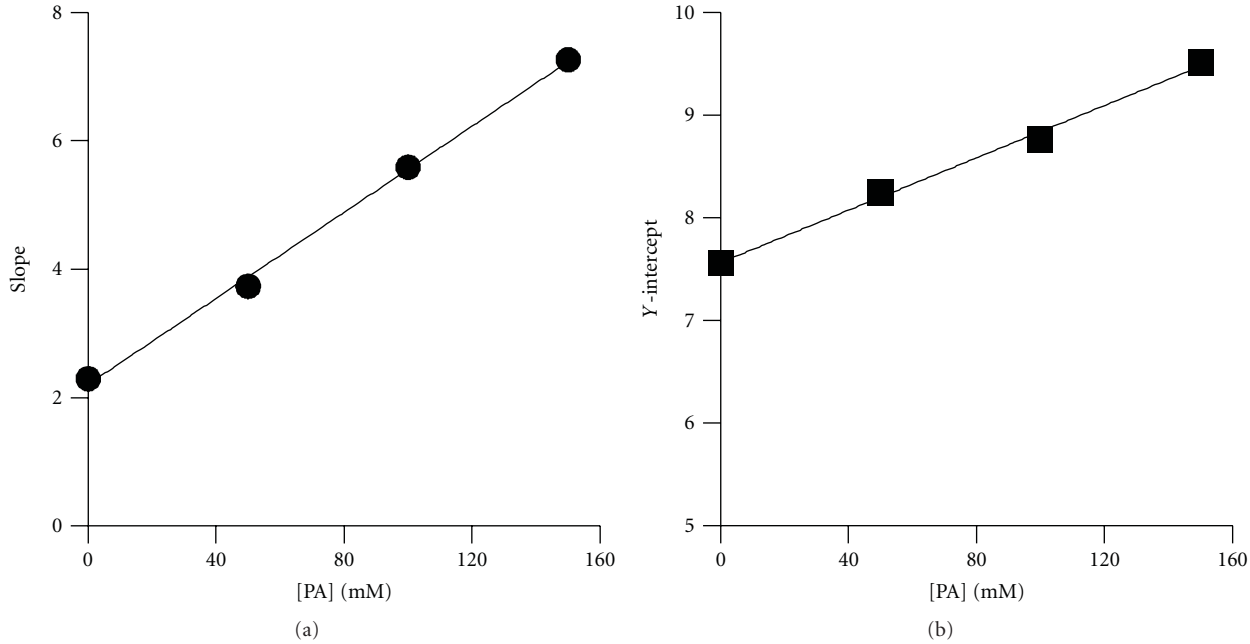


Figure 4: Secondary replots. (a) Plot of Slope versus [PA]. All data were collected from Lineweaver-Burk plots. The replot was plotted based on (2). (b) Plot of Y-intercept versus [PA]. The replot was plotted based on (3).

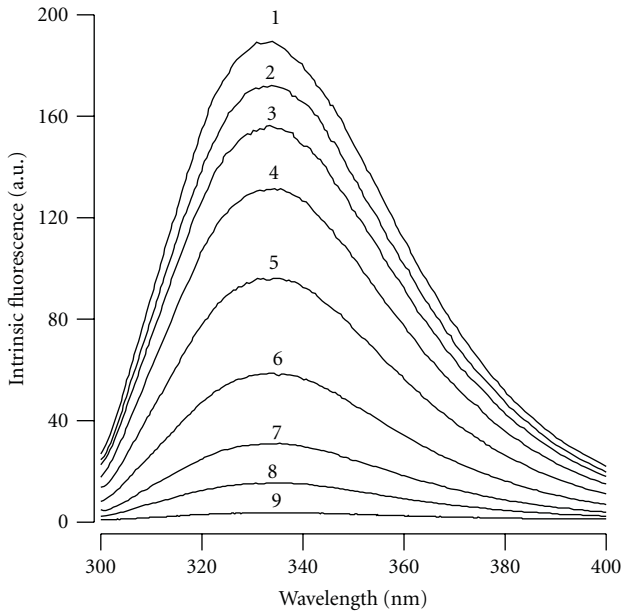


Figure 5: Intrinsic fluorescence changes by PA. Tyrosinase was incubated with PA for 3 h before measurements. Label 1 represents the native state. Labels 2 through 9 indicate PA concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 400 mM, respectively.

the assay buffer, the residual activity was higher than 60% even at 800 mM PA ($n = 3$), implying that PA reversibly binds to tyrosinase (Figure 1(b)). The phenomenon of slight activation by PA at the low concentration was also observed in Figure 1(b).

To confirm the reversibility of PA-mediated inhibition, plots of the remaining activity versus [E] were constructed (Figure 2). The results showed straight lines passing through the origin, indicating the PA-mediated reversible inhibition, as predicted in results of Figure 1.

3.2. Lineweaver-Burk Plot Analysis of Tyrosinase Inhibition by PA. We adapted Lineweaver-Burk plot analysis to elucidate inhibition type and mechanism of PA on tyrosinase. The results showed changes in both the apparent $V_{\max}$ and the K_m, indicating that PA induced a mixed type of inhibition (Figure 3). The secondary replots of Slope versus (PA) and Y-intercept versus (PA) were linearly fitted (Figures 4(a) and 4(b)), showing that PA has a single inhibition site or a single class of inhibition site on tyrosinase. Using (1)–(3), the α-value was calculated to be 9.05 ± 1.4 ($n = 2$), and the K_i was 65.84 ± 1.10 mM ($n = 2$).

3.3. Effect of PA on Tyrosinase Tertiary Structure Spectrofluorimetry Studies. Next, we measured the intrinsic fluorescence changes of tyrosinase in the presence of PA. We found that the intrinsic fluorescence of tyrosinase is changed significantly accompanying a quenching effect, which gradually decreased with no significant shift of the maximum peak wavelength (Figure 5). At 400 mM, PA completely quenched the fluorescence. For calculating binding affinity, a double reciprocal plot was evaluated according to (4) as shown in Figure 6. The result revealed a linear relationship, we calculated the binding constant to be $K = 0.068 \pm 0.03\ \text{mM}^{-1}$, and the binding number was $n = 1.01 \pm 0.65$ by using (4).

To compare the intrinsic fluorescence result and further to elucidate the changes in tyrosinase hydrophobicity due to alterations of the active site shape by PA, the ANS-binding fluorescence changes were monitored in the presence

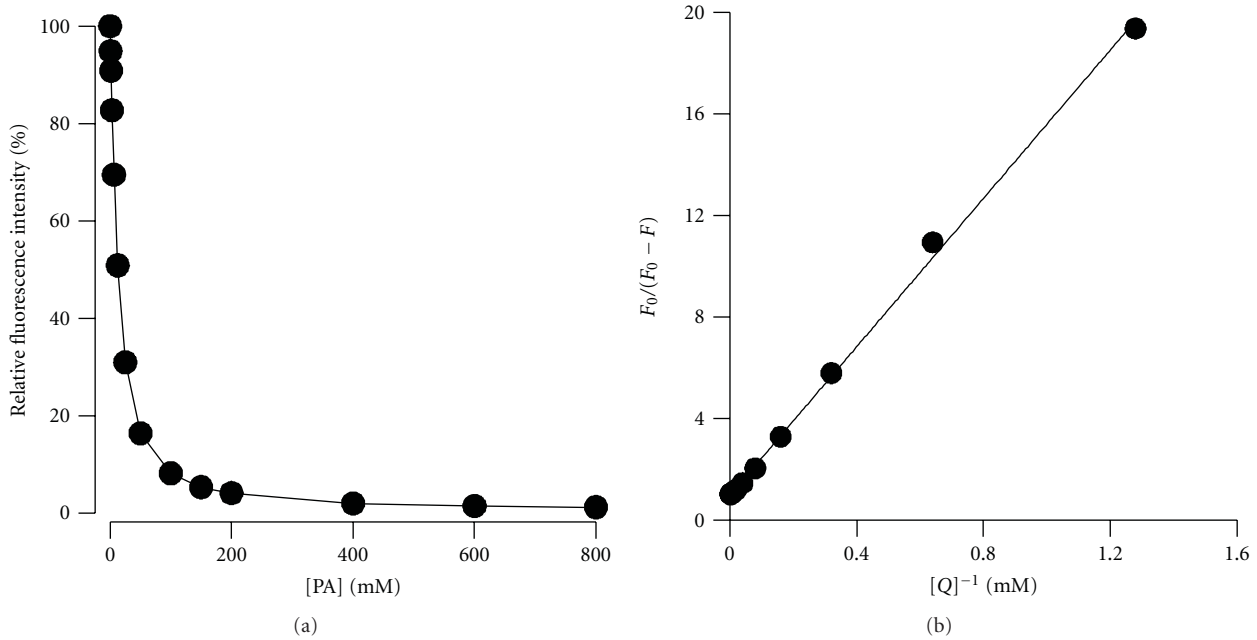


Figure 6: Plot of maximum fluorescence intensity versus [PA]. (a) Tyrosinase was incubated with various concentrations of PA (1.56 to 800 mM). (b) Double reciprocal plot of $F_0/(F_0 - F)$ versus $[Q]^{-1}$. Data was treated according to (4). F_0 maximum native fluorescence intensity; F maximum fluorescence intensity of sample; Q quencher PA.

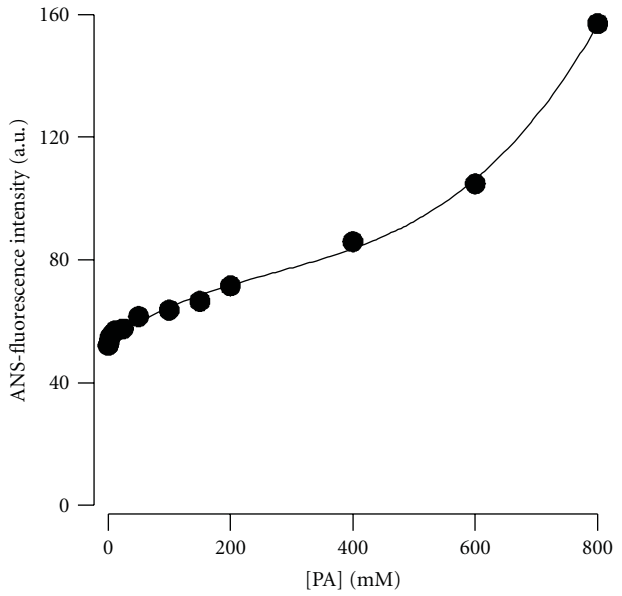


Figure 7: Changes in ANS-binding fluorescence of tyrosinase at different PA concentrations. ANS (40 μM) was incubated with tyrosinase for 30 min to label the hydrophobic enzyme surfaces prior to fluorescence measurements. Data are presented as the means ($n = 2$).

of PA (Figure 7). PA gradually increased the ANS-binding fluorescence of tyrosinase in a dose-dependent manner, an indication that binding to inhibitor exposed the hydrophobic surfaces within the tyrosinase, which might be mainly caused from the active site.

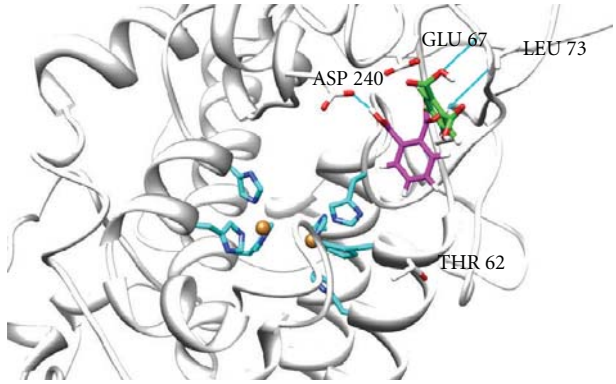


Figure 8: Computational docking simulation of binding between tyrosinase and PA. The modeled 3D structure of tyrosinase using SWISS-MODEL to assemble 556 amino acids selected with a homology-modeling protocol. Two copper ions (balls) coordinated with six histidines, as indicated the blue colors in the figure. The pink stick is PA as docked by Dock6.3. The green stick is PA as docked by AutoDock4.2.

3.4. Computational Prediction of 3D Tyrosinase Structure and Docking Simulation of PA Binding. Because the crystallographic structure of tyrosinase is not available, we selected a template structure from the PDB (2zmxA) to simulate the 3D structure of tyrosinase. In the predicted structure of tyrosinase, a binding pocket is indicated with two coppers: one is coordinated to HIS38, HIS54, and HIS63, and the other is coordinated to HIS190, HIS194, and HIS216, respectively (Figure 8). The docking simulation of binding between PA and tyrosinase was successful in producing a significant

score (binding energy for Dock6.3 was −27.22 kcal/mol). We searched for PA-binding residues within tyrosinase that were close to each other and found the most important residues at THR62, GLU67, LEU73, and ASP240 predicted from Dock6.3. In the same way, AutoDock4.2 was also applied to probe docking sites. As a result, PA-binding residues were predicted as GLU67 and LEU73 with a relatively low score (binding energy for AutoDock4.2 was −0.97 kcal/mol). We found that LEU73 has been commonly predicted from both programs. The docking simulations provided informative data for the PA as a tyrosinase inhibitor by identifying binding residues near to active site pocket, which might directly affect the L-DOPA substrate docking and catalysis by inducing regional structure changes.

4. Discussion

Previous studies have recognized an apparently potent inhibitory effect of compounds with hydroxyl groups on tyrosinase [29–32]. In this context, we hypothesized that PA could be a potent tyrosinase inhibitor due to the structural aspect with two hydroxyl groups. To confirm our supposition, we simulated the docking between PA and tyrosinase and conducted kinetic studies. As a result, we found that PA was directly involved with tyrosinase inhibition not via copper chelation but via mixed-type manner. Experimentally, our kinetic studies consistently confirmed all results and the model described in Scheme 1. The inhibitory mechanism of PA was partly similar to those of copper chelators but still has different aspect that PA did not directly bind to coppers at the active site [33, 34]. V_{max} value changes were predicted by docking simulation that PA might not compete with L-DOPA for docking because the binding sites are not located in the active site pocket. However, the K_m changes occurred in more complex manner: L-DOPA did not directly compete with PA but the L-DOPA accessibility for docking to coppers at the active site could be affected by the PA-docking-induced conformational changes resulting in the tertiary shape of active site, and this conformational change was confirmed by monitoring the hydrophobic surface changes of tyrosinase. This is due to the reason that PA docking site is very near to the active site where L-DOPA oxidation is occurred. Thus, the mixed-type inhibition of tyrosinase by PA was observed.

Using computational simulations, we predicted that PA bound directly with several residues (among them LEU73 is the most significant) of tyrosinase. This result is consistently matched to the data in Figure 6 where the number of binding site is calculated as approximately one. LEU73 residue is thought to be involved in the first stage of PA binding. Because the PA binding site is quite different to the L-DOPA binding site, it is not overlapped, but relatively in close proximity, which may allow PA binding to interact with the enzyme-substrate intermediate or another step in catalysis (V_{max} changes), and inducing detectable hydrophobic changes in the active site may result in retarding L-DOPA accession (K_m changes). Overall, the experimental data fit very well with data predicted by the simulations.

Taken together, our study provides new insight into the role of several residues in tyrosinase and provides useful information regarding the 3D structure of tyrosinase. A combination of inhibition kinetics and computational modeling may facilitate the testing of potential tyrosinase inhibitors and the prediction of their inhibitory mechanisms. The present study also suggested that the strategy of predicting tyrosinase inhibition based on hydroxyl groups and orientation may prove useful for the screening of potential tyrosinase inhibitors.

Abbreviations

DOPA: 3,4-dihydroxyphenylalanine
PA: Phthalic acid
ANS: 1-anilinonaphthalene-8-sulfonate.

Acknowledgments

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Research Article

Towards Al^{3+}-Induced Manganese-Containing Superoxide Dismutase Inactivation and Conformational Changes: An Integrating Study with Docking Simulations

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Superoxide dismutase (SOD, EC 1.15.1.1) plays an important antioxidant defense role in skins exposed to oxygen. We studied the inhibitory effects of Al^{3+} on the activity and conformation of manganese-containing SOD (Mn-SOD). Mn-SOD was significantly inactivated by Al^{3+} in a dose-dependent manner. The kinetic studies showed that Al^{3+} inactivated Mn-SOD follows the first-order reaction. Al^{3+} increased the degree of secondary structure of Mn-SOD and also disrupted the tertiary structure of Mn-SOD, which directly resulted in enzyme inactivation. We further simulated the docking between Mn-SOD and Al^{3+} (binding energy for Dock 6.3: −14.07 kcal/mol) and suggested that ASP152 and GLU157 residues were predicted to interact with Al^{3+}, which are not located in the Mn-contained active site. Our results provide insight into the inactivation of Mn-SOD during unfolding in the presence of Al^{3+} and allow us to describe a ligand binding via inhibition kinetics combined with the computational prediction.

1. Introduction

Superoxide dismutases (SOD, EC 1.15.1.1) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide [1–3]. They play an important antioxidant defense role in skins exposed to oxygen. In this regard, for the treatment of systemic inflammatory diseases including skin ulcer lesions, the topical application of free Mn-SOD or Cu, Zn-SOD extracted from bovine, bacterial, and other species was dramatically effective in skin lesions [4]. It has been reported that significant increase in the levels of SOD occurs in vitiligo patients due to the increased oxidative stress [5]. The involvement of oxidative stress in chronic idiopathic urticaria associated with SOD was also reported [6]: the activity of SOD was markedly increased in lesional skin as compared with skin of healthy subjects, indicating that oxidative stress is crucially involved in chronic idiopathic urticaria and suggesting that oxidative stress is secondary to the development of inflammation. The earlier reports [7, 8] suggested that the activity of activator protein-1, which is associated with tumor promotion, was reduced in Mn-SOD transgenic mice overexpressing Mn-SOD in the skin, suggesting that Mn-SOD reduced tumor incidence by suppressing activator protein-1 activation.

The mechanism of Mn-SOD catalysis is very important, and the mechanism therefore needs to be investigated from different sources using various kinetic methods. The information regarding the tertiary structure and the structural integrity of the active site of Mn-SOD is little known and in this regard, investigation on structure-function relationships

in this enzyme including docking of a ligand is important. In this study, we applied Al^{3+} to understand Mn-SOD structural changes and inhibition mechanisms. As a result, we proposed an inhibitory effect of Al^{3+} on Mn-SOD and suggest the mechanisms of combination between inhibition kinetics and computational prediction to depict the Al^{3+} action in the catalysis of Mn-SOD.

2. Materials and Methods

2.1. Materials. Aluminum chloride crystal ($AlCl_3 \cdot 6H_20$), Pyrogallol, and ANS were purchased from Sigma-Aldrich (USA). EDTA and Tris were from Fluka (Switzerland). The crude form of Mn-SOD (from *Thermus thermophilus*) was purchased from BioTech Company (China). We further purified Mn-SOD using the ÄKTAFPLC system (GE Healthcare, USA); a single band was obtained on both SDS-PAGE and native nonreducing PAGE gels. All other reagents used were local products of analytical grade. 10 mM Tris-HCl buffer (pH 8.2) was used during preparing all samples in this study.

2.2. Mn-SOD Assay. The assay for Mn-SOD was performed spectrophotometrically [9]. The activity of SOD was calculated according to the procedures of pyrogallol's autoxidation, which could be monitored by the change in absorbance at 325 nm per min. Reactions were performed in a typical reaction volume of 1 mL to which 10 μL of enzyme solution was added to measure Mn-SOD activity. The activity and absorption were measured with a Perkin Elmer Lambda Bio U/V spectrophotometer.

2.3. Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra of Mn-SOD at different Al^{3+} concentrations were recorded on a Jasco J-810 Spectropolarimeter in the region of 190–250 nm at room temperature. The sample cell path length was 0.1 cm. CD measurements were carried out according to the provider's instructions. The final spectrum was obtained on the average of three scans. Blanks were collected and subtracted from the appropriate samples in data processing.

2.4. Intrinsic and ANS-Binding Fluorescence Measurements. Mn-SOD was denatured by incubation in 10 mM Tris-HCl (pH 8.2) containing various concentrations of Al^{3+} for 3 h, 25°C. The fluorescence emission spectra were measured with a Jasco FP750 spectrofluorometer with the use of a 1 cm path-length cuvette. An excitation wavelength of 280 nm was used for the tryptophan fluorescence measurements, and the emission wavelength ranged between 300 and 410 nm. The changes of the ANS-binding fluorescence intensity for Mn-SOD were studied by labeling with 40 μM ANS for 30 min prior to measurement. An excitation wavelength of 380 nm was used for the ANS-binding fluorescence, and the emission wavelength ranged from 400 to 650 nm.

2.5. Determination of the Binding Constant and the Number of Binding Sites. According to a previous report [10], when small molecules are bound to equivalent sites on a macromolecule, the equilibrium between free and bound molecules are given by the following equation to evaluate the binding constant (K) and number of binding sites (n):

$$\frac{F_0}{F_0 - F} = \frac{1}{n} + \frac{1}{K}\frac{1}{[Q]}, \tag{1}$$

where F_0 and F are the relative steady-state fluorescence intensities in the absence and presence of quencher, respectively. $[Q]$ is the quencher (Al^{3+}) concentration. The values for K and n can be derived from the intercept and slope of a plot based on (1).

2.6. In Silico Docking of Mn-SOD and Al^{3+}. The known 3D structure of Mn-SOD was obtained from PDB data base (ID: 3MDS). Among the many tools available for *in silico* protein-ligand docking, DOCK6.3 was applied because of its automated docking capability. The program performed ligand docking using a set of predefined 3D grids of the target protein and used a systemic search technique [11]. The original structure of Al^{3+} was derived from the PubChem database (Compound ID: 104727, http://www.pubchem.org/). To prepare for the docking procedure, the following steps were taken: (1) conversion of 2D structures to 3D structures, (2) calculation of charges, (3) addition of hydrogen atoms, and (4) location of pockets. For these steps, we used the fconverter program of the J-Chem package (http://www.chemaxon.com/) and OpenBabel (http://openbabel.org/).

3. Results

3.1. Effect of Al^{3+} on the Activity of Mn-SOD: Inactivation Kinetics. We assayed Mn-SOD at the equilibrium and the kinetic states in the presence of Al^{3+}. Mn-SOD was significantly inactivated by Al^{3+} with a dose-dependent manner (Figure 1). When the Al^{3+} concentration was increased to 0.8 mM, the activity of Mn-SOD was completely abolished. The IC_{50} value was measured as 0.19 mM ($n = 2$).

To evaluate the inactivation kinetics and rate constants, time interval measurements were performed. The different time courses of Mn-SOD in the presence of 0.2, 0.3, and 0.4 mM Al^{3+}, respectively, were recorded (Figure 2(a)). The enzyme activity was gradually decreased with time intervals and this implied that Al^{3+} may induce Mn-SOD tertiary structural change due to the fact that the activity of Mn-SOD was synchronized with the conformational changes. The microscopic inactivation rates constants (k_a) were properly calculated from the semilogarithmic plots (Figure 2(b)) where the reactions were plotting as two-phasic courses (fast, k_1 and slow, k_2). The rate constants for 0.2, 0.3, and 0.4 mM Al^{3+} were obtained as $k_1 = 7.39$, 7.83, and $13.3 \times 10^{-3}\,s^{-1}$, respectively, and $k_2 = 0.58$, 1.25, and $1.73 \times 10^{-3}\,s^{-1}$, respectively. These results suggested that the Mn-SOD inactivation by Al^{3+} followed the first-order kinetic process, and the enhancing Al^{3+} concentration could promote the inactivation rate.

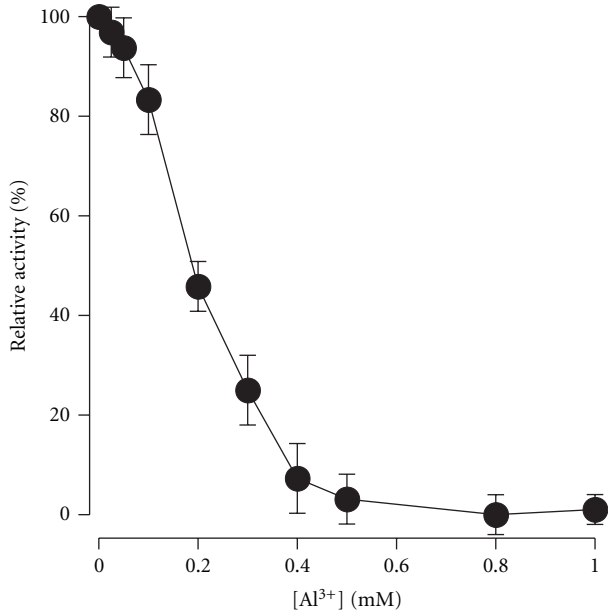

Figure 1: Inactivation of Mn-SOD in the presence of Al^{3+}. Data and bars are presented as means ($n = 2$). Mn-SOD was incubated with various concentrations of Al^{3+} for 3 h, and then added to the assay system in the presence of the corresponding concentrations of Al^{3+}. The final concentration of Mn-SOD was 1.25 μM.

3.2. Al^{3+}-Induced Secondary Structural Changes of Mn-SOD Measured by CD. To compare the enzyme activity changes with the secondary structural changes, we performed the Far-UV circular dichroism (CD) spectroscopy. As the concentration of Al^{3+}increased, the overall amount of secondary structure decreased gradually in a dose-dependent manner: specifically, the measurements for both 208 and 222 nm indicated that overall helical contents were decreased with increasing Al^{3+} concentration (Figure 3). Interestingly, the overall secondary structure of Mn-SOD was mostly sustained at lower than 0.3 mM Al^{3+} but the activity was drastically abolished by Al^{3+} in this range as shown in Figure 1.

3.3. Effect of Al^{3+} on the Tertiary Structure of Mn-SOD: Spectrofluorimetry Studies. Next, tertiary structural changes of Mn-SOD in the presence of Al^{3+} were also measured by intrinsic and ANS-binding fluorescences measurements. The intrinsic fluorescence changes showed that Al^{3+}might induce the unfolding of Mn-SOD which was monitored by the decrease of intrinsic fluorescence spectra (Figure 4). Based on the quenching effect of $Al^{3+}$and (1), we deduced a double reciprocal plot revealing a linear relationship (Figure 5). From this data, we calculated the binding constant as $K = 5.4 \pm 0.8 \times 10^3\ M^{-1}$ and the binding number as $n = 1.5 \pm 0.3$ according to plotting results and (1). These results revealed that Al^{3+} has a strong binding affinity for tyrosinase in the absence of substrate and that there are one or two possible binding sites.

The kinetics of Mn-SOD unfolding was also monitored (Figure 6(a)). The data of the semilogarithmic plots showed that the unfolding process also followed the first-order

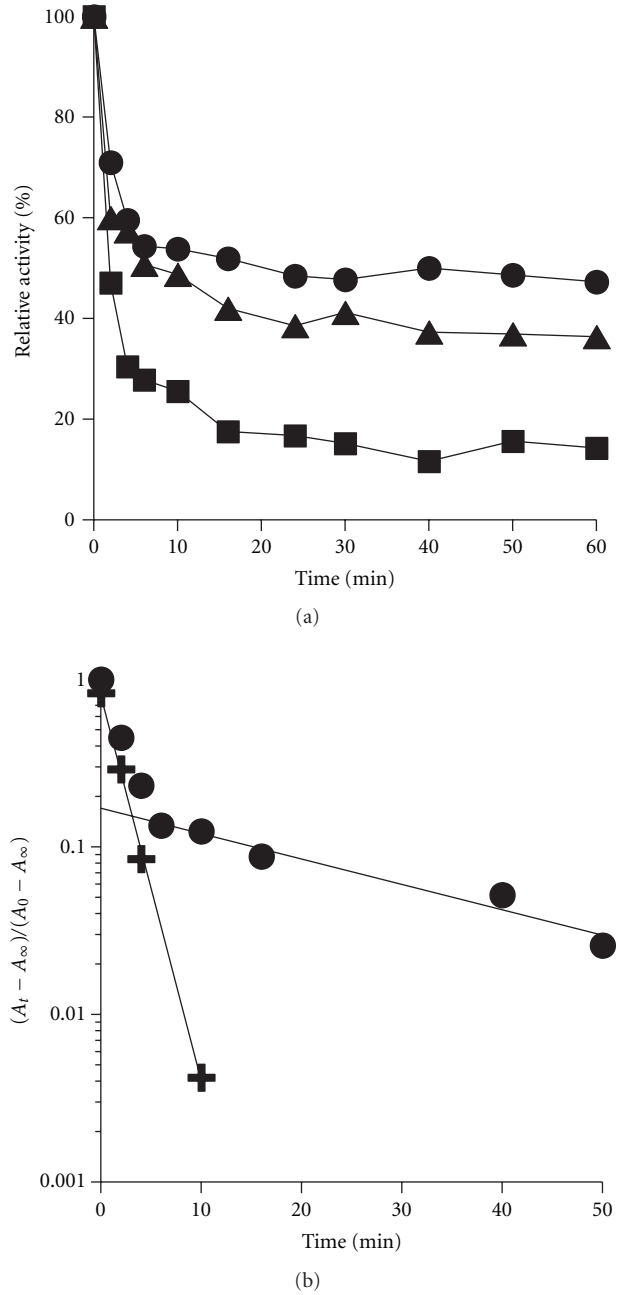

Figure 2: Inactivation kinetics of Mn-SOD in the presence of Al^{3+}. (a) The Al^{3+} concentrations were 0.2 (•), 0.3 (▲), and 0.4 mM (■), respectively. The reaction occurred at 25°C. The final concentration of Mn-SOD was 1.25 μM. (b) The semilogarithmic plot. The Al^{3+} concentration was 0.2 mM. (•) Experimental points. (+) Points obtained by subtracting the contribution of the slow phase from the data in the curve (- - -).

kinetics where the reactions were plotting as two-phasic courses (fast, ku_1 and slow, ku_2). The rates constants were calculated as $ku_1 = 2.89$ and $ku_2 = 0.21 \times 10^{-3}\ s^{-1}$ (Figure 6(b)). These results indicated that unfolding process of Mn-SOD was synchronized with the activity inactivation with the same order reaction, which was comparative to the results in Figure 2.

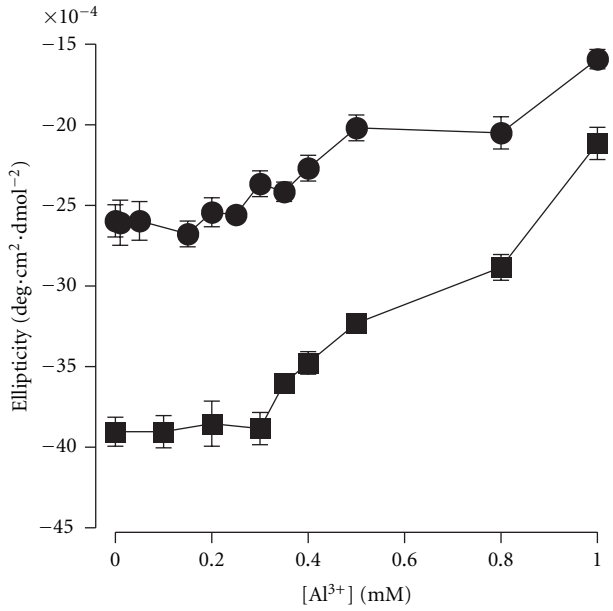

Figure 3: Far-ultraviolet CD spectra of Mn-SOD in the presence of different Al^{3+} concentrations. (a) Mn-SOD was incubated with Al^{3+} solutions for 3 h before measurement at 25°C. Blanks were collected and subtracted from the sample spectra in data processing. (b) CD spectra changes of Mn-SOD at 208 (•) and 222 (■) nm. Data indicate mean values ($n = 2$). The final Mn-SOD concentration was 20 μM.

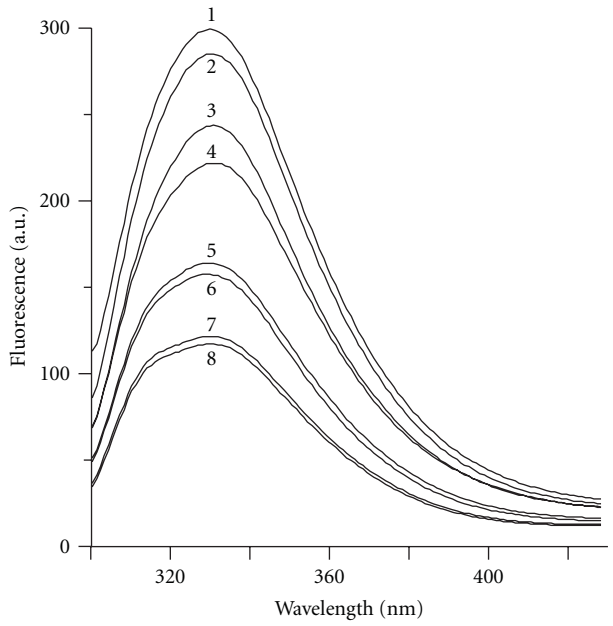

Figure 4: Intrinsic fluorescence changes of Mn-SOD by Al^{3+}. Intrinsic fluorescence spectra changes. Mn-SOD was incubated with Al^{3+} for 3 h before being measured. The final Mn-SOD concentration was 1.6 μM. Curves from 1 to 8 represent 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 Al^{3+} mM, respectively.

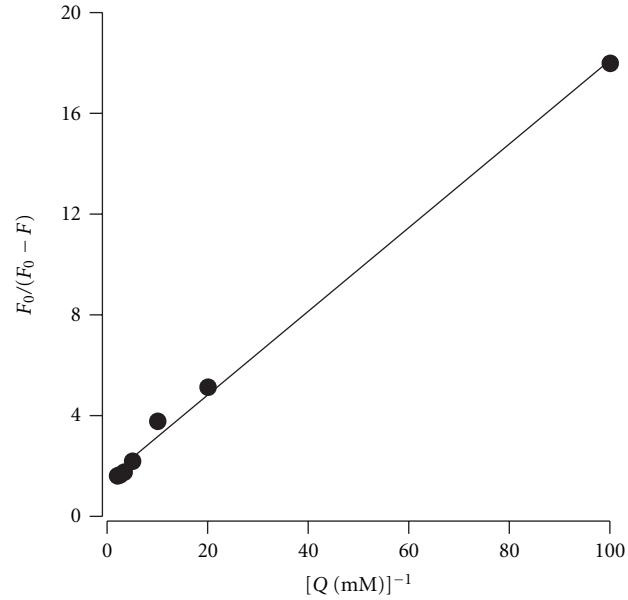

Figure 5: Double reciprocal plot of $F_0/(F_0 - F)$ versus $[Q]^{-1} \cdot F0$, native maximum fluorescence intensity; F: maximum fluorescence intensity of sample; Q: quencher Al^{3+}.

In the next step, we monitored the hydrophobicity changes of Mn-SOD in the presence of Al^{3+}. The ANS-fluorescence intensities were changed by overall range of Al^{3+} concentrations (Figure 7), indicating that hydrophobic surfaces of Mn-SOD were exposed during Al^{3+}-mediated unfolding. In general, ANS dye can bind to hydrophobic amino acid residues, thus, it is used to monitor the tertiary structural disruption of the enzyme in the presence of inactivator. Our results showed that increase Al^{3+} concentration caused the Mn-SOD ANS-fluorescence intensity increase in a concentration-dependent manner.

3.4. Computational Docking Simulation for Mn-SOD and Al^{3+}. Because the crystallographic structure of Mn-SOD from *Thermus thermophilus* has been elucidated (PDB ID: 3MDS), we easily constructed the 3D structure of Mn-SOD. The docking between Mn-SOD and Al^{3+} by using Dock6.3 was successful with significant score (−14.07 kcal/mol) and we searched for Al^{3+} binding residues of Mn-SOD. We found that the most important expected binding residues interacting with Al^{3+} were ASP152 and GLU157 residues (box in Figure 8). The docking simulation provided the supportive information for the inactivation of Mn-SOD by Al^{3+} where the binding site is not located in the manganese-containing active site pocket (Figure 9). We found that Al^{3+}-induced inactivation of Mn-SOD is not due to the replacement of manganese or chelating from the active site.

4. Discussion

Several biological effects of Al^{3+} have been reported [12–15]: the results were mostly focused on the toxic effects such as the involvement of oxidative stress, deregulation of cell signaling,

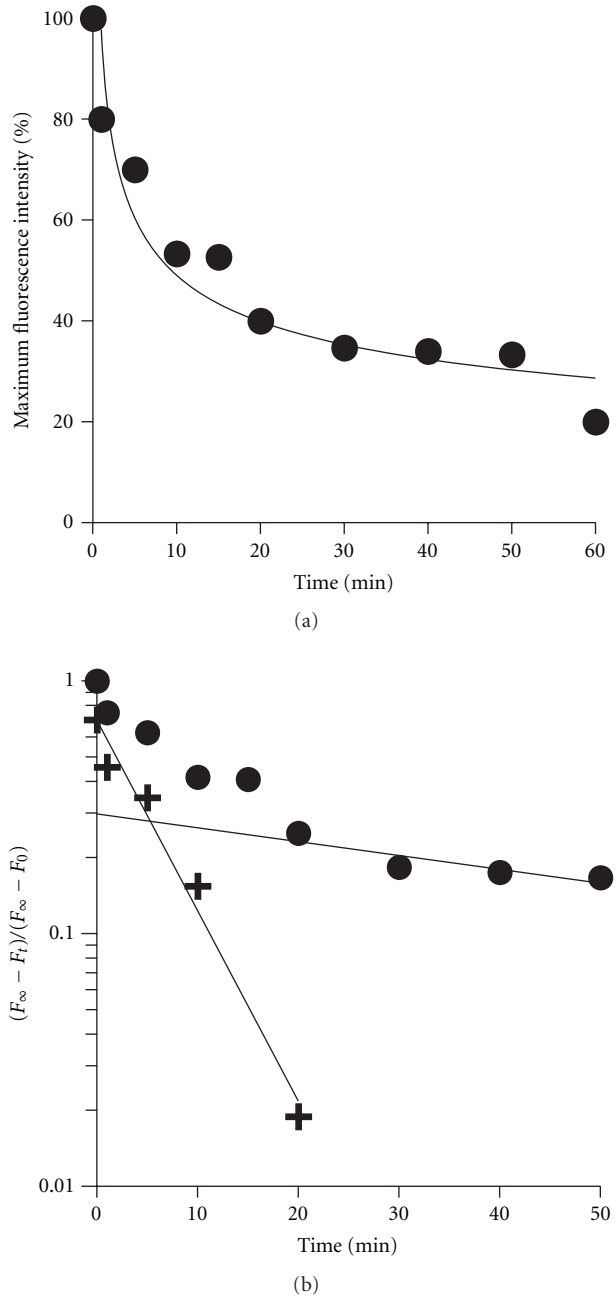

Figure 6: The kinetics of Al^{3+}-induced fluorescence spectra changes. (a) Plot of maximum intensity versus time (min). Intrinsic fluorescence spectra changes were measured in response to 0.2 mM Al^{3+} for various time intervals. The final Mn-SOD concentration was 1.6 μM. (b) A semilogarithmic plot. (•) Experimental points. (+) Points obtained by subtracting the contribution of the slow phase from the data in the curve (- - -). F_t: maximum intensity at various time interval; F_∞: maximum intensity at equilibrated state; F_0: maximum intensity at initial state.

membrane biophysics alterations, and the neurotoxicity in neurotransmission. On the contrary, a study reported that Al^{3+} can promote faster wound healing in response to skin injury [16] when it is prepared as the template to generate large uniform membranes with differing nanopore sizes.

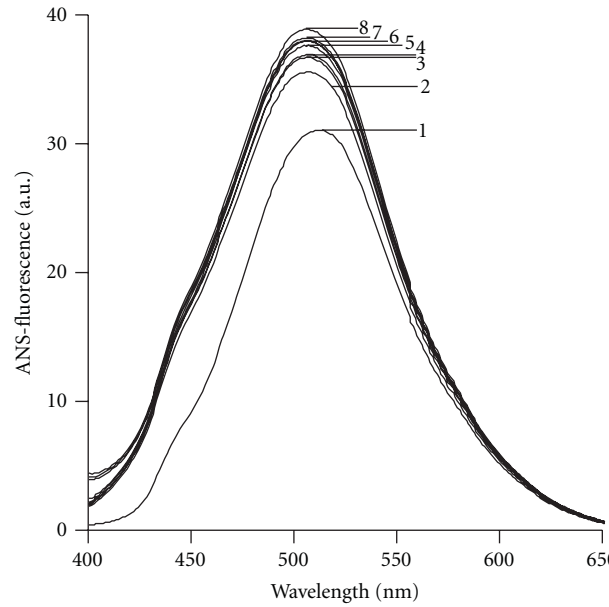

Figure 7: The ANS-binding fluorescence changes of Mn-SOD by Al^{3+}. ANS-binding fluorescence spectra in the presence of various concentrations of Al^{3+}. ANS (40 μM) was incubated for 30 min to label the hydrophobic surface of Mn-SOD prior to measurement. Other conditions were as for Figure 4. Curves from 1 to 8 indicate in 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 Al^{3+} mM, respectively.

Our investigation suggested a possible cytotoxic effect of Al^{3+} induced by Mn-SOD inhibition and it may sequentially induce the oxidative damage. Our data consistently supports the previous report that Al^{3+} acted as a toxic material for skin fibroblasts [17].

The changes of enzyme activity and structure in various ions have been extensively studied. For the case of Al^{3+}, it has been applied to several enzymes [18–20] to test its effects on the activities. The biological effect of Al^{3+} has been gradually elucidated and in this regard, we mainly focused on the changes in Mn-SOD activity and structures in Al^{3+} solutions in the present study, and we found that Al^{3+} worked as an inactivator to Mn-SOD accompanying with kinetic unfolding processes both in activity and structures. The relative activity and the conformational changes were synchronized in overall concentration of Al^{3+}. The activity of Mn-SOD was conspicuously observed when the secondary structure change has not yet occurred. The tertiary structural change of Mn-SOD by Al^{3+} was confirmed by the result of exposing the hydrophobic surface. Even at low concentration of Al^{3+}, the overall structure of Mn-SOD was changed and this directly affected the structural shape of active site pocket, regardless of Mn-SOD active center site is very impact and stable due to manganese contained inside. Al^{3+} binding site is distinctive to the substrate binding site of Mn-SOD. Al^{3+} did not directly compete with substrate but it affect the catalysis with a dose-dependent manner, implying that Al^{3+} binding site is near to substrate docking site. The computational simulations supported this observation that Al^{3+} can form a ligand-binding complex directly with ASP152 and GLU157 residues of Mn-SOD, and these amino acid residues are

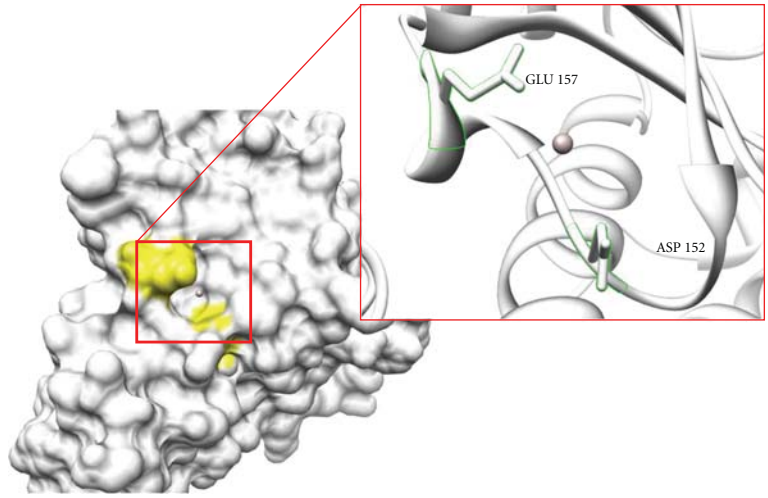

Figure 8: Computational docking simulations between Mn-SOD and Al^{3+}. 3D structure of Mn-SOD was constructed from PDB (ID: 3MDS), and the red box indicates the predicted binding sites for Al^{3+} via Dock6.3. Right red box shows the Al^{3+} binding residues.

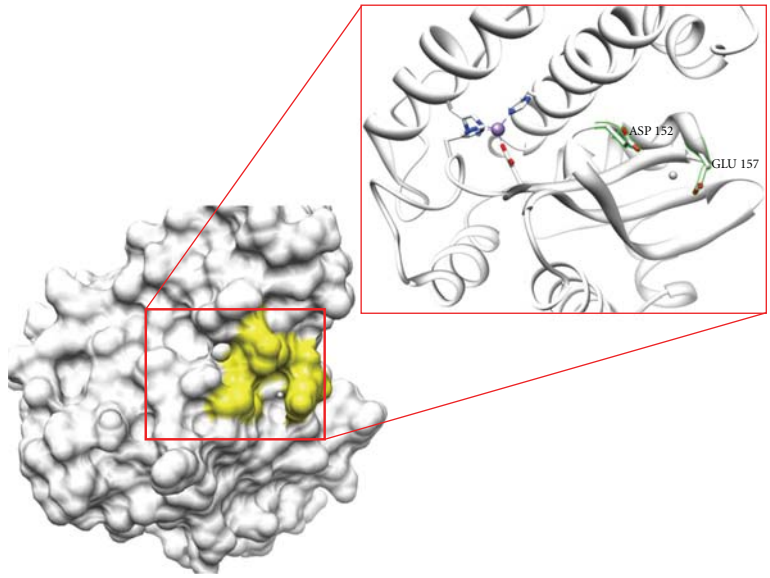

Figure 9: A comparison of active site and Al^{3+} docking site. Left part shows active site containing manganese metal (gray ball) and right part indicates Al^{3+} binding site.

not located in the active site. Therefore, the conformational changes were observed firstly prior to the occurrence of activity loss since the active site is relative compact and stable via manganese presence and Al^{3+} docking site is relative flexible part of Mn-SOD.

According to the results observed in the present study, we deduced the mechanisms of Mn-SOD response to Al^{3+}: (i) Al^{3+} ligand-binding to Mn-SOD causes fist-order kinetic inactivation which was synchronized with conformational changes; (ii) Al^{3+} also induced the decrease of secondary structure at relatively high concentration but compared to the activity and tertiary structural changes, the secondary structure was less sensitive to Al^{3+} than the tertiary structure; (iii) interestingly, the result of computational simulation support our supposition that Mn-SOD was bound to the near of active site, not in the inner site of active site pocket.

In conclusion, Mn-SOD from extremophile such as *Thermus thermophilus* was tend to be very stable against the changes of temperature and pH, as well as denaturants addition such as urea and guanidine hydrochloride. However, we found that Mn-SOD from *Thermus thermophilus* was conspicuously denatured by Al^{3+}. Taken together, the inhibition kinetics combined with the computational prediction allowed us to elucidate into the relationship between enzymatic reaction and structural changes of Mn-SOD from *Thermus thermophilus* and provides greater insight regarding the folding of Mn-SOD as well as the cytotoxicity of Al^{3+}.

Conflict of Interests

There is no conflict of interests in this study.

List of Abbreviations

Mn-SOD: Manganese-containing superoxide dismutase SOD
ANS: 1-anilinonaphthalene-8-sulfonate.

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Research Article

Computational Approach to Identify Enzymes That Are Potential Therapeutic Candidates for Psoriasis

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Psoriasis is well known as a chronic inflammatory dermatosis. The disease affects persons of all ages and is a burden worldwide. Psoriasis is associated with various diseases such as arthritis. The disease is characterized by well-demarcated lesions on the skin of the elbows and knees. Various genetic and environmental factors are related to the pathogenesis of psoriasis. In order to identify enzymes that are potential therapeutic targets for psoriasis, we utilized a computational approach, combining microarray analysis and protein interaction prediction. We found 6,437 genes (3,264 upregulated and 3,173 downregulated) that have significant differences in expression between regions with and without lesions in psoriasis patients. We identified potential candidates through protein-protein interaction predictions made using various protein interaction resources. By analyzing the hub protein of the networks with metrics such as degree and centrality, we detected 32 potential therapeutic candidates. After filtering these candidates through the ENZYME nomenclature database, we selected 5 enzymes: DNA helicase (RUVBL2), proteasome endopeptidase complex (PSMA2), nonspecific protein-tyrosine kinase (ZAP70), I-kappa-B kinase (IKBKE), and receptor protein-tyrosine kinase (EGFR). We adopted a computational approach to detect potential therapeutic targets; this approach may become an effective strategy for the discovery of new drug targets for psoriasis.

1. Introduction

Psoriasis is a common inflammatory disease affecting more than 25 million people in North America and Europe. It is associated with arthritis, myopathy, enteropathy, spondylotic joint disease, and atopic dermatitis. This disease is characterized by well-demarcated lesions on the skin of the elbows, knees, and scalp. It is an autoimmune disease triggered by an activated cellular immune system resulting from a combination of genetic and environmental factors. It is also frequently inherited and is passed from one generation to the next [1].

Many factors trigger psoriasis, including bacterial pharyngitis, stress, and various medications (e.g., lithium and β-blockers). Perturbation of epidermal keratinocytes is considered an activating signal in psoriasis [2], and in regions with psoriasis lesions, keratinocyte proliferation is increased along with inflammation and angiogenesis [3]. Recent studies have reported that the interaction between T-cells and keratinocytes gives rise to a cytokine soup dominated by Th1-type and Th17-type cytokines such as interleukin- (IL-) 12, IL-17, interferon- (IFN-) γ, and tumor necrosis factor (TNF) [4]. In addition, keratinocytes stimulated with IL-20 upregulate a variety of inflammatory genes, including monocyte chemotactic protein-1 (MCP-1) and myeloid-related protein-14 (MRP-14) [5, 6].

Some genetic studies have reported a strong association between psoriasis and human leukocyte antigen- (HLA-) C, particularly with the HLA-Cw0602 allele. Individuals who are homozygous have a 2.5-fold higher risk of developing psoriasis than those who are heterozygous [7]. Moreover, a genomewide association study revealed that polymorphisms in genes related to IL-23 and nuclear transcription factor κB (NFκB) signaling are associated with psoriasis [8]. TNF is currently considered a major target in psoriasis pathogenesis,

because much higher levels of TNF are found in lesional skin than in normal skin [9]. Treatment with TNF antagonists elicits a response in patients with psoriasis. However, despite the success of anti-TNF-α therapies, the involvement of TNF-α in disease pathogenesis is not yet fully understood. Furthermore, these drugs have clinical nonresponse rates that range from 20% to 50% in patients with psoriasis [10]. Therefore, there is a need for new and effective drug targets and compounds.

New research initiatives have been undertaken to collect high-throughput mRNA expression and protein-protein interaction (PPI) data from different organisms. This important source of biological information has been effectively employed in the search for new drugs [11]. Systematic analysis using bioinformatics has enabled researchers to extract and manipulate biological information with the goal of understanding the pathogenesis of disease. In particular, the combined analysis of gene expression and PPI may help identify candidates that are potential therapeutic targets. Recent studies analyzing protein interaction networks have been carried out in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* [12, 13]; such studies have confirmed that topological metrics of protein interaction networks are useful for predicting essential target proteins. These studies have also been expanded to organisms of medical importance, such as the malaria parasite [14], as a starting point for the discovery of new drug targets. In humans, the analysis of PPIs has also been useful in detecting important proteins, such as hub proteins, when the interactions were predicted using a homologous approach [15].

To better understand the pathogenesis of psoriasis and to identify potential therapeutic targets, we performed a microarray analysis comparing lesional and nonlesional psoriatic skin and a protein interaction network analysis that was constructed using differentially expressed genes obtained from the microarray data. We identified potential therapeutic or drug target candidates by analyzing the protein interaction network with the metrics of degree and centrality. We then selected the enzymes from the candidates and detected nonsynonymous single-nucleotide polymorphisms (SNPs) in the enzyme genes that could cause structural changes in the proteins. These putative enzyme targets are a starting point for the discovery of new psoriasis drugs.

2. Materials and Methods

2.1. Microarray Analysis Related to Psoriasis. Microarray data from psoriasis patients were downloaded from Gene Expression Omnibus (GEO), which is a public database of centrally archived raw microarray data [16]. We used 2 microarray datasets (GDS2518 and GDS3539) generated using Affymetrix human genome microarrays, which have more than 4 million gene expression measurements. The GDS2518 dataset contained transcriptome data of lesional and nonlesional skin from 13 patients with plaque-type psoriasis [17]. The GDS3539 dataset contained similar data from 33 patients [18]. In order to identify genes that are differentially expressed in psoriasis patients, we compared lesional and nonlesional skin data to microarray datasets.

2.2. Identification of Differentially Expressed Genes from Transcriptome. We removed probe redundancy because 1 gene has several probes on a single microarray chip. After removing the redundancy, the average expression profiles were calculated for the probe clusters having multiple expression profiles. From each of the given microarray datasets, we obtained differentially expressed genes (DEGs) by unpaired two class analysis (Sigma = 2.4, Q-value = 0.0001) by using significance analysis for microarray (SAM) [19]. We then combined the DEGs obtained from GDS2518 and GDS3539.

2.3. PPI Resources. Predictions of PPI have been applied in various studies in order to understand the mechanism of disease development, find key proteins related to species lethality, and search for drug targets between a host and pathogen [20]. PPI resources were assembled from a combination of several experimental protein interaction databases. The protein interaction resources include 6 databases: DIP [21], BIND [22], IntAct [23], MINT [24], HPRD [25], and BioGrid [26]. We performed a redundancy test to remove identical protein sequences from the interaction databases. The databases contain 116,773 proteins and 229,799 interactions.

2.4. Protein Network Prediction from the DEGs. There are computational methods for predicting PPIs such as gene neighborhood [27], gene fusion [28], phylogenetic profile [29], and interolog [30]. In particular, the interolog approach is widely used to predict PPIs when the sequences of target proteins are known. In the interolog approach, the interaction of 2 query proteins is predicted when both have homologousproteins that are already known to interact [30]. A protein network of DEGs selected from the microarrays was predicted from homologous interactions. To find homologous interactions among the DEGs, we converted the DEGs to proteins, and we aligned these with proteins from the interaction resources using PSI-BLAST [31] with a minimal cutoff of 40% sequence identity, 70% length coverage, and an E-value of 0.0001.

2.5. Detection of Essential Proteins with Topological Metrics and Selecting for Enzymes. The protein network analysis has been applied to find essential proteins, such as hubs, that are related to a disease or biological pathway [11]. To find essential proteins, we performed a protein network analysis with topological metrics such as degree and betweenness centrality using Perl program. Proteins with a high number of interaction partners in the network were regarded as degree-based hubs. Proteins with many shortest paths between other proteins were regarded as betweenness-based hubs. Proteins that have high betweenness scores mainly exist in the middle of the protein network. If the protein is removed from the network, the proportion of unreachable protein pairs and the mean shortest path length between all pairs of reachable nodes are increased [32]. We selected the essential proteins that have the top 1% of total degree and 1% of total betweenness in the protein network as hubs. Finally, the proteins were filtered through the list

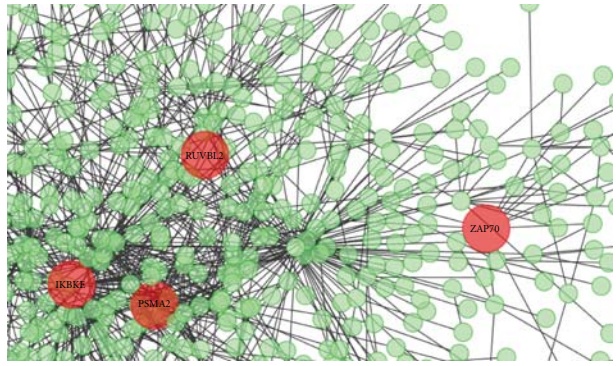
of enzyme proteins stored in the ENZYME nomenclature database (http://expasy.org/enzyme/).

2.6. Structural Changes Induced by Nonsynonymous SNPs. Nonsynonymous single-nucleotide polymorphisms (nsSNPs) have been implicated in various diseases; nsSNPs can alter protein function [33], destabilize core protein structure, and reduce protein solubility [34]. Because polymorphisms are an important genetic factor in psoriasis [7], we checked for nsSNPs in the selected enzymes using the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), and we predicted the structural changes in the enzymes caused by the nsSNPs through structure modeling and stability analysis. On the basis of homology modeling, three-dimensional structural models of the target enzymes were built using SWISS-MODEL [35]. The SWISS-MODEL program automatically provides an all-atom model using alignments between the query sequence and known homologous structures. For homology modeling, the known homologous structures of enzymes from the Protein Data Bank (PDB) (http://www.pdb.org/) were used as the structural templates. Further, the stability of the enzyme structure was checked using the CPUSAT program [36].

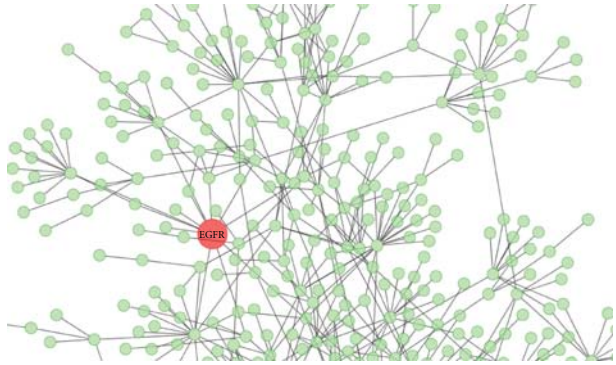
3. Results and Discussion

3.1. Psoriasis Microarray Coupled with SAM Analysis. We compared the microarray datasets GDS2518 and GDS3539 from regions with and without lesions in psoriasis patients. Using SAM (Sigma 2.6 cutoff value with a 0.0001 Q-value), 6,437 candidate genes were found, and 3,264 genes were upregulated while 3,173 genes were downregulated. Among the listed candidates were several distinct genes that have been known to be associated with psoriasis; these genes coded for TNF-α, IFN-γ receptor 1, IL-8, and IL-20 (data not shown). In this study, we used bioinformatic analyses in which several methods were integrated, including PPI and structure prediction, to detect novel candidate proteins except well-known genes.

3.2. Identification of Putative Therapeutic Candidates. There are several ways to obtain candidate genes for disease analysis, including cDNA microarray and proteomics approaches such as mass spectrometry. Although we could obtain many upregulated or downregulated genes in psoriasis using high-throughput omics approaches, it was very difficult to choose the important genes. However, PPI mapping can help find potential targets among these candidates. Recent studies have successfully applied this approach to discover drug targets using computational predictions of protein networks in the bacterium *Mycobacterium tuberculosis* and in humans [37–39]. Therefore, we predicted the protein interaction network of psoriasis-related proteins using homologous interaction prediction. We separately constructed 2 protein interaction networks using 3,264 upregulated genes and 3,173 downregulated genes in psoriasis. The protein interaction network constructed from the upregulated genes contained 1,310 proteins and 1,934 interactions, and the network constructed from the downregulated genes contained 985 proteins and 1,205 interactions, and they are shown in Figure 1. In order to find potential therapeutic candidates, we calculated the number of interacting partners (degree) [40] and betweenness centrality [41] for each protein. These 2 topological metrics have been shown to improve the detection of essential proteins in protein networks [42]. Betweenness centrality correlates more closely with essentiality than degree, thereby exposing critical nodes that usually belong to the group of scaffold proteins or proteins involved in crosstalk between signaling pathways. This metric has also been proposed in the new paradigm of network pharmacology as a good method for investigating potential drug targets [43]. In the protein network, we selected the top 1% of the degree and centrality rank as hub proteins because this cutoff value identifies the group with the highest probability to be an essential group of proteins. We chose 17 proteins in the upregulated gene protein interaction network and 15 proteins in the downregulated gene protein interaction network. The protein list and network properties are described in Tables 1 and 2. We found the 5 enzymes by filtering



(a)



(b)

Figure 1: Protein interaction networks constructed by DEGs in psoriasis. The node indicates protein and the edge indicates protein-protein interaction. The red nodes are essential enzymes predicted by topological metrics such as degree and betweenness centrality. (a) Protein interaction network constructed from genes upregulated in microarray data from regions with and without lesions in psoriasis patients. The network consisted of 1,310 proteins and 1,934 interactions. (b) Protein interaction network constructed from downregulated genes. The network consisted of 985 proteins and 1,205 interactions.

Table 1: Selected hub proteins in the protein interaction network constructed from upregulated genes.

Gene symbol	*Refseq ID*	*Full name (enzyme number)*	*Topological metrics*
CDKN1A	NP_000380.1	Cyclin dependent kinase inhibitor 1A	centrality: 64419
RUVBL2	**NP_006657.1**	**RuvB like 2 (3.6.4.12: DNA helicase)**	**centrality: 50925**
COPS6	NP_006824.2	COP9 constitutive photomorphogenic homolog subunit 6	centrality:60596
EWSR1	NP_001156757.1	Ewing sarcoma breakpoint region 1	centrality: 62682
FTSJ1	NP_036412.1	FtsJ homolog 1	degree: 27
SFN	NP_006133.1	14-3-3 sigma	centrality: 50372
GRB2	NP_002077.1	Grb2	centrality: 397096
CCDC85C	NP_001138467.1	C14orf65 protein	centrality: 76862
EIF6	NP_002203.1	Eukaryotic translation initiation factor 6	centrality: 61909
ARF6	NP_001654.1	ADP ribosylation factor 6	centrality: 45220
NFKB1	NP_001158884.1	NFKB1	degree: 18
PCNA	NP_002583.1	Proliferating cell nuclear antigen	centrality: 46316
PINX1	NP_060354.4	Pin2 interacting protein X1	centrality: 51027
PSMA2	**NP_002778.1**	**Proteasome subunit alpha type 2 (3.4.25.1: Proteasome endopeptidase complex)**	**degree: 17**
VDAC1	NP_003365.1	VDAC1	degree: 18
ZAP70	**NP_001070.2**	**ZAP70 (2.7.10.2: Nonspecific protein-tyrosine kinase)**	**centrality: 50140**
IKBKE	**NP_001180250.1**	**IKKE (2.7.11.10: I-kappa-B kinase)**	**centrality: 412760**

the group of detected 32 proteins through the ENZYME nomenclature database. Among the upregulated genes, 4 enzymes were selected as putative targets: DNA helicase (RUVBL2), proteasome endopeptidase complex (PSMA2), non-specific protein-tyrosine kinase (ZAP70), and I-kappa-B kinase (IKBKE). From the downregulated genes, EGF receptor (EGFR) was selected.

3.3. Experimental Evidence of the Predicted Targets Related to Psoriasis. The predicted enzymes were directly or indirectly related to psoriasis. The predicted enzymes have several significant features. IKBKE was already known as a psoriasis-related protein that is essential for the regulation of antiviral signaling and inflammatory pathways. TNF-α, which exists on upstream of IKBKE signaling pathway, has been used as a psoriasis drug target. TNF-α inhibitors such as etanercept are used for treating rheumatoid and psoriatic arthritis. The abnormal activation of T-cells is known to be a factor in the development of psoriasis. Therefore, T-cell infiltration and the inhibition of cytokines are the major modalities for the treatment of psoriasis [1].

ZAP70 plays a role in T-cell development and lymphocyte activation [44]. This enzyme, which is phosphorylated at tyrosine residues upon T-cell antigen receptor (TCR) stimulation, functions in the initial step of TCR-mediated signal transduction in combination with the Src family kinases Lck and Fyn [45]. Mutations in this enzyme cause selective T-cell defects and a severe combined immunodeficiency disease characterized by a selective absence of CD8-positive T-cells [46]. Furthermore, a significant change in ZAP70 expression was reported during the course of chronic lymphocytic leukemia [47].

We found EGFR, which plays a role in morphogenesis by modulating cell adhesion, as a downregulated target. EGFR promotes keratinocyte antimicrobial defenses in a differentiation-dependent manner. Ligands of the EGF family regulate autocrine keratinocyte proliferation, and IL-1 family cytokines have epithelial defense responses [48].

Two proteasome-related enzymes were also found among the upregulated target candidates. RUVBL2 is a DNA helicase that is essential for homologous recombination and DNA double-strand break repair. Recent research reported that the depletion of RUVBL2 leads to tumor growth arrest and that it is overexpressed in a majority of hepatocellular carcinomas [49]. Moreover, the enzyme levels are strictly controlled by a posttranslational mechanism involving proteasomal degradation of newly synthesized proteins [50]. PSMA2 is a proteasome subunit alpha type 2 and is a member of the peptidase T1A family. Proteasome-related enzymes may be valuable as therapeutic candidates since a proteasome blockade causes the overexpression of the suppressor of cytokine signaling (SOCS) 3protein, which inhibits the IFN-α response which are overexpressed in psoriasis [51].

This may be reasonable because these enzymes are important regulators in the mechanism of development of psoriasis. Although more information on the biological functions of these interesting enzymes is needed, they are potential candidates for future drug screening and therapeuticor drug target development.

3.4. Structural Stability Analysis of Enzymes Using nsSNPs. We predicted the structural changes in ZAP70 caused by nsSNPs with structural modeling and stability analysis because mutations can cause selective T-cell defects [46]. Known homologous structures of ZAP70, PDB entries 1M61 and 1U59 (100% and 98.9% sequence identity), were used as structural templates. Currently, 19 nsSNPs in the dbSNP database have been reported to cause nonsense or missense changes in ZAP70. Using the CUPSAT program, we found 10 nsSNPs that cause unstable structural changes.

Table 2: Selected hub proteins in the protein interaction network constructed from downregulatedgenes.

Gene symbol	*Refseq ID*	*Full name (enzyme number)*	*Topological metrics*
EIF1B	NP_005866.1	Translation factor sui1 homolog	centrality: 41540
RNPS1	NP_006702.1	RNA binding protein S1, serine-rich domain	degree: 21
EGFR	**NP_005219.2**	**EGF receptor (2.7.10.1: Receptor protein-tyrosine kinase)**	**centrality: 83945**
NINL	NP_079452.3	KIAA0980 protein	centrality: 53502
HTT	NP_002102.4	Huntingtin	centrality: 99972
HMGB1	NP_002119.1	High mobility group box 1	centrality: 43032
APP	NP_000475.1	Amyloid beta A4 protein	centrality: 51716
RIF1	NP_001171134.1	Rap1 interacting factor 1	degree: 17
PLSCR4	NP_001121778.1	Phospholipid scramblase 4	centrality: 60954
BCL6	NP_001124317.1	B cell lymphoma 6 protein	degree: 18
TBP	NP_001165556.1	TATA box binding protein	centrality: 40738
SUMO1	NP_001005781.1	SMT3 suppressor of mif two 3 homolog 1	degree: 21
UNC119B	NP_001074002.1	Unc-119 homolog B	centrality: 42366
NCOR1	NP_001177367.1	Nuclear receptor corepressor 1	degree: 16
UTP14C	NP_067677.4	UTP14, U3 small nucleolarribonucleoprotein, homolog C	centrality: 60679

Table 3: Nonsynonymous SNPs of ZAP70.

rsNumber	*DNA substitution*	*Amino-acid substitution*	*Overall stability*	*Predicted ΔΔG (kcal/mol)*
rs56077145	**106C > G**	**Leu36Val**	**Destabilising**	**−1.03**
rs113994172	239C > A	Pro80Gln	Stabilising	0.82
rs56264206	308A > T	Asn103Ile	Stabilising	0.71
rs55845489	**309C > A**	**Asn103Lys**	**Destabilising**	**−0.36**
rs55679020	311G > C	Arg104Pro	Stabilising	0.11
rs111771234	550T > A	Phe115Ile	Stabilising	0.67
rs55964305	**524G > T**	**Arg175Leu**	**Destabilising**	**−0.67**
rs56403250	572C > T	Pro191Leu	Stabilising	0.08
rs76059124	**653C > T**	**Ala218Val**	**Destabilising**	**−1.86**
rs113310375	**1274T > C**	**Val356Ala**	**Destabilising**	**−2.01**
rs113994174	**1393C > T**	**Arg465Cys**	**Destabilising**	**−0.81**
rs56059280	**1468G > A**	**Asp490Asn**	**Destabilising**	**−0.29**
rs104893674	**1554C > A**	**Ser518Arg**	**Destabilising**	**−0.56**
rs56189815	1568G > T	Trp523Leu	Stabilising	3.36
rs56146954	**1580T > G**	**Val527Gly**	**Destabilising**	**−4.09**
rs113994175	1714A >T	Met572Leu	Stabilising	0.17
rs55803111	1781G > A	Arg594Gln	Stabilising	1.11
rs56326640	**1783G > T**	**Ala595Ser**	**Destabilising**	**−0.36**
rs56250717	1826G > A	Gly609Asp	—	—

The nsSNPs are shown in Table 3. In particular, Val527Gly had the lowest unstable energy value. The native structure and changed structures are shown in Figure 2. Although there is no experimental evidence which shows that nsSNPs regulate the expression level in T-cells, stability analysis of the target structure can give us more biological information to understand the enzymatic mechanism and to evaluate the drug effect.

