RESEARCH ARTICLE

Integrated Transcriptome Analysis of microRNA and mRNA in Mouse Skin Derived Precursors (SKPs) and SKP Derived Fibroblast (SFBs) by RNA-Seq

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Abstract: Background: Skin-derived precursors (SKPs) display the characteristics of self-renewal and multilineage differentiation.

Objective: The study aimed to explore the molecular mechanisms of mouse SKPs differentiation into SKP-derived fibroblasts (SFBs).

Methods: We compared the microRNA (miRNA) profile in mouse SKPs and SFBs by RNA sequencing. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed to validate the miRNA expression. The integrated analysis of miRNA and mRNA expression data was performed to explore the potential crosstalk of miRNA-mRNA in SKP differentiation.

Results: 207 differentially expressed miRNAs and 835 miRNA target genes in the gene list of integrated mRNA expression profiling were identified. Gene Ontology (GO) enrichment analysis revealed that cell differentiation and cell proliferation process were significantly enriched. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed the target genes were significantly most enriched in the cytokine-cytokine receptor interaction, cancer pathways and axon guidance signaling pathway. The most upregulated and downregulated target genes were involved in the Wnt, Notch, cytokine-cytokine receptor interaction, TGF- β , p53 and apoptotic signaling pathway. The miRNA-mRNA regulatory networks and 507 miRNA-mRNA pairs were constructed. Seven miRNAs (miR-486-3p, miR-504-5p, miR-149-3p, miR-31-5p, miR-484, miR-328-5p and miR-22-5p) and their target genes *Wnt4*, *Dