

Research Article

Regulating the Wnt/ β -catenin Signaling Pathway Promotes Repigmentation in Vitiligo Using Fire Needle Therapy

Yan-Li Xu,^{1,2} Guang-Mei Sun,² Jin-Mei Zhang,² Yu-Han Ma,¹ Guang-Zhi Li,² Lu Zhang,² Fang Cheng,² and Bao-Xiang Zhang ²

¹School of Clinical Medicine, Shandong Second Medical University, 7166 Baotong West Street, Weifang, Shandong 261053, China

²Department of Dermatology, Yidu Central Hospital of Weifang, 5168 Jiangjunshan Road Qingzhou, Weifang, Shandong 262500, China

Correspondence should be addressed to Bao-Xiang Zhang; zhangbx666@126.com

Received 10 January 2024; Revised 23 February 2024; Accepted 29 February 2024; Published 11 March 2024

Academic Editor: Nawaf Al Mutairi

Copyright © 2024 Yan-Li Xu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background. Vitiligo is a dermatological disorder characterized by the depletion of melanocytes. The key to its treatment lies in the promotion of melanin regeneration. The Wnt/ β -catenin signalling pathway assumes a pivotal role in this regenerative process. Fire needle therapy (FNT), a traditional Chinese medical technique, has emerged as a promising intervention. **Methods.** We analyzed gene expression associated with the Wnt/ β -catenin signalling pathway in both normal skin and the lesions of patients with vitiligo in the Gene Expression Omnibus database (GEO database). Furthermore, we evaluated the gene expression of this pathway and assessed the effects of FNT on pigmentation and its influence on the Wnt/ β -catenin signalling pathway in a vitiligo mouse model. **Results.** Compared to that of the normal skin, the mRNA levels of *WNT10A*, *WNT10B*, *WNT2*, *WNT2B*, *WNT3*, *WNT3A*, *WNT4*, *WNT5B*, *WNT7B*, and *LEF1* displayed significant decreases in the lesions of patients with vitiligo. Moreover, vitiligo mouse skin lesions improved following FNT, and the number of melanocytes and melanin levels increased. In addition, posttreatment, genes associated with the Wnt/ β -catenin signalling pathway were revealed to be upregulated. **Conclusion.** FNT may play an important role in promoting pigmentation in vitiligo through modulation of the Wnt/ β -catenin signalling pathway. This adds to the growing body of literature supporting the use of FNT to treat vitiligo. This, in turn, may inform clinical practice and improve patient outcomes.

1. Introduction

Vitiligo is an autoimmune disorder characterized by the progressive loss of melanocytes in the epidermis with an estimated global prevalence of between 0.5% and 2% [1]. The typical lesion of vitiligo is well-demarcated, nonscaly milky white macules. It can occur anywhere in the body. The disorder can significantly affect the patient's quality of life, potentially leading to the development of mental health conditions, such as anxiety [2] and depression [3]. The current primary treatment strategies for vitiligo involve the systemic administration of glucocorticoids, local application of calcineurin inhibitors, and vitamin D3 derivatives. In addition, localized phototherapy is widely employed, including, narrowband ultraviolet B (NBUVB) and 308-nm

excimer laser therapy [4]. However, these treatments often fall short of their desired efficacy. Hence, the exploration of novel treatments for vitiligo is imperative.

Clinical observations during the treatment of vitiligo have highlighted perifollicular repigmentation patterns, where repigmentation spots emerge around hair follicles and gradually expand. This suggests that the hair follicle bulge serves as a melanocyte reservoir [5]. Melanocyte stem cells within the hair follicle may be induced to proliferate, differentiate, and migrate, thus replenishing the depigmented epidermis [6]. The Wnt/ β -catenin signalling pathway, also known as canonical Wnt signalling, plays a pivotal role in cell development and proliferation [7]. It is central to the proliferation and differentiation of these melanocyte stem cells (McSCs) [8, 9]. The differentiation of McSCs in both

epidermal and hair pigmentation processes necessitates the activation of the Wnt/ β -catenin signalling [10, 11]. Decreased β -catenin expression across the epidermal layer of vitiligo lesions has been observed by immunofluorescence staining assays [12]. Moreover, Wnt signalling regulates hair follicle signalling in the epidermis by coordinating dynamic intercellular communication between the epidermal and dermal layers [13]. This indicates that the activation of the Wnt signalling pathway might play a role in vitiligo repigmentation.

Fire needle therapy (FNT) is an ancient practice in traditional Chinese medicine and is utilized as a symptomatic treatment for vitiligo. FNT employs a slender needle which can withstand high temperatures. The needles are heated over an open flame until red-hot and are then swiftly inserted into vitiligo lesions [14]. Clinically, the technique has exhibited notable therapeutic outcomes [15]. In a mouse model of androgenetic alopecia treatment, fire needles stimulated hair growth by activating the Wnt/ β -catenin signalling pathway, thereby promoting the proliferation and differentiation of hair follicle melanocyte stem cells [16]. The hair follicle bulge, as a melanocyte reservoir, plays a significant role in vitiligo repigmentation. Currently, research exploring the relationship among the Wnt/ β -catenin signalling pathway, hair follicle melanocyte stem cells, and the pigment deposition induced by fire needles in vitiligo is lacking. We hypothesize that FNT may promote the recoloration of vitiligo lesions through this pathway. We tested this hypothesis by comparing the gene expression associated with the Wnt/ β -catenin signalling pathway in normal skin to that in the lesions of patients with vitiligo in the Gene Expression Omnibus database (GEO database). The database is an international public repository of functional genomic datasets, including high-throughput microarray, and next-generation sequencing data, all submitted by the research community. The data is indexed, cross-linked, and searchable [17]. Furthermore, we used a vitiligo mouse model to investigate the impact of FNT on pigmentation and its influence on the Wnt/ β -catenin signalling pathway.

2. Methods

2.1. Bioinformatics Analysis. We procured normalized total RNA expression levels from skin samples of vitiligo patients through the GEO database (with the accession numbers GSE75819 and GSE65127). This study conducted a differential gene expression analysis focusing on the Wnt/ β -catenin signalling pathway in vitiligo lesions compared to a control group, and the results were validated using the bootstrap hypothesis test.

2.2. Establishment of the Vitiligo Model and Groups. C57BL/6J male mice (7 weeks old) were purchased from the Animal Center at Weifang Medical College, Weifang, China. All research procedures were approved by the Weifang Medical Ethics Committee (project number 2022SDL004).

The vitiligo model was created following established protocols [18]. Briefly, mice were subcutaneously immunized in the hind footpad once a week for two weeks with TRP2-180 (50 μ g; Anaspec, Fremont, USA), lipopolysaccharide (LPS) (5 μ g; InvivoGen, San Diego, USA), and CpG-oligodeoxynucleotide (CpG-ODN) (5 μ g; InvivoGen). Subsequently, mice were intradermally injected twice in the tail with a one-week interval. After the final injection, the emergence of depigmentation in the tail area was witnessed over a span of four weeks.

The mice were randomly divided into four groups: Blank ($n=10$), Iwr-1 ($n=10$), Fire needle ($n=10$), and Sham ($n=10$). The fire needle group underwent fire needles once weekly for four weeks. In contrast, the Iwr-1 group received Iwr-1 (TargetMOL, Wellesley Hills, MA, USA) injections into the depigmented tail skin once weekly for four weeks. As previously reported [10], a 10 mM stock solution of Iwr-1 in DMSO was diluted with phosphate-buffered saline (PBS) to 0.1 mM and subsequently administered by injection into the tail skin of mice (4 μ g per cm²). The aim was to inhibit the Wnt signal pathway. The Sham treatment group did not receive any interventions post-modelling, while the Blank group remained procedure-free.

2.3. Fire Needle Therapy. The fire needle (1.5 inches in length, 0.25 mm in diameter) was applied to vitiligo lesions. Mice were immobilized without anaesthesia using a specialized apparatus after sterilizing the tail skin. An alcohol lamp was positioned close to the tail skin lesion. The fire needle tip and body were heated until they turned red. The designated point was swiftly pricked with the needle which was then promptly withdrawn. The fire needle was used at a depth of 0.5–1 mm, with a stimulation frequency of 30 times per square centimetre.

2.4. Haematoxylin and Eosin (H&E) Staining and Fontana-Masson Staining. The mice were euthanized using cervical dislocation, and their tail skin was shaved. Subsequently, the lesional skin and tail skin were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4 μ m) were stained with H&E. Fontana-Masson staining was carried out following the instructions provided for the Masson-Fontana melanin staining solution (Shangbao, Shanghai, China). After deparaffinization, tissue sections were hydrated and then exposed to the working Fontana silver ammonia solution at a temperature of 56°C for 40 minutes. Following this, the sections underwent treatment with sodium hypochlorite solution (Shangbao) for one minute, and finally, they were exposed to neutral red solution for five minutes.

2.5. Immunofluorescence. The tissue sections underwent deparaffinization, rehydration, and antigen retrieval by boiling in an EDTA buffer solution with a pH of 9.0 (Servicebio, Hubei, China). Skin sections were blocked with 5% BSA for one hour at 25°C. Subsequently, they were incubated overnight at 4°C with rabbit anti-DCT

(dilution 1:100; Bioworld, Dublin, OH, USA) and rabbit anti-beta catenin (dilution 1:500; Servicebio, China) antibodies. After rinsing with PBS, the tissue was subjected to incubation with Dylight 488-tagged donkey anti-rabbit secondary antibodies (diluted at 1:300; Bioworld) at 37°C for 1 h. Subsequently, DAPI (Beyotime, Jiangsu, China) was applied for 5 min. Photographs of all stained sections were taken using a fluorescence microscope (Leica, Germany) fitted with a Leica DMI4000B camera.

2.6. Western Blot. Mouse skin tissues were collected and lysed in RIPA buffer (Epizyme, Shanghai, China) containing a protease inhibitor cocktail (Bimake, Houston, TX, USA). Protein samples, ranging from 25 to 40 µg per lane, were separated using a 10% SDS-PAGE gel and subsequently transferred onto PVDF membranes. After blocking with Rapid Block Buffer (Beyotime, Jiangsu, China) for 15 min at 25°C, the membranes were incubated with specific primary antibodies at the following dilutions: rabbit anti-TRP1 antibody (1:1000; Abcam), rabbit anti-DCT antibody (1:1000; Abcam), rabbit anti-MITF antibody (1:1000; Abcam), rabbit anti-Wnt3a antibody (1:500; Zen Bioscience, Chengdu, China), rabbit anti-Wnt3 antibody (1:500; Zen Bioscience, Chengdu, China), rabbit anti-β-catenin antibody (1:500; Zen Bioscience, China), and rabbit anti-GAPDH antibody (1:10,000; Zen Bioscience, China). Primary antibody incubation was performed at 25°C. Subsequently, the membranes were exposed to a goat anti-rabbit secondary IgG antibody conjugated to HRP (1:10,000; Beyotime, Jiangsu, China) for 50 min at 25°C. The ECL western blot detection system (Epizyme, China) was employed to generate signals captured on X-ray film.

2.7. Quantitative Real-Time PCR (qPCR). Total RNA was extracted from vitiligo mice tail lesions using TRIzol reagent (Vazyme, Nanjing, China), according to the manufacturer's instructions to ensure efficient RNA isolation. The HiScript III RT SuperMix (Vazyme) was used for cDNA synthesis. Quantitative real-time PCR was conducted employing the SYBR qPCR Master Mix (Vazyme), following the protocol provided by the manufacturer. The GAPDH gene was utilized as the reference gene, and data analysis was conducted by employing the $2^{-\Delta\Delta CT}$ method (Table 1).

2.8. Statistical Analysis. The data were expressed as the mean ± standard deviation (SD). Statistical analyses were conducted using SPSS Statistics version 22.0. Two-group comparisons were performed using a *t*-test, and for comparisons involving more than two groups, a one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was applied. $p < 0.05$ was considered statistically significant.

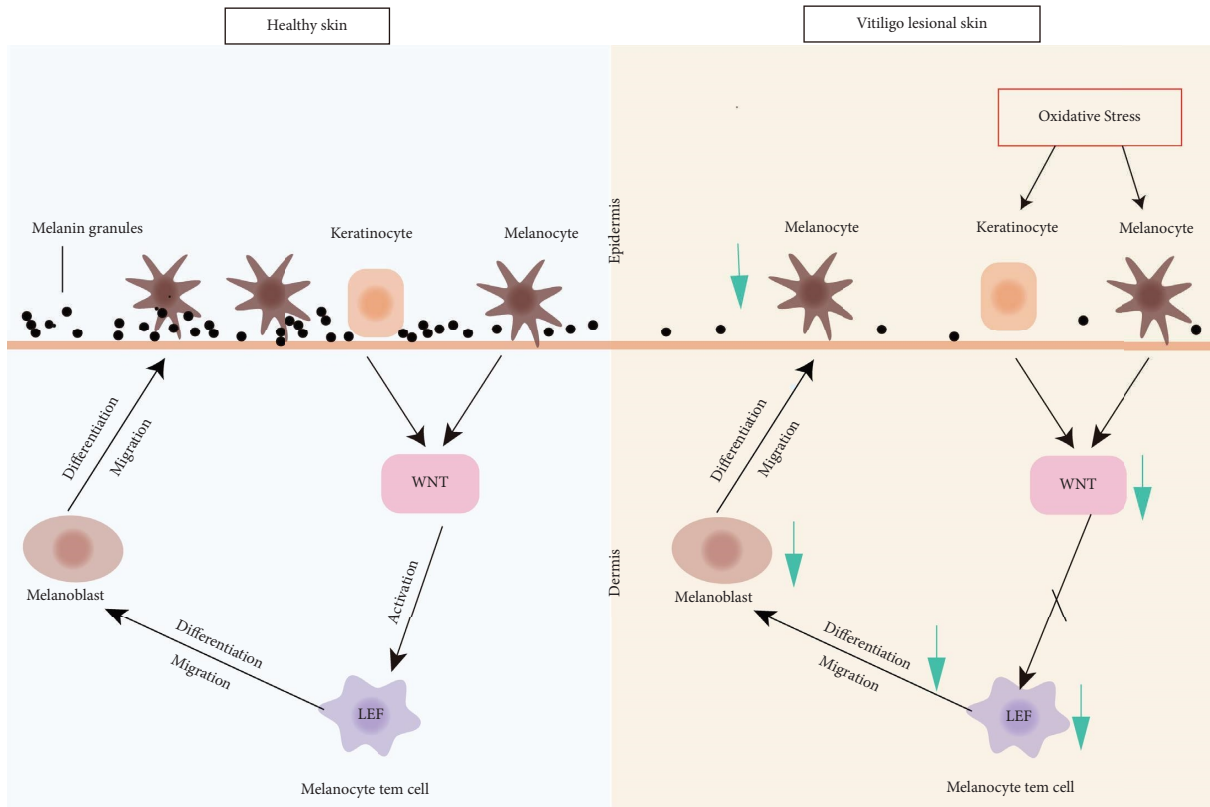
3. Results

3.1. Gene Expression Analysis of the Wnt/β-Catenin Signalling Pathway in Lesions from Patients with Vitiligo. To comprehensively examine the status of the Wnt/β-catenin

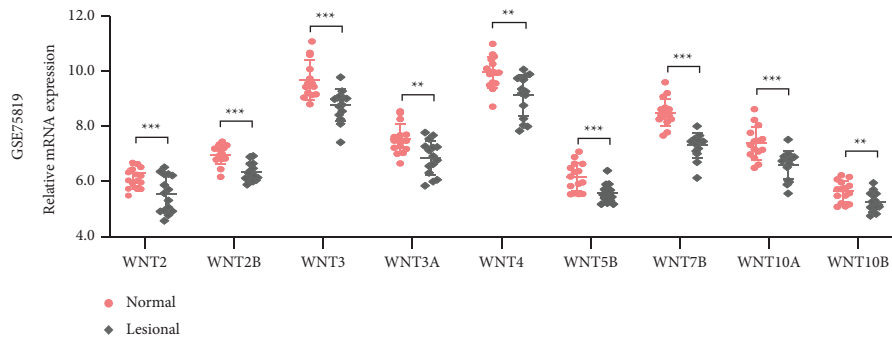
TABLE 1: Primer used for quantitative real-time PCR.

Target gene	primer sequences (5' to 3')
<i>Mitf</i>	F: CAAATGGCAAATACGTTACCCG
<i>Mitf</i>	R: CTCCTTTTTTATGTTGGGAAGGT
<i>Wnt10b</i>	F: GAAGGGTAGTGGTGAGCAAGA
<i>Wnt10b</i>	R: GGTACAGCCACCCCATTC
<i>Wnt3</i>	F: CTCGCTGGCTACCCAATTTG
<i>Wnt3</i>	R: CTTCACACCTTCTGCTACGCT
<i>Wnt3a</i>	F: CTCCTCTCGGATACCTCTTAGTG
<i>Wnt3a</i>	R: GCATGATCTCCACGTAGTTCCTG
<i>Ctnnb1</i>	F: ATGGAGCCGGACAGAAAAGC
<i>Ctnnb1</i>	R: CTTGCCACTCAGGGAAGGA
<i>Lef1</i>	F: AGAAATGAGAGCGAATGTCGTAG
<i>Lef1</i>	R: CTTTGCACGTTGGGAAGGA
<i>Trp1</i>	F: CCCCTAGCCTATATCTCCCTTTT
<i>Trp1</i>	R: TACCCTCGTGGGGATAATGGC
<i>Trp2</i>	F: CTCAAGGGTATGTTGAAGCAGT
<i>Trp2</i>	R: GTTGTGGGCTTACCACACT
<i>Tyr</i>	F: CTCTGGGCTTAGCAGTAGGC
<i>Tyr</i>	R: GCAAGCTGTGGTAGTCGCT

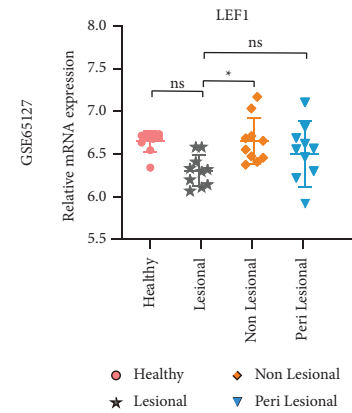
signalling pathway in vitiligo lesions, we analyzed publicly available gene array data from the GEO database (Accession number: GSE75819). This data included mRNA expression levels of altered components within the Wnt/β-catenin signalling pathway in skin biopsies obtained from both normal skin and vitiligo lesions in 15 patients. Upon comparing the mRNA expression levels with those of normal skin samples, we discerned a marked reduction in the transcript levels of numerous pivotal components in the lesions of vitiligo. Notably, the mRNA levels of *WNT10A*, *WNT10B*, *WNT2*, *WNT2B*, *WNT3*, *WNT3A*, *WNT4*, *WNT5B*, and *WNT7B* displayed significant decreases in vitiligo lesions skin (Figure 1(a)). We explored a further gene array dataset from the GEO database (Accession number: GSE65127). In this gene array, we analyzed the gene expression patterns of the Wnt/β-catenin signalling pathway in lesional, peri-lesional, and nonlesional skin samples from 10 vitiligo patients and 10 healthy controls. Interestingly, our analysis revealed that the mRNA levels of *WNT10B* and *WNT11* were significantly diminished in lesional samples compared to those in healthy skin samples. Notably, a distinctive decrease was also observed in the expression of *LEF1* mRNA in lesional vitiligo skin when compared to nonlesional vitiligo samples and healthy skin samples (Figure 1(b)). To further assess the stability of the results, the bootstrap hypothesis test was used for internal validation. The bootstrap hypothesis test is known for its efficacy in estimating the distribution of a statistic based on random resampling with replacement. We conducted a bootstrap hypothesis test to perform the sampling 1000 times to assess the differences between the main genes, *WNT2*, *WNT2B*, *WNT3*, *WNT3A*, *WNT4*, *WNT5B*, *WNT7B*, *WNT10A*, *WNT10B*, *WNT11*, and *LEF1*. We found stable gene expression results in dataset GSE75819. However, genes *WNT10B* and *WNT11* were excluded from dataset GSE65127 due to unstable expression. The difference between the *LEF1* expression levels in the lesion and



(a)



(b)



(c)

Figure 1: Continued.

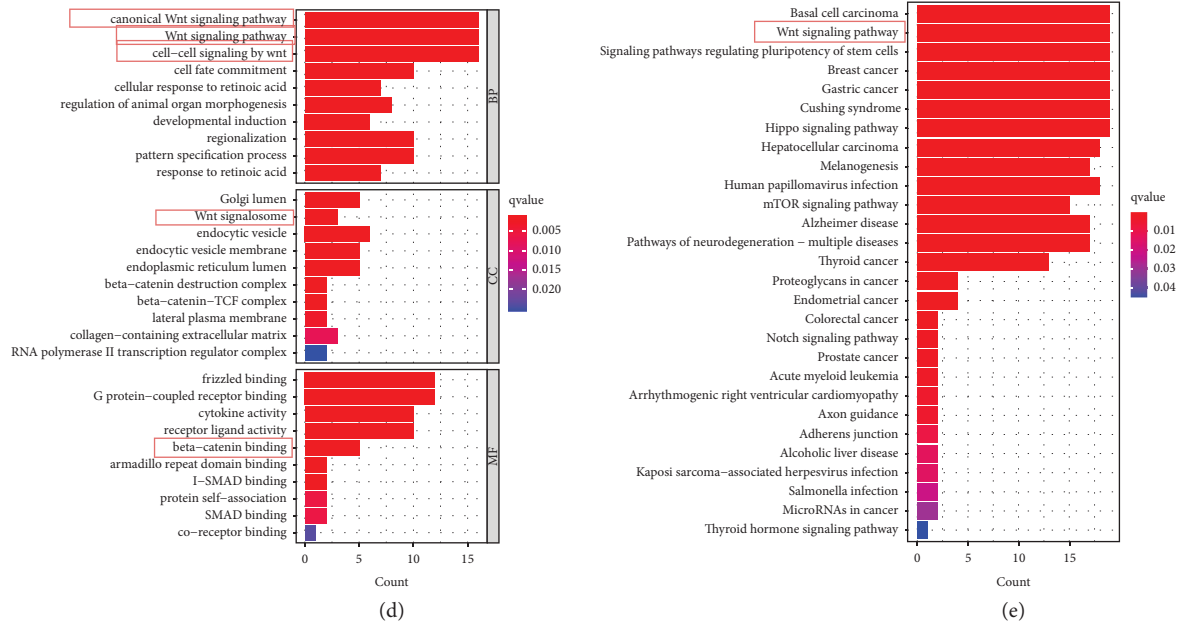


FIGURE 1: Expression of Wnt/ β -catenin signalling pathway in skin lesions of vitiligo. (a) Mechanism diagram. In normal skin, keratinocytes and melanocytes stimulate the Wnt pathway, promoting differentiation and proliferation of melanocyte stem cells. This dynamic process ensures a continual turnover of epidermal melanocyte pools. In vitiligo lesions, oxidative stress diminishes Wnt pathway activity in both melanocytes and keratinocytes. This reduction may impede the differentiation and proliferation of melanocyte stem cells, consequently contributing to active depigmentation of the skin and rendering resistance to repigmentation strategies. (b) Scatter plot showing the expression level of Wnt signal path-related down-regulated genes in vitiligo skin, compared with that of normal skin. The expression values were obtained from the NCBI GEO (accession number: GSE75819). Fifteen biopsies were taken from normal skin and vitiligo skin. (c) Scatter plot showing the expression levels of down-regulated genes associated with the Wnt signalling pathway in vitiligo skin, compared with that of healthy skin, perilesional skin, or non-lesional skin. The expression values were acquired from the NCBI GEO (accession number: GSE65127). Ten samples of healthy skin and ten from patients with vitiligo were collected from lesional, peri-lesional and non-diseased skin. Gene ontology enrichment (d) and kyoto encyclopedia of genes and genomes enrichment (e), the red wireframe represents the pathway associated with the Wnt signal pathway. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Significant differences are indicated. ns $p > 0.05$ no significant difference.

nonlesion groups was statistically significant (Table 2). This analysis was performed using the R (4.3.2). Furthermore, our exploration encompassed gene ontology (GO) enrichment (Figure 1(c)) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment (Figure 1(d)) analysis of the down-regulated genes. This analysis underscored the significant involvement of the Wnt/ β -catenin signalling pathway in the intricate developmental processes underlying vitiligo.

3.2. Establishment of a Vitiligo Mouse Model and Reduced Wnt/ β -Catenin Pathway-Related Gene Expression in Lesions. Mice received two immunization cycles using TRP2, LPS, and CpG-ODN sequentially delivered to the hind footpad and tail skin at one-week intervals (Figure 2(a)). A distinct depigmented skin lesion appeared near the tail injection site four weeks postfinal immunization (Figure 2(b)). Histological examination with H&E staining of the lesional tail skin sections showed a notable decrease in melanocytes and melanin content relative to controls (Figure 2(c)). In addition, we noted a notable reduction in the expression of melanogenesis-associated genes (*Trp1*, *Trp2*, *Mitf*) and genes associated with the Wnt/ β -catenin pathway (*Wnt3*, *Wnt3a*,

TABLE 2: The results of bootstrap hypothesis test.

	Mean bootstrap estimate	95% CI	<i>p</i> value
<i>WNT10A</i>	0.70	0.36–1.05	0.0009
<i>WNT10B</i>	0.34	0.12–0.56	0.0084
<i>WNT2</i>	0.68	0.34–1.01	0.0011
<i>WNT2B</i>	0.57	0.36–0.77	0
<i>WNT3</i>	0.57	0.42–1.23	0.0008
<i>WNT3A</i>	0.60	0.24–0.96	0.0057
<i>WNT4</i>	0.75	0.33–1.17	0.0038
<i>WNT5B</i>	0.54	0.26–0.80	0.001
<i>WNT7B</i>	1.45	1.16–1.75	0
<i>LEF1</i>	0.47	0.25–0.69	0.0021

Wnt10b, *Lef1*, and *Cttnb1*) within vitiligo lesion areas, as corroborated by quantitative PCR (QPCR) analysis (Figure 2(d)).

3.3. Effects of FNT on Vitiligo Repigmentation. We treated the vitiligo mice with a fire needle once weekly for four weeks in the fire needle group. Concurrently, subcutaneous injections of Iwr-1 were given weekly into the tails of the vitiligo mice to inhibit Wnt/ β -catenin signalling. Compared to the Sham group and the Iwr-1 group, the lesion

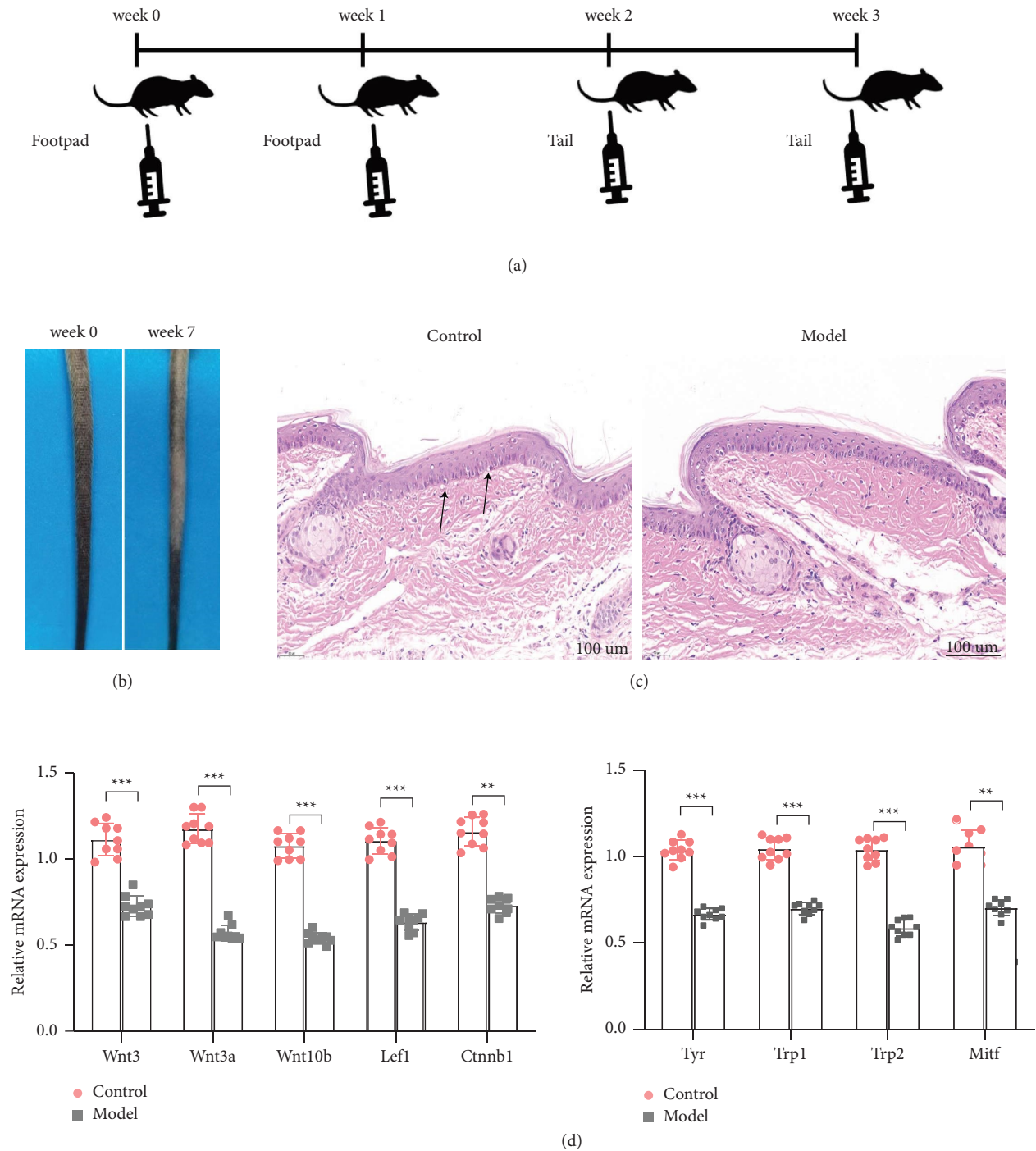


FIGURE 2: Development of the vitiligo mouse model and the expression of related genes. (a) Immunization schedule. C57BL/6J mice were immunized subcutaneously twice at a one-week interval into the hind footpad with TRP2-180, LPS and CpG ODN. One week after the same adjuvants were injected intradermally into the tail, again at a one-week interval (Figure 2(a)). After four weeks of final immunization, we observed depigmented skin lesions around the tail injection site. (b) Developed depigmented skin lesions in the tail at week seven. (c) Representative pictures show haematoxylin and eosin staining in the tail sections of the control and vitiligo mouse model (Scale bar = 100 μ m). (d) The mRNA expression levels of *Trp1*, *Trp2*, *Tyr*, *Mitf*, *Wnt3*, *Wnt3a*, *Wnt10b*, *Lef1*, *Ctnnb1* in the vitiligo model mouse skin and control mouse skin by qRT-PCR. Data are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences are indicated. ns $p > 0.05$ no significant difference.

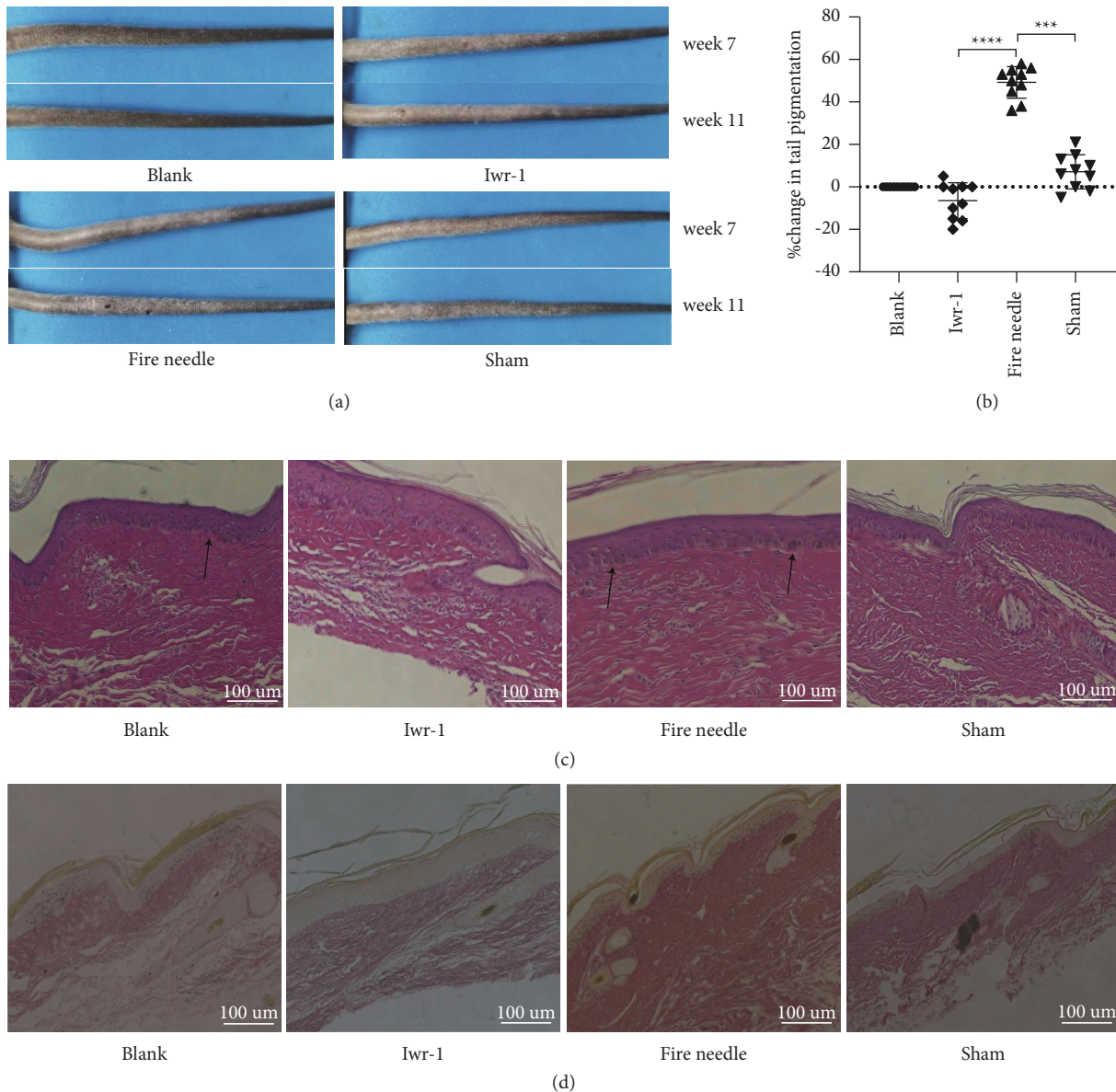


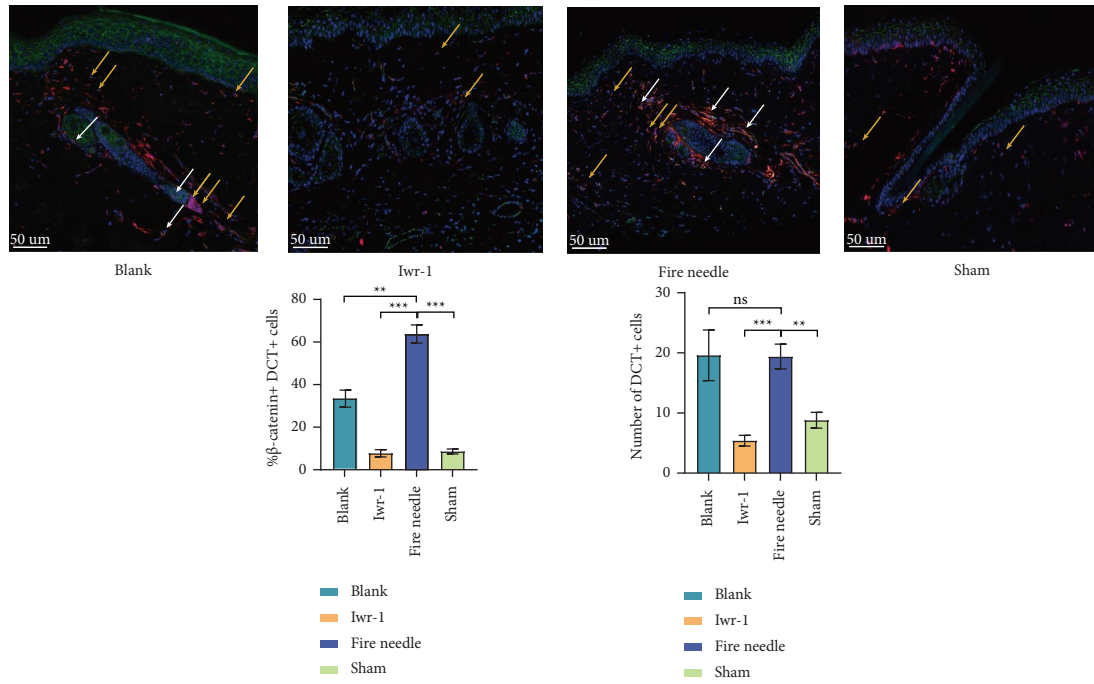
FIGURE 3: Fire needling induces vitiligo repigmentation. (a) Representative images showing the changes before and after treatment. The mice were divided into four groups: the blank group, the inhibitor (Iwr-1) group, the fire needle group, and the sham treatment group. (b) Comparison of the final percentage change in pigmentation among the blank group, Iwr-1 group, fire needle group, and sham group. (c) Haematoxylin and eosin staining was performed on the tail skins of mice in each group (bar = 100 μm). (d) Fontana-Masson staining showing the amount and distribution of melanin in each group (bar = 100 μm).

area of the mice treated with the FNT exhibited pronounced repigmentation. In contrast, lesions in the Iwr-1 group either marginally expanded or remained unchanged (Figures 3(a) and 3(b)).

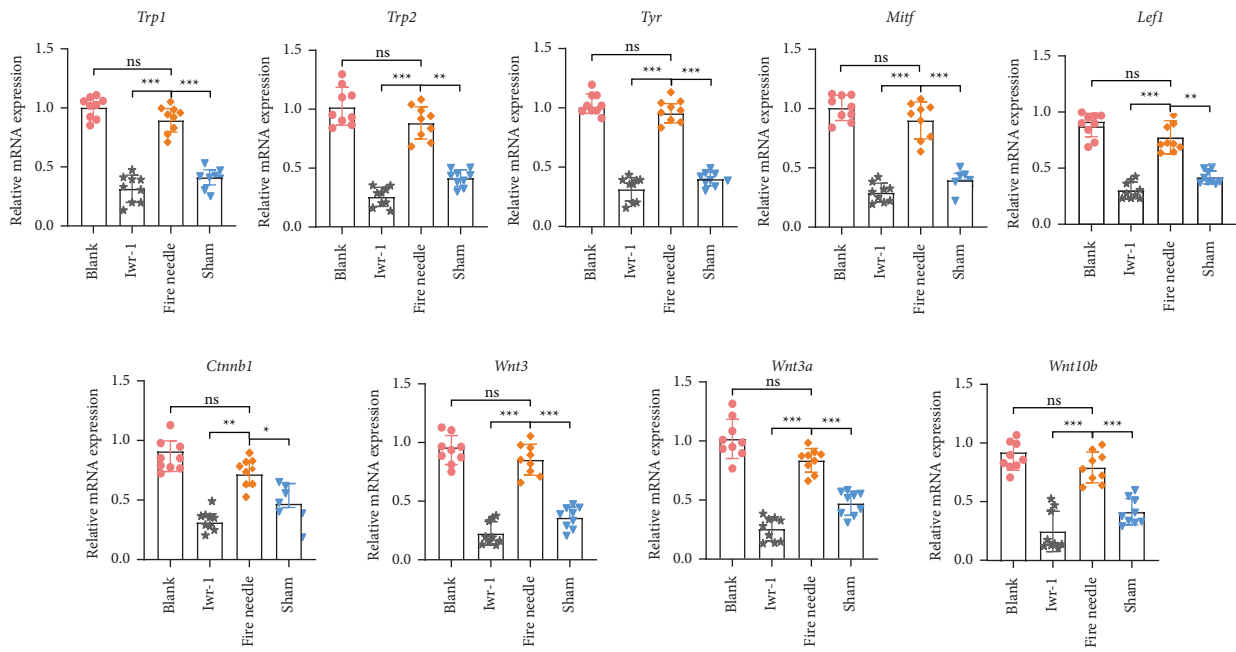
Histological evaluations using H&E and Fontana-Masson staining conclusively showed that FNT enhances melanocyte proliferation and melanin production (Figures 3(c) and 3(d)).

3.4. Effect of Fire Treatment on the Wnt/ β -Catenin Signalling Pathway. We analyzed the expression of the Wnt/ β -catenin signalling pathway after fire FNT in mice model. Dual immunofluorescence staining showed nuclear β -catenin

accumulation in the melanocyte marker DCT (Figure 4(a)). We noticed a significant rise in DCT+ β -catenin + cells in the lesions of fire needle-treated mice relative to mice in the Sham group. These cells were primarily located around hair follicles. Interestingly, the Iwr-1 group showed no DCT+ β -Catenin + expression, while the Sham group exhibited DCT+ β -catenin-cells, pointing to the presence of melanocytes but the absence of β -catenin. We conducted a detailed analysis of the expression levels of melanogenesis-associated genes (Trp1, Trp2, Tyr, Mitf) and genes related to Wnt/ β -catenin signalling (Wnt3, Wnt3a, Wnt10b, Lef1, Ctnnb1) (Figure 4(b)). The results showed elevated levels of these genes in the treatment group relative to the Sham group, while the inhibitor group



(a)



(b)

Figure 4: Continued.

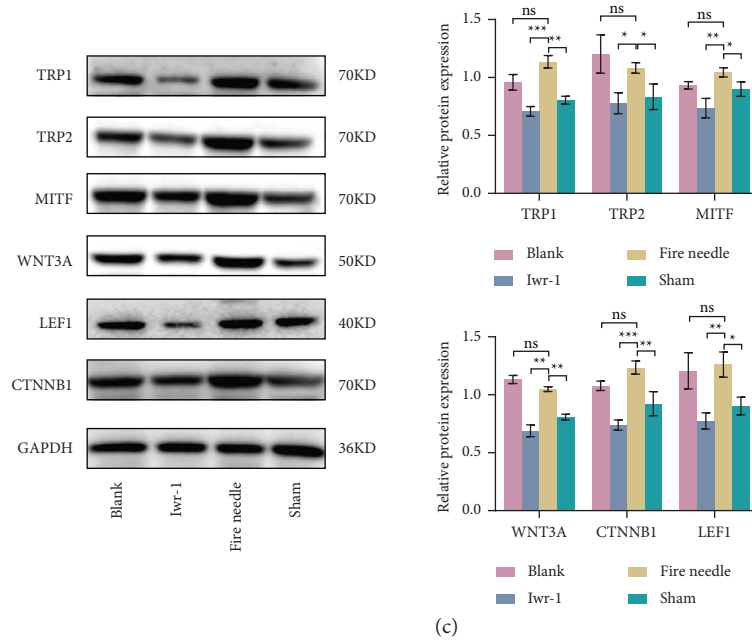


FIGURE 4: Effect of FNT on the Wnt/ β -catenin signalling pathway. (a) Dual immunostaining reveals the accumulation of β -catenin in the migrated DCT (+) melanocytes within each vitiligo lesion skin, as indicated by arrowheads and related quantifications. The yellow arrow represents DCT (+) β -catenin (-) $n=5$, scale bar = 50 μm . (b) qRT-PCR analysis was performed on key factors of the Wnt/ β -catenin pathway and genes related to melanin synthesis in the blank group, inhibitor group, treatment group, and sham group. (c) The protein expressions of TRP1, TRP2, MITF, WNT3, β -catenin, and LEF1 in the tail skin were detected using western blot analysis and quantifications of the protein expressions. The statistical significance was represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significantly different as indicated. ns $p > 0.05$ no significant difference. There were five samples in each group for the qRT-PCR test.

exhibited a notable reduction. Additionally, protein concentrations of TRP1, TRP2, MITF, Wnt3, CTNNB, and LEF1 were higher in the treatment group (Figure 4(c)).

4. Discussion

Vitiligo is an autoimmune skin disease that targets melanocytes, resulting in the appearance of depigmented white patches on the skin [19]. There are multiple mechanisms believed to be responsible for the loss of melanocytes, including genetic predisposition, environmental triggers, and immune-mediated responses [20]. Current therapies for vitiligo primarily focus on the promotion of the accumulation of pigment in affected areas. Clinically, the most common pattern of repigmentation in human vitiligo is a perifollicular distribution, characterized by the presence of black pigment dots surrounding hair follicles. This suggests that hair follicles are the main source of repigmentation [5, 21]. Moreover, McSCs in hair follicles can differentiate into epidermal melanocytes in mice after injuries or UVB treatment [22]. These studies indicate that follicular melanocyte stem cells (McSCs) function as supplementary reservoirs for melanocytes, playing a crucial role in vitiligo repigmentation.