4. Conclusion

In this study, we attempted to explore potential therapeutic candidates for psoriasis by utilizing bioinformatic methods. We have presented essential proteins from upregulated and downregulated genes in psoriasis; some of these findings have been supported by experimental evidence reported in the literature. Of particular interest are the predicted

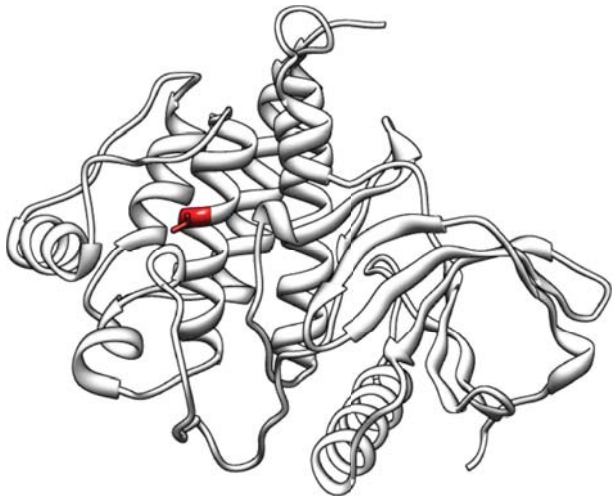


Figure 2: Protein structure of ZAP70 and structural variations by nsSNPs. We predicted the structural changes in ZAP70 caused by nsSNPs through structural modeling and stability analysis because mutations can cause selective T-cell defects [46]. In order to predict the structure of ZAP70, we used PDB entries 1M61 and 1U59 as the structural templates, which have 100% and 98.9% sequence identity, respectively, which were found to be suitable structural templates. We found 19 nsSNPs from the dbSNP database that cause nonsense or missense changes in ZAP70. From these nsSNPs, 10 nsSNPs were found to lead to unstable structural changes by using CUPSAT. In particular, Val527Gly had the lowest unstable energy value (Predicted $\Delta\Delta G$: −4.09). The red region shows the location of Val527Gly in the middle of the alpha-helix structure.

essential enzymes that are important proteins related to the pathogenesis of psoriasis; these enzymes can be explored as therapeutic or drug target candidates. Further studies should be conducted to determine the role of these candidate enzymes in psoriasis and to explore agonists of the upregulated candidates or antagonists of downregulated candidates as drug targets by exploiting the property of multiple targeting. These results will aid future drug discovery efforts, enabling drug development in a more timely and cost-effective manner.

Abbreviations

SAM: Significance analysis of microarray
PPI: Protein-protein interactions
SNP: Single-nucleotide polymorphism.

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Research Article

Computational Simulations to Predict Creatine Kinase-Associated Factors: Protein-Protein Interaction Studies of Brain and Muscle Types of Creatine Kinases

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Creatine kinase (CK; EC 2.7.3.2) is related to several skin diseases such as psoriasis and dermatomyositis. CK is important in skin energy homeostasis because it catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine. In this study, we predicted CK binding proteins via the use of bioinformatic tools such as protein-protein interaction (PPI) mappings and suggest the putative hub proteins for CK interactions. We obtained 123 proteins for brain type CK and 85 proteins for muscle type CK in the interaction networks. Among them, several hub proteins such as NFKB1, FHL2, MYOC, and ASB9 were predicted. Determination of the binding factors of CK can further promote our understanding of the roles of CK in physiological conditions.

1. Introduction

Creatine kinase (CK) (ATP: creatine kinase N-phosphotransferase, EC 2.7.3.2) is thought to be crucial for intracellular transport and the storage of high energy phosphate because it catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine, which leads to the creation of phosphocreatine and MgADP [1]. CK plays an important role in the cellular energy metabolism of vertebrates, and it is widely distributed in tissues that require a lot of energy [2]. Several types of CK are expressed in various tissues: the muscle and brain types of CK are the most common, and three different isoenzymes that include CK-MM (the muscle type homodimer), CK-BB (the brain type homodimer), and CK-MB (the muscle plus brain type heterodimer) originate from these two common types. CK is an important serum marker for myocardial infarction. Various types of CKs (the muscle, brain, and mitochondrial types) are thought to be important not only in the diagnosis of myocardial infarction, cardiac hypertrophy, and muscular dystrophy but also for studies of some other serious diseases, including Alzheimer's disease, Parkinson's disease, and psoriasis [3–8].

CK-BB is associated with several pathologies, including neurodegenerative and age-related diseases. Recently, Chang et al. [9] reported an important role for CK-BB in osteoclast-mediated bone resorption, which was found using a proteomics approach. They found that CK-BB is greatly increased during osteoclastogenesis and suggested that it represents a potential target for antiresorptive drug development. CK-BB interacts with the potassium-chloride cotransporter 3, which is involved in the pathophysiology of hereditary motor and sensory neuropathy with agenesis of the corpus callosum [10]. Previous studies [11, 12] have reported that CK-BB is involved in Alzheimer's disease (AD) as an oxidatively modified protein. This suggests that oxidatively damaged CK-BB may be associated with aging and age-related neurodegenerative disorders such as AD.

CK-MM is a good model to use for studying folding pathways because of several characteristics: (i) it is a dimer

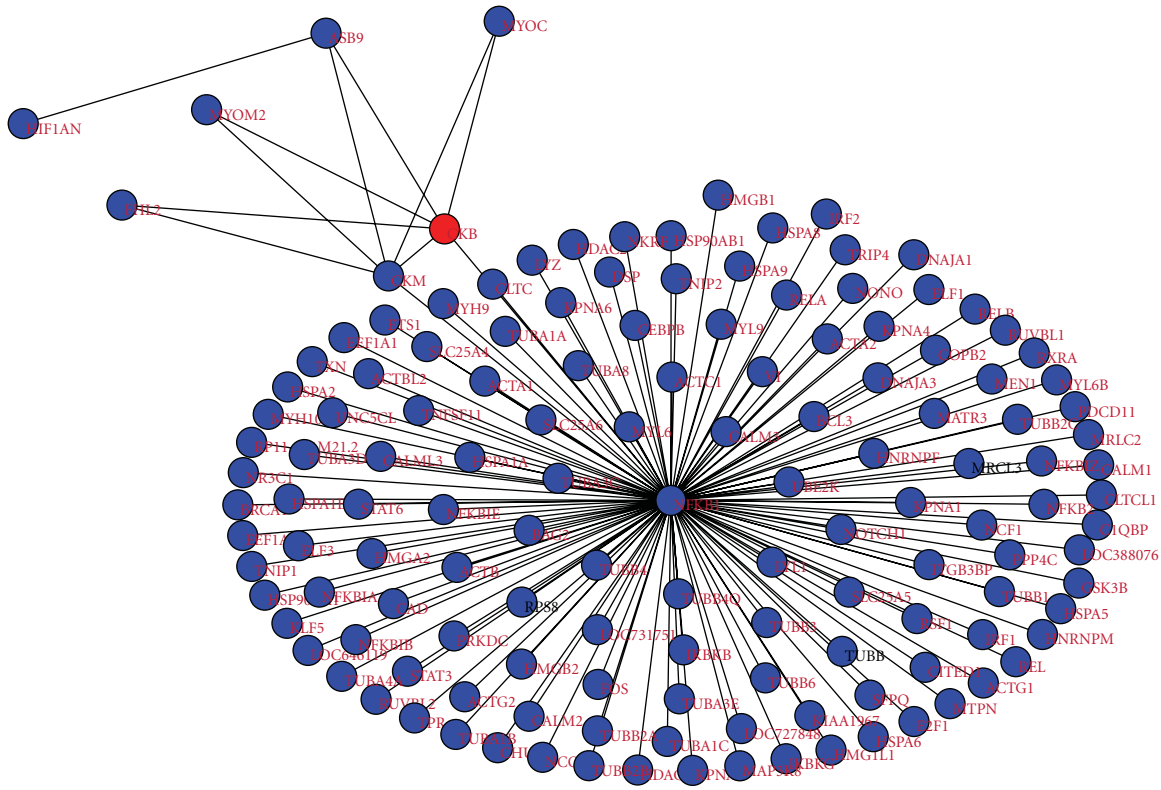

Figure 1: PPI map for CKB as a target hub protein with the 80% identity. Labels with red color indicate the hub protein of targeting. The image was made by the aiSee program (http://www.aisee.com/).

that consists of two identical subunits, each with an N-terminal domain with about 100 residues and a C-terminal domain with about 250 residues connected by a long linker [13]; (ii) extensively denatured CK can be renatured spontaneously with restoration of its enzymatic activity in the absence of any external assistance [14]; (iii) its folding pathway is complicated and involves several intermediates [15, 16]; (iv) conformational changes of the secondary and tertiary structures can be easily measured by monitoring activity changes [14, 15]; (v) protein-protein interactions, including molecular chaperones, are observed during refolding [17, 18].

In this study, we obtained computational predictions of the binding proteins by using two types of CK (CK-BB and CK-MM) as hub proteins in bioinformatic algorithms. As a result, we obtained 208 protein lists in the interaction networks via application of both muscle and brain types of CK. Determination of the binding factors and functions of CK can further promote our understanding of the physiological roles of CK.

2. Materials and Methods

2.1. PPI Mappings: PEIMAP and PSIMAP Algorithms. We present the functionally classified protein-protein interactions on the basis of the cell cycle, cell transport, oxidoreductase, and apoptosis. PPI resources were assembled from a combination of several experimental protein interaction databases. The protein interaction resources included six databases: DIP [19], BIND [20], IntAct [21], MINT [22], HPRD [23], and BioGrid [24]. We performed a redundancy test to remove identical protein sequences from the interaction databases. The databases contain 116,773 proteins and 229,799 interactions.

PPI prediction uses most of the major types of PPI algorithms. They are (1) Protein Structural Interactome MAP (PSIMAP), a method that uses the structural domain of the SCOP (Structural Classification of Proteins) database [25] and (2) Protein Experimental Interactome MAP (PEIMAP), a common method that uses public resources of experimental protein interaction information such as HPRD, BIND, DIP, MINT, IntAct; and BioGrid. The basic procedure of PSIMAP is to infer interactions between proteins by using their homologs. Interactions among domains or proteins for known PDB (Protein Data Bank) structures are the basis for the prediction. If an unknown protein has a homolog to a domain, then PSIMAP assumes that the query has the probability to interact with its homolog's partners. This concept is called "homologous interaction." The original interaction between two proteins or domains is based on the Euclidean distance. Therefore, PSIMAP gives a structure-based interaction prediction [26]. PEIMAP was constructed by combining several experimental protein-protein interaction databases. We carried out a redundancy check to remove identical protein sequences from the source interaction

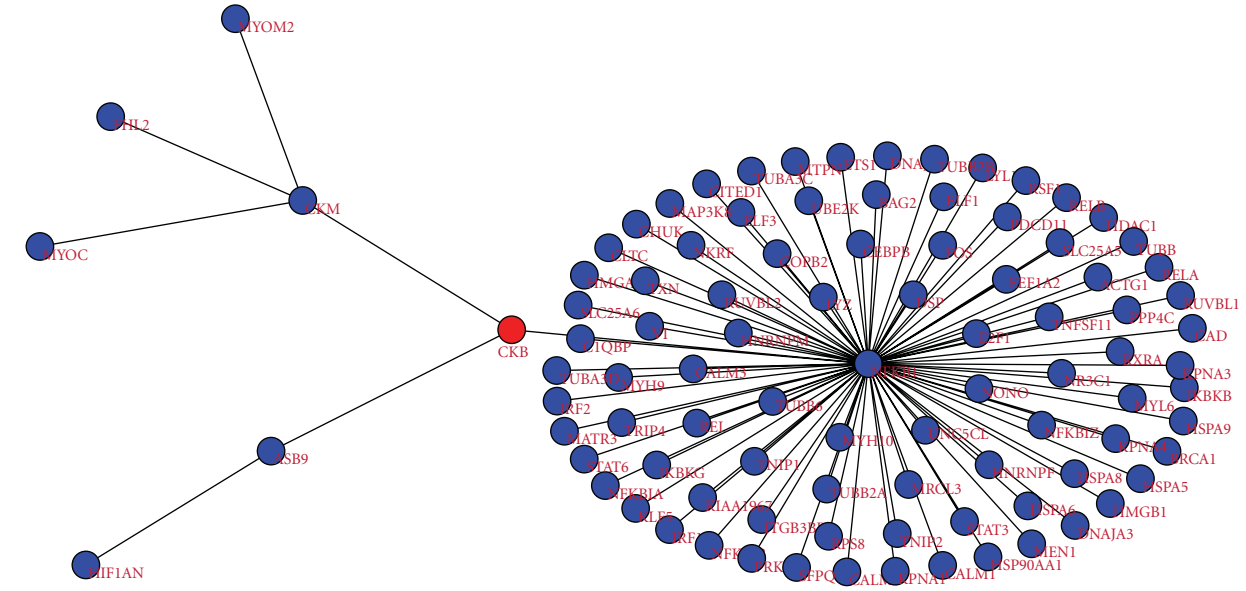

Figure 2: PPI map for CKB as a target hub protein with the 100% identity. The methodological conditions were the same as for Figure 1 except the identity.

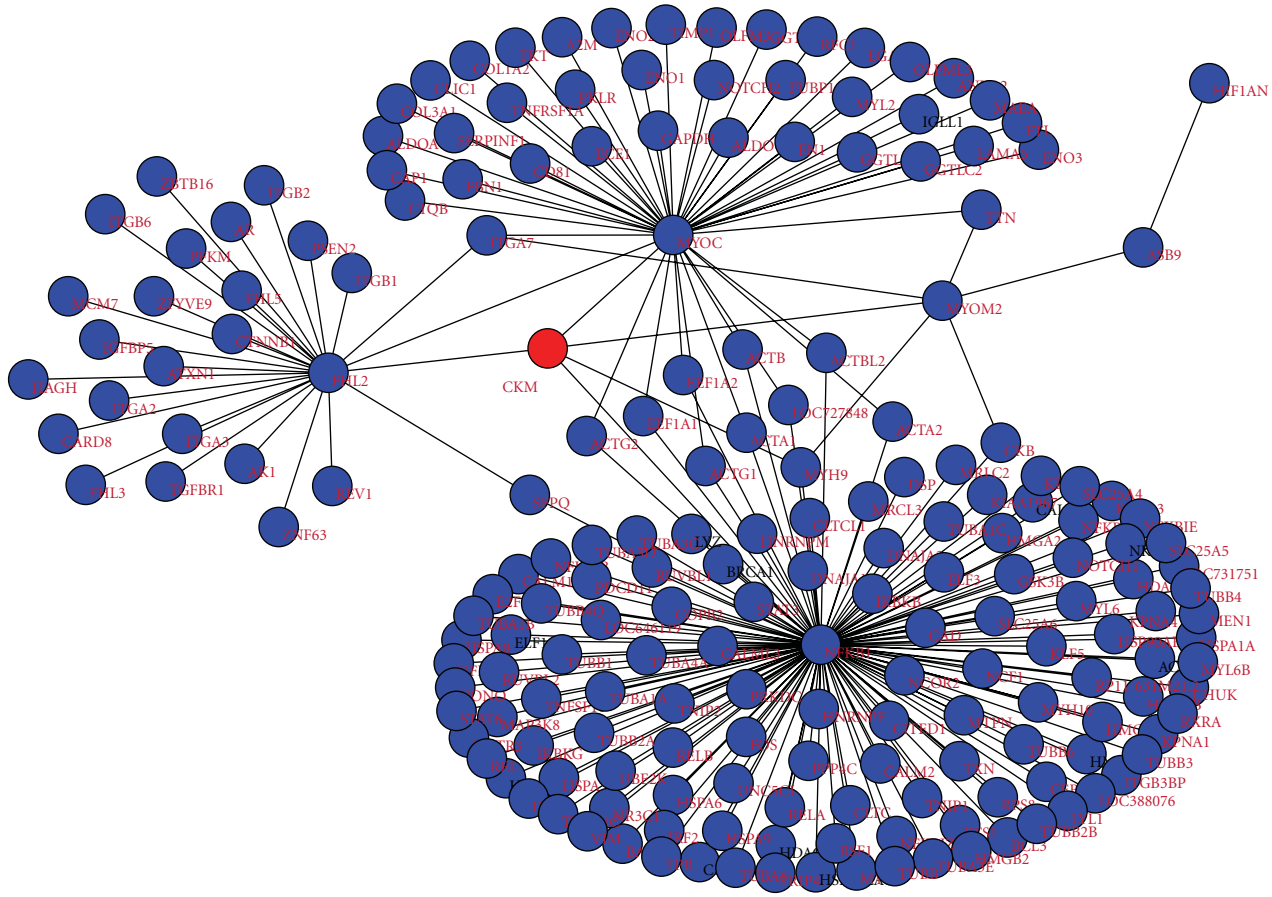

Figure 3: PPI map for CKM as a target hub protein with the 80% identity. Labels with red color indicate the hub protein of targeting. The image was made by the aiSee program (http://www.aisee.com/).

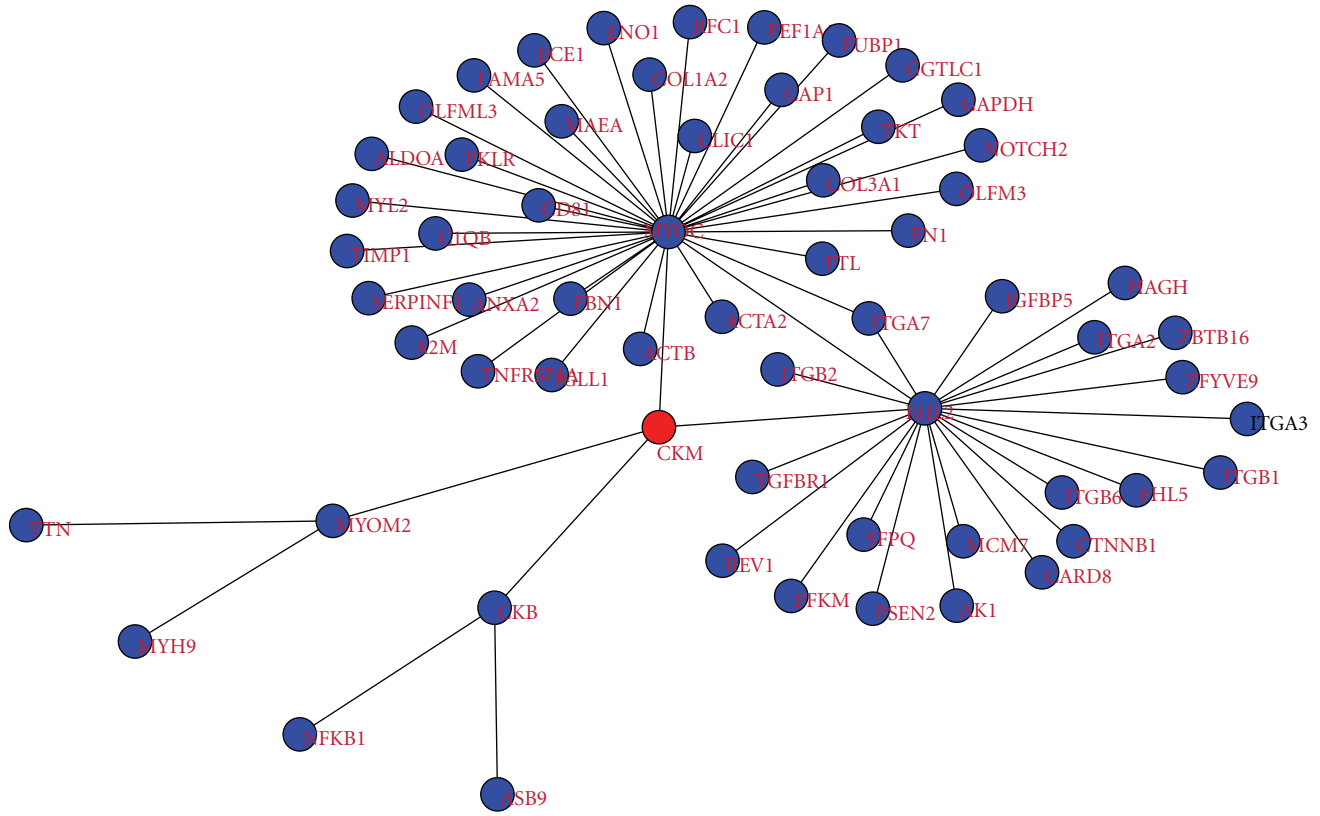

Figure 4: PPI map for CKM as a target hub protein with the 100% identity. The methodological conditions were the same as for Figure 3 except the identity.

databases. The image was made by the Pajak2.00 program (http://vlado.fmf.uni-lj.si/pub/networks/pajek/).

3. Results and Discussion

We identified potential candidates through protein-protein interaction predictions made using various protein interaction resources. By analyzing the hub protein of the networks with metrics such as degree and centrality, we detected 123 potential candidates for CKB interacting (direct or indirect) factors and 85 candidates for CKM.

In Figure 1, interacting factors such as NFKB1 (NP_003989, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), MYOC (NP_000252; myocilin, trabecular meshwork inducible glucocorticoid response), MYOM2 (NP_003961; myomesin (M-protein) 2, 165 kDa), FHL2 (NP_001034581, four-and-a-half LIM domains 2), HIF1AN (NP_060372, hypoxia-inducible factor 1, alpha subunit inhibitor), ASB9 (NP_076992, ankyrin repeat and SOCS box-containing 9), and CKM (NP_001815, creatine kinase, muscle) were elucidated. Interestingly, NFKB1 was detected as a hub protein interacting with CK-BB in our results. In Figure 2, we obtained results similar to those from Figure 1, where NFKB1, MYOC, MYOM2, FHL2, HIF1AN, ASB9, and CKM were detected as interacting factors that were directly or indirectly associated with CKB. NFKB1, CKM, and ASB9 interacted with CKB directly.

In the same way, we detected the CKM-associated proteins as shown in Figure 3 with 80% sequence identity. As a result, we found that CKB, FHL2, MYOC, ASB9, HIF1AN, NFKB1, TTN (NP_596870, titin), MYH9 (NP_002464, myosin, heavy chain 9, non-muscle), and ITGA7 (NP_002197, integrin, alpha 7) mainly interacted with CKM at 80% sequence identity. At the level of 100% identity, we found that MYOM2, CKB, FHL2, and MYOC directly interacted with CKM as shown in Figure 4. In addition to these factors, complete lists of factors that interacted with CKB and CKM in a direct or indirect manner are shown in Tables 1 and 2. After overlapping the results from Figures 1 to 4, we found that NFKB1, FHL2, and MYOC were still detected as hub proteins in Figure 5.

NFKB1 (also known as p50 or NF-kappaB) is a well-known transcription regulator that is responsible for the expression and regulation of many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis, and apoptosis [27–31]. It translocates into the nucleus and stimulates the expression of many genes involved in various biological functions. NFKB1 is also associated with a number of inflammatory diseases such as lymphoma [32], Alzheimer disease [33], psoriatic arthritis [34], breast cancer [35, 36], and rheumatoid arthritis [37]. Activation of NFKB1 requires binding of NF-kappaB essential modulator (NEMO) to ubiquitinated substrates [38]. With respect to an association with CK, it has been reported that NFKB1 is mostly associated with myocardial ischemia/reperfusion.

Table 1: Gene lists for the analyses of the PEIMAP and PSIMAP using CK-BB as a hub protein with 100% identity.

Gene ID	Gene symbol	Full name
6256	RXRA	Retinoid X receptor, alpha
3309	HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)
3320	HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
6778	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced
3146	HMGB1	High-mobility group box 1
3301	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1
4221	MEN1	Multiple endocrine neoplasia I
3312	HSPA8	Heat shock 70 kDa protein 8
3840	KPNA4	Karyopherin alpha 4 (importin alpha 3)
2274	FHL2	Four-and-a-half LIM domains 2
4792	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
57805	KIAA1967	KIAA1967
3185	HNRNPF	Heterogeneous nuclear ribonucleoprotein F
203068	TUBB	Tubulin, beta
6774	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
4670	HNRNPM	Heterogeneous nuclear ribonucleoprotein M
1997	ELF1	E74-like factor 1 (ets domain transcription factor)
113457	TUBA3D	Tubulin, alpha 3D
1999	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
5591	PRKDC	Protein kinase, DNA-activated, catalytic polypeptide
708	C1QBP	Complement component 1, q subcomponent binding protein
2274	FHL2	Four-and-a-half LIM domains 2
3313	HSPA9	Heat shock 70 kDa protein 9 (mortalin)
8600	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11
3659	IRF1	Interferon regulatory factor 1
84617	TUBB6	Tubulin, beta 6
7280	TUBB2A	Tubulin, beta 2A
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
8517	IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
7295	TXN	Thioredoxin
10318	TNIP1	TNFAIP3 interacting protein 1
4793	NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
3065	HDAC1	Histone deacetylase 1
3551	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
1152	CKB	Creatine kinase, brain
4069	LYZ	Lysozyme (renal amyloidosis)
140462	ASB9	Ankyrin repeat and SOCS box-containing 9
4653	MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response
6774	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
3660	IRF2	Interferon regulatory factor 2
7278	TUBA3C	Tubulin, alpha 3c
4221	MEN1	Multiple endocrine neoplasia I
5966	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)
1147	CHUK	Conserved helix-loop-helix ubiquitous kinase
55922	NKRF	NFKB repressing factor
2113	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
64332	NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

Table 1: Continued.

Gene ID	Gene symbol	Full name
51773	RSF1	Remodeling and spacing factor 1
5971	RELB	v-rel reticuloendotheliosis viral oncogene homolog B
1832	DSP	Desmoplakin
347733	TUBB2B	Tubulin, beta 2B
2353	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
9325	TRIP4	Thyroid hormone receptor interactor 4
4435	CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1
22984	PDCD11	Programmed cell death 11
790	CAD	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
1326	MAP3K8	Mitogen-activated protein kinase kinase kinase 8
1917	EEF1A2	Eukaryotic translation elongation factor 1 alpha 2
9172	MYOM2	Myomesin (M-protein) 2, 165 kDa
10856	RUVBL2	RuvB-like 2 (E. coli)
1158	CKM	Creatine kinase, muscle
808	CALM3	Calmodulin 3 (phosphorylase kinase, delta)
672	BRCA1	Breast cancer 1, early onset
801	CALM1	Calmodulin 1 (phosphorylase kinase, delta)
293	SLC25A6	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
3310	HSPA6	Heat shock 70 kDa protein 6 (HSP70B′)
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
136319	MTPN	Myotrophin
2274	FHL2	Four-and-a-half LIM domains 2
9093	DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3
4628	MYH10	Myosin, heavy chain 10, non-muscle
4221	MEN1	Multiple endocrine neoplasia I
6774	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
3839	KPNA3	Karyopherin alpha 3 (importin alpha 4)
57805	KIAA1967	KIAA1967
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
1869	E2F1	E2F transcription factor 1
55662	HIF1AN	Hypoxia-inducible factor 1, alpha subunit inhibitor
79155	TNIP2	TNFAIP3 interacting protein 2
9532	BAG2	BCL2-associated athanogene 2
6421	SFPQ	Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
10627	MRCL3	Myosin regulatory light chain MRCL3
7431	VIM	Vimentin
672	BRCA1	Breast cancer 1, early onset
2274	FHL2	Four-and-a-half LIM domains 2
4221	MEN1	Multiple endocrine neoplasia I
672	BRCA1	Breast cancer 1, early onset
4221	MEN1	Multiple endocrine neoplasia I
3836	KPNA1	Karyopherin alpha 1 (importin alpha 5)
3093	UBE2K	Ubiquitin-conjugating enzyme E2K (UBC1 homolog, yeast)
805	CALM2	Calmodulin 2 (phosphorylase kinase, delta)
5970	RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
9782	MATR3	Matrin 3
8600	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11
8607	RUVBL1	RuvB-like 1 (E. coli)

Table 1: Continued.