The Wnt/ β -catenin signalling pathway can regulate a variety of biological processes, including regulating stem cell pluripotency, cell migration, self-regeneration, and cell fate determination [23, 24]. This pathway is central to melanocyte biology. The multifunctional kinase GSK3 β is displaced upon activation of the Wnt receptor complex. As

a result, cytosolic β -catenin undergoes stabilisation and is translocated to the nucleus. In the nucleus, β -catenin forms complexes with the lymphoid-enhancing factor-1/T-cell factor transcription factor, leading to an upregulation of MITF expression consequently promoting melanogenesis [24, 25]. In healthy skin, keratinocytes and melanocytes activate the Wnt pathway, promoting the differentiation and proliferation of melanocyte stem cells and ensuring a continuous renewal of the epidermal melanocyte population [26]. Through the analysis of publicly available gene array data from patients with vitiligo, we found that the gene expression of *WNT10A*, *WNT10B*, *WNT2*, *WNT2B*, *WNT3*, *WNT3A*, *WNT4*, *WNT5B*, *WNT7B*, and *LEF1* were down-regulated in the vitiligo skin lesions. This suggests the possibility that Wnt signalling is inhibited. Previous research using immunofluorescence staining assays supports the idea that β -catenin expression is reduced in the entire epidermal layer of vitiligo lesions [12]. Vitamin D analogues enhance repigmentation in vitiligo by protecting melanocytes from oxidative damage through Wnt/ β -catenin pathway activation [27]. Ex vivo studies have shown that Wnt agonists can stimulate resident stem cells to differentiate into pre-melanocytes in vitiligo lesion [26]. Consequently, targeting the Wnt signalling pathway holds potential in relation to vitiligo treatment. NB-UVB, an effective and common approach for vitiligo management, operates by activating melanocyte stem cells, which are considered the reservoir of melanocytes, during the therapeutic process. This mechanism is believed to be mediated by the action of the Wnt signalling

[10]. During the wound healing process, melanocytes are recruited to the wound site, resulting in pigmentation. This phenomenon is primarily attributed to the influence of the Wnt signalling on McSCs [28]. Here, we observed a reduction in lesion size and an increase in pigmentation post-FNT in our vitiligo mouse model. Importantly, we noted an increase in melanocytes and melanin in vitiligo lesions. This supports the idea that bulge McSCs relocate from the hair follicle to the epidermis following a fire needle.

FNT, an external treatment modality used in traditional Chinese medicine with historical roots dating back to the time of the Yellow Emperor (475–221 BC), has shown promise in promoting repigmentation in vitiligo [29]. FNT is distinguished by its simplicity and affordability. Although the treatment requires weekly hospital visits, increasing patient attendance, its integration with conventional treatments including topical medications and phototherapy significantly boosts outcomes and shortens therapy duration. Systematic reviews and meta-analyses have shown that treatment including fire needles had a significantly therapeutic effect in a shorter time than traditional methods without fire needles [30, 31]. Moreover, the cost-effectiveness of FNT coupled with its synergistic benefits when used alongside other treatments, elevates its clinical utility [32]. In terms of safety, several patients experienced minor adverse reactions, such as local itching, erythema, and, less commonly, blistering at the puncture sites. These reactions may be due to the procedure, the individual condition of the patient, or an infection caused by contact with foreign bodies after the procedure. Systematic evaluations have confirmed that the incidence of these adverse effects is comparable to control groups including topical medications and phototherapy [32, 33], indicating a favorable safety profile for FNT. In our study, the mice in the fire needle group showed redness at the sites of needle insertion after treatment. Bleeding and scab formation may occur in few cases; however, the scabs may fall off within one week.

FNT acts as a controlled injury method, drawing parallels with contemporary treatments such as CO2 fractional laser and microneedling [34, 35]. In addition, Wnt ligands in epithelial cells activate Wnt signalling in epidermal melanocytes during wound healing [28]. Interestingly, our study found that there is an increase in the expression levels of WNT3A, β -CATENIN, and LEF1 proteins, as well as the mRNA levels of *Wnt3*, *Wnt3a*, *Wnt10b*, *Lef1*, and *Ctnnb1* after fire needle. This suggests that FNT may promote the proliferation and differentiation of McSCs by activating the Wnt/ β -catenin signal pathway, increasing the production of melanin, and promoting the recovery of vitiligo. This research contributes to the growing body of evidence supporting the efficacy of traditional Chinese medicine in the treatment of vitiligo.

Previous studies have shown the mouse model displays epidermal depigmentation while melanocytes remain within the hair follicles, and the model may be studied in the mechanisms of repigmentation [18]. However, our mouse model cannot fully reflect the complexity and heterogeneity of the human condition which is a limitation of our study. For example, the epidermis of human skin is thick and

multilayered, with a prominent stratum corneum, whereas mouse skin has a thinner and less stratified epidermis [36]. Therefore, the response to FNT may differ. The depth and density of FNT should be adjusted according to individual conditions for clinical use. While our vitiligo models offer valuable insights into the mechanism of FNT, it is essential to corroborate the findings in human-based systems to ensure their clinical relevance which need to be further studied.

In conclusion, our study provides novel evidence that FNT effectively regulates the Wnt/ β -catenin signalling pathways to promote pigmentation in vitiligo. This research contributes to the growing body of evidence supporting the efficacy of traditional Chinese medicine in the treatment of vitiligo.

Abbreviations

FNT:	Fire needle therapy
NBUVB:	Narrowband ultraviolet B
McSCs:	Melanocyte stem cells
GEO:	Database gene expression omnibus database
TRP2-180:	Tyrosinase-related protein 2–180
CpG-ODN:	CpG-oligodeoxynucleotide
LPS:	Lipopolysaccharide
H&E:	Haematoxylin and eosin
DCT:	Dopachrome tautomerase.

Data Availability

All authors confirm that the data supporting the findings of this study are available within the article.

Ethical Approval

All research procedures were approved by the Weifang Medical Ethics Committee (project no. 2022SDL004).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Yan-Li Xu conducted most of the experiments, analyzed the results, and wrote the manuscript. Guang-Mei Sun supervised and designed the study and analyzed the results. Jin-Mei Zhang wrote the manuscript and discussed the results. Yu-Han Ma conducted experiments and discussed the results. Guang-Zhi Li, Lu Zhang, and Fang Cheng discussed the results. Bao-Xiang Zhang supervised and designed experiments and revised the manuscript.

Acknowledgments

This study was supported by Scientific Research and Innovation Fund of Yidu Central Hospital of Weifang (ydky2021ms09) and the Technological Development Project of Medical and Health of Shandong Province (202004121432).

References

- [1] Y. Wang, S. Li, and C. Li, "Clinical features, immunopathogenesis, and therapeutic strategies in vitiligo," *Clinical Reviews in Allergy and Immunology*, vol. 61, no. 3, pp. 299–323, 2021.
- [2] A. Kussainova, L. Kassym, A. Akhmetova et al., "Vitiligo and anxiety: a systematic review and meta-analysis," *PLoS One*, vol. 15, no. 11, Article ID e0241445, 2020.
- [3] O. Osinubi, M. J. Grainge, L. Hong et al., "The prevalence of psychological comorbidity in people with vitiligo: a systematic review and meta-analysis," *British Journal of Dermatology*, vol. 178, no. 4, pp. 863–878, 2018.
- [4] M. Bohm, J. A. Schunter, K. Fritz et al., "S1 Guideline: diagnosis and therapy of vitiligo," *JDDG: Journal der Deutschen Dermatologischen Gesellschaft*, vol. 20, no. 3, pp. 365–378, 2022.
- [5] S. A. Birlea, G. E. Costin, D. R. Roop, and D. A. Norris, "Trends in regenerative medicine: repigmentation in vitiligo through melanocyte stem cell mobilization," *Medicinal Research Reviews*, vol. 37, no. 4, pp. 907–935, 2017.
- [6] N. B. Goldstein, M. I. Koster, L. G. Hoaglin et al., "Narrow band ultraviolet B treatment for human vitiligo is associated with proliferation, migration, and differentiation of melanocyte precursors," *Journal of Investigative Dermatology*, vol. 135, no. 8, pp. 2068–2076, 2015.
- [7] D. Bastakoty and P. P. Young, "Wnt/ β -catenin pathway in tissue injury: roles in pathology and therapeutic opportunities for regeneration," *The FASEB Journal*, vol. 30, no. 10, pp. 3271–3284, 2016.
- [8] Y. S. Choi, Y. Zhang, M. Xu et al., "Distinct functions for wnt/ β -catenin in hair follicle stem cell proliferation and survival and interfollicular epidermal homeostasis," *Cell Stem Cell*, vol. 13, no. 6, pp. 720–733, 2013.
- [9] X. Wang, Y. Liu, J. He, J. Wang, X. Chen, and R. Yang, "Regulation of signaling pathways in hair follicle stem cells," *Burns & trauma*, vol. 10, Article ID tkac022, 2022.
- [10] T. Yamada, S. Hasegawa, Y. Inoue et al., "Wnt/ β -Catenin and kit signaling sequentially regulate melanocyte stem cell differentiation in UVB-induced epidermal pigmentation," *Journal of Investigative Dermatology*, vol. 133, no. 12, pp. 2753–2762, 2013.
- [11] N. B. Goldstein, A. Steel, C. C. Barbulescu et al., "Melanocyte precursors in the hair follicle bulge of repigmented vitiligo skin are controlled by RHO-GTPase, KCTD10, and CTNNB1 signaling," *Journal of Investigative Dermatology*, vol. 141, no. 3, pp. 638–647, 2021.
- [12] S. J. Zhao, H. Jia, X. L. Xu et al., "Identification of the role of wnt/ β -catenin pathway through integrated analyses and in vivo experiments in vitiligo," *Clinical, Cosmetic and Investigational Dermatology*, vol. 14, pp. 1089–1103, 2021.
- [13] X. Lin, X. Meng, and J. Lin, "The possible role of Wnt/ β -catenin signalling in vitiligo treatment," *Journal of the European Academy of Dermatology and Venereology*, vol. 37, no. 11, pp. 2208–2221, 2023.
- [14] S. Yang, W. Sun, and C. Zou, "Recent development of fire needle therapy in the treatment of vitiligo," *Journal of Clinical Acupuncture and Moxibustion*, vol. 30, no. 04, pp. 77–78, 2014.
- [15] H. Jiang, X. Long, Y. Chen, and W. Wang, "Systematic evaluation and meta-analysis of randomized controlled trials of fire needle combined phototherapy for the treatment of vitiligo," *BioMed Research International*, vol. 2022, Article ID 6984149, 11 pages, 2022.
- [16] X. F. X. W. Han, L. Chang, X. Chen et al., "Fire-acupuncture induces proliferation and differentiation of melanocyte stem cells in mouse hair follicle by activating Wnt/ β -catenin signal," *Chinese Journal of Dermatovenereology*, vol. 34, no. 6, pp. 634–639, 2020.
- [17] T. Barrett, S. E. Wilhite, P. Ledoux et al., "NCBI GEO: archive for functional genomics data sets--update," *Nucleic Acids Research*, vol. 41, no. D1, pp. D991–D995, 2012.
- [18] S. You, Y. H. Cho, J. S. Byun, and E. C. Shin, "Melanocyte-specific CD8+ T cells are associated with epidermal depigmentation in a novel mouse model of vitiligo," *Clinical and Experimental Immunology*, vol. 174, no. 1, pp. 38–44, 2013.
- [19] K. Ezzedine, V. Eleftheriadou, M. Whittton, and N. van Geel, "Vitiligo," *The Lancet*, vol. 386, no. 9988, pp. 74–84, 2015.
- [20] J. Chen, S. Li, and C. Li, "Mechanisms of melanocyte death in vitiligo," *Medicinal Research Reviews*, vol. 41, no. 2, pp. 1138–1166, 2021.
- [21] M. Ghasemi, A. Bajouri, S. Shafiiyan, and N. Aghdami, "Hair follicle as a source of pigment-producing cells for treatment of vitiligo: an alternative to epidermis?" *Tissue engineering and regenerative medicine*, vol. 17, no. 6, pp. 815–827, 2020.
- [22] W. C. Chou, M. Takeo, P. Rabbani et al., "Direct migration of follicular melanocyte stem cells to the epidermis after wounding or UVB irradiation is dependent on Mc1r signaling," *Nature Medicine*, vol. 19, no. 7, pp. 924–929, 2013.
- [23] A. Banerjee, G. Jothimani, S. V. Prasad, F. Marotta, and S. Pathak, "Targeting Wnt signaling through small molecules in governing stem cell fate and diseases," *Endocrine, Metabolic & Immune Disorders Drug Targets*, vol. 19, no. 3, pp. 233–246, 2019.
- [24] J. Wu, J. P. Saint-Jeannet, and P. S. Klein, "Wnt-frizzled signaling in neural crest formation," *Trends in Neurosciences*, vol. 26, no. 1, pp. 40–45, 2003.
- [25] H. Wang, Y. Zhong, N. Li et al., "Transcriptomic analysis and validation reveal the pathogenesis and a novel biomarker of acute exacerbation of chronic obstructive pulmonary disease," *Respiratory Research*, vol. 23, no. 1, p. 27, 2022.
- [26] C. Regazzetti, F. Joly, C. Marty et al., "Transcriptional analysis of vitiligo skin reveals the alteration of WNT pathway: a promising target for repigmenting vitiligo patients," *Journal of Investigative Dermatology*, vol. 135, no. 12, pp. 3105–3114, 2015.
- [27] L. Tang, W. Fang, J. Lin, J. Li, W. Wu, and J. Xu, "Vitamin D protects human melanocytes against oxidative damage by activation of Wnt/ β -catenin signaling," *Laboratory Investigation*, vol. 98, no. 12, pp. 1527–1537, 2018.
- [28] Q. Sun, P. Rabbani, M. Takeo et al., "Dissecting Wnt signaling for melanocyte regulation during wound healing," *Journal of Investigative Dermatology*, vol. 138, no. 7, pp. 1591–1600, 2018.
- [29] C. S. M. D. Branch, "Expert consensus on traditional Chinese medicine treatment of vitiligo," *Chinese Journal of Dermatovenereology of Integrated Traditional and Western Medicine*, vol. 16, no. 2, pp. 191–192, 2017.
- [30] M. Huang, L. Tang, X. Li et al., "Clinical observation on therapeutic effect of filiform fire needle for vitiligo: a retrospective study," *Dermatologic Therapy*, vol. 33, no. 6, Article ID e14415, 2020.
- [31] Y. Luo, W. Qian, T. Dai et al., "A new therapy for vitiligo using fire needles: a systematic review of evidence from 3618 subjects," *Evidence-based Complementary and Alternative Medicine*, vol. 2020, Article ID 8492097, 19 pages, 2020.
- [32] L. L. Zhang, X. X. Wang, J. Q. Yuan, X. J. Zhu, and H. L. Jing, "Meta-analysis on the efficacy of fire needle in the treatment of steady vitiligo," *Chinese Journal of Applied Mechanics*, vol. 30, no. 11, pp. 98–103, 2021.

- [33] X. Li, "Clinical effect of milli-fire needle combined with Zitong Xiaobai tablets in treatment of localized vitiligo: an analysis of 60 cases," *Hunan Journal of Traditional Chinese Medicine*, vol. 33, no. 01, pp. 12–15, 2017.
- [34] Y. Hu, X. Qi, Y. Hu et al., "Effects of CO2 fractional laser therapy on peripheral blood cytokines in patients with vitiligo," *Dermatologic Therapy*, vol. 32, no. 4, Article ID e12992, 2019.
- [35] Y. M. E. H. Neinaa, S. S. Lotfy, N. R. Ghaly, and N. N. Doghaim, "A comparative study of combined micro-needling and narrowband ultraviolet B phototherapy versus their combination with topical latanoprost in the treatment of vitiligo," *Dermatologic Therapy*, vol. 34, no. 2, Article ID e14813, 2021.
- [36] H. D. Zomer and A. G. Trentin, "Skin wound healing in humans and mice: challenges in translational research," *Journal of Dermatological Science*, vol. 90, no. 1, pp. 3–12, 2018.