Gene ID	Gene symbol	Full name
4627	MYH9	Myosin, heavy chain 9, nonmuscle
23421	ITGB3BP	Integrin beta 3 binding protein (beta3-endonexin)
140462	ASB9	Ankyrin repeat and SOCS box-containing 9
4841	NONO	Non-POU domain containing, octamer-binding
9276	COPB2	Coatomer protein complex, subunit beta 2 (beta prime)
4221	MEN1	Multiple endocrine neoplasia I
1213	CLTC	Clathrin, heavy chain (Hc)
292	SLC25A5	Solute carrier family 25 (Mitochondrial carrier; adenine nucleotide translocator), member 5
4066	LYL1	Lymphoblastic leukemia-derived sequence 1
64332	NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
5531	PPP4C	Protein phosphatase 4 (formerly X), catalytic subunit
8091	HMGA2	High-mobility group AT-hook 2
6202	RPS8	Ribosomal protein S8
1051	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
222643	UNC5CL	Unc-5 homolog C (C. elegans)-like
4790	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
71	ACTG1	Actin, gamma 1
3312	HSPA8	Heat shock 70 kDa protein 8
9782	MATR3	Matrin 3
3320	HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1
4637	MYL6	Myosin, light chain 6, alkali, smooth muscle and nonmuscle
2908	NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
4793	NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
688	KLF5	Kruppel-like factor 5 (intestinal)
672	BRCA1	Breast cancer 1, early onset

During reperfusion, the absence of poly(ADP-ribose) polymerase-1 (PARP-1) leads to a reduction of myocardial apoptosis, which is associated with reduced NFKB1 activation [39, 40], and proteasome inhibition ablates activation of NFKB1 in myocardial reperfusion and reduces reperfusion injury [41]. Myocardial injury was assessed by measuring the serum levels of CK, and CK was reduced in serum along with reduction of NFKB1 activation.

FHL2 is a member of the human four-and-a-half-LIM-only protein family, which consists of the members FHL1, FHL2, FHL3, FHL4, and ACT. These proteins function in various cellular processes, including regulation of cell survival, transcription, and signal transduction [42]. FHL2 contains an LIM domain, one of the protein-protein interaction motifs, which allows specific proteins to combine with certain partners. The specificity of a protein-protein interaction can be obtained by an interaction code predicted by conserved amino acid sequences. The interaction of FHL2 with transcription factors and other proteins involved in cancer development was examined. Since transcription factors control all fundamental developmental and homeostatic processes, transcriptional cofactors such as FHL2 are likely to contribute to human carcinogenesis and are of clinical importance in various forms of cancer [43], including leukemia [44]. With respect to an association with CK, Chung et al. [45] reported that FHL2 (developmentally enhanced phosphotransfer enzyme-anchoring protein) amalgamated the myofibrillar CK metabolic signaling circuit, providing an energetic continuum between mitochondria and the nascent contractile machinery in a murine embryonic stem cell cardiac differentiation model. They reported that CK-M clustered around developing myofibrils, sarcolemma, and the perinuclear compartment, whereas CK-B was tightly associated with myofibrillar alpha-actinin, forming wire-like structures extending from the nuclear compartment to the sarcolemma. FHL2 was also increased in myocardial ischemia-reperfusion injury, where IL-6 and IL-8 mRNA are upregulated in human cardiac myocytes [46].

Recently, ASB9 was found to interact with ubiquitous mitochondrial CK [47]. The ankyrin repeat domains of ASB9 can associate with the substrate binding site of CK in a SOCS box-independent manner. The overexpression of ASB9 induces ubiquitination of CK. ASB9 reduces CK activities and cell growth and negatively regulates cell growth. ASB9 is a member of the ankyrin repeat and is a suppressor of the cytokine signaling (SOCS) box protein family. It can interact with the SOCS box domain of the elongin B-C adapter complex and can further complex with the cullin and ring box proteins to form E3 ubiquitin ligase complexes [48]. These complexes may be involved in specific substrate-recognition for ubiquitination and degradation and mediate the substrate-recognition of the E3 ubiquitin ligases.

Table 2: Gene lists for the analyses of the PEIMAP and PSIMAP using CK-MM as a hub protein with 100% identity.

Gene ID	Gene symbol	Full name
1889	ECE1	Endothelin-converting enzyme 1
5981	RFC1	Replication factor C (activator 1) 1, 145 kDa
226	ALDOA	Aldolase A, fructose-bisphosphate
2335	FN1	Fibronectin 1
9372	ZFYVE9	Zinc finger, FYVE domain containing 9
60	ACTB	Actin, beta
3688	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
7273	TTN	Titin
2274	FHL2	Four-and-a-half LIM domains 2
4853	NOTCH2	Notch homolog 2 (Drosophila)
2512	FTL	Ferritin, light polypeptide
1192	CLIC1	Chloride intracellular channel 1
2274	FHL2	Four-and-a-half LIM domains 2
5313	PKLR	Pyruvate kinase, liver and RBC
302	ANXA2	Annexin A2
7704	ZBTB16	Zinc finger and BTB domain containing 16
2200	FBN1	Fibrillin 1
27332	ZNF638	Zinc finger protein 638
92086	GGTLC1	Gamma-glutamyltransferase light chain 1
713	C1QB	Complement component 1, q subcomponent, B chain
3029	HAGH	Hydroxyacylglutathione hydrolase
5664	PSEN2	Presenilin 2 (Alzheimer disease 4)
7086	TKT	Transketolase (Wernicke-Korsakoff syndrome)
4176	MCM7	Minichromosome maintenance complex component 7
1152	CKB	Creatine kinase, brain
1499	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa
140462	ASB9	Ankyrin repeat and SOCS box-containing 9
9457	FHL5	Four-and-a-half LIM domains 5
4653	MYOC	Myocilin, trabecular meshwork inducible glucocorticoid response
3029	HAGH	Hydroxyacylglutathione hydrolase
7704	ZBTB16	Zinc finger and BTB domain containing 16
3675	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
226	ALDOA	Aldolase A, fructose-bisphosphate
3689	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
3688	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
302	ANXA2	Annexin A2
92086	GGTLC1	Gamma-glutamyltransferase light chain 1
3679	ITGA7	Integrin, alpha 7
2023	ENO1	Enolase 1, (alpha)
9172	MYOM2	Myomesin (M-protein) 2, 165 kDa
1158	CKM	Creatine kinase, muscle
4790	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
2	A2M	Alpha-2-macroglobulin
3688	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
56944	OLFML3	Olfactomedin-like 3
1281	COL3A1	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
2274	FHL2	Four-and-a-half LIM domains 2
3688	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)

Table 2: Continued.

Gene ID	Gene symbol	Full name
118427	OLFM3	Olfactomedin 3
22900	CARD8	Caspase recruitment domain family, member 8
3488	IGFBP5	Insulin-like growth factor binding protein 5
7132	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
226	ALDOA	Aldolase A, fructose-bisphosphate
3675	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
51455	REV1	REV1 homolog (S. cerevisiae)
6421	SFPQ	Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)
302	ANXA2	Annexin A2
4633	MYL2	Myosin, light chain 2, regulatory, cardiac, slow
8880	FUBP1	Far upstream element (FUSE) binding protein 1
2274	FHL2	Four-and-a-half LIM domains 2
3688	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
27332	ZNF638	Zinc finger protein 638
3673	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
203	AK1	Adenylate kinase 1
4627	MYH9	Myosin, heavy chain 9, non-muscle
5213	PFKM	Phosphofructokinase, muscle
140462	ASB9	Ankyrin repeat and SOCS box-containing 9
7076	TIMP1	TIMP metallopeptidase inhibitor 1
5176	SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1
3694	ITGB6	Integrin, beta 6
59	ACTA2	Actin, alpha 2, smooth muscle, aorta
4176	MCM7	Minichromosome maintenance complex component 7
10487	CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)
7046	TGFBR1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53 kDa)
3543	IGLL1	Immunoglobulin lambda-like polypeptide 1
5313	PKLR	Pyruvate kinase, liver and RBC
51455	REV1	REV1 homolog (S. cerevisiae)
10296	MAEA	Macrophage erythroblast attacher
3911	LAMA5	Laminin, alpha 5
2597	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
975	CD81	CD81 molecule
92086	GGTLC1	Gamma-glutamyltransferase light chain 1
1915	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
5664	PSEN2	Presenilin 2 (Alzheimer disease 4)
1278	COL1A2	Collagen, type I, alpha 2

The interaction between CK and MYOC has not been elucidated. However, MYOC has a cytoskeletal function, and this implies that it may interact with CK somehow. MYOC is expressed in many ocular tissues including the trabecular meshwork [49], which is a specialized eye tissue that is essential in regulating intraocular pressure. MYOC mutations have been identified as the cause of hereditary juvenile-onset open-angle glaucoma [50].

Researchers could apply computational prediction by PPI mapping to help determine target proteins. Since the next step in the functional study of interesting proteins/genes is a time- and cost-consuming process, the number of target proteins is limited; hence, for the right choice, computational prediction on the basis of database information could be critical at this step. Functional studies can be further conducted using a mouse model and a large number of clinical samples. Final confirmation and CK mechanisms could then be more clearly evaluated for developing drugs to effectively treat CK-related diseases.

The functions of most of the candidate proteins predicted in this study have not been well reported in skin diseases or in the pathogenesis of other diseases. We provide new

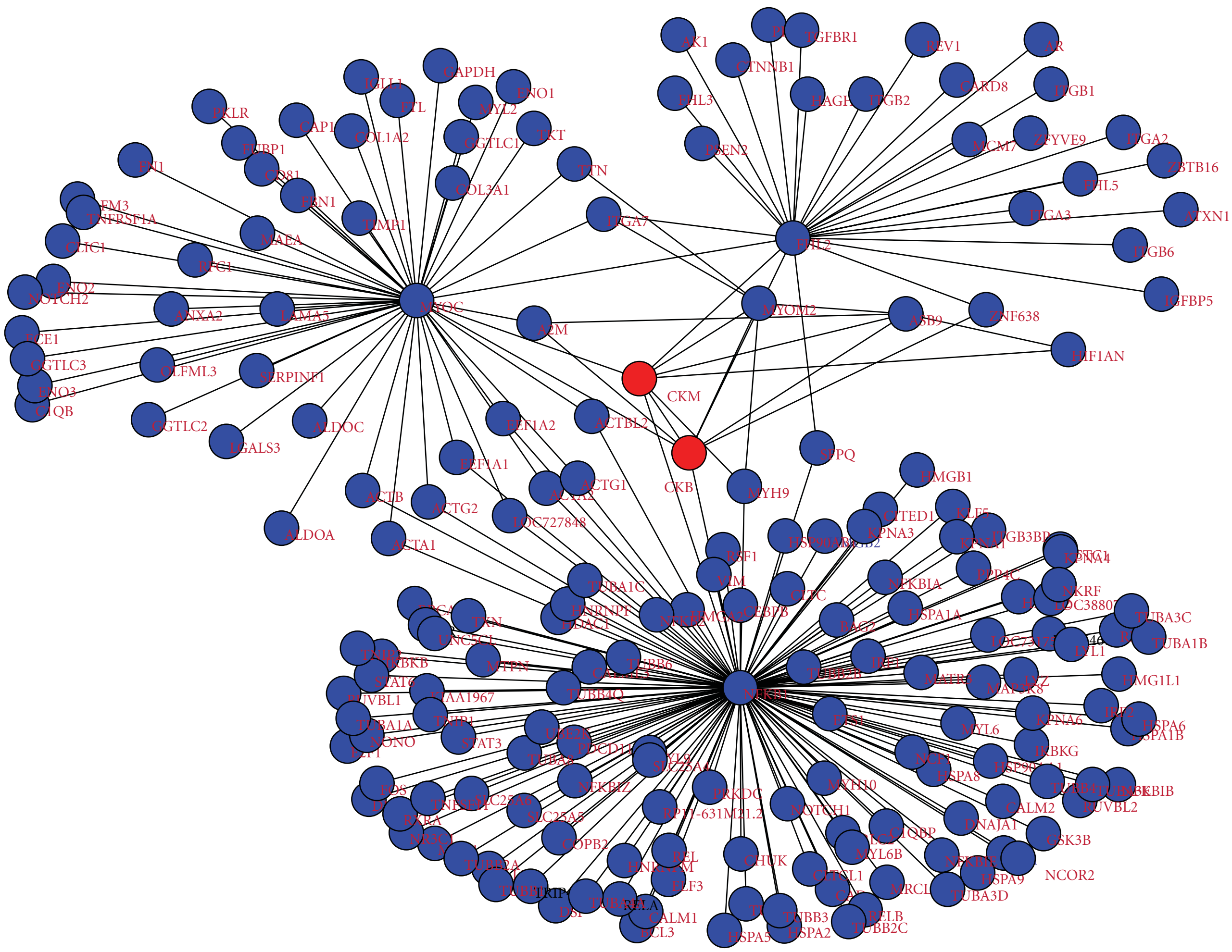

Figure 5: Overlapping map between CKB and CKM PPI maps. Data were input by using the results from Figures 1 to 4.

information regarding these candidate proteins' interaction with CK, as well as the involvement of several hub proteins such as NFKB1, FHL2, ASB9, and MYOC. Although we do not suggest a direct role of any candidate protein in skin diseases, we provide candidate proteins to be targeted in further studies of CK-associated diagnostic markers and/or treatment of corresponding skin conditions. Furthermore, we also provide some insights into understanding the responses of CK in skin.

Abbreviations

PPI: Protein-protein interaction
CK-MM: Muscle type homodimer
CK-BB: Brain type homodimer.

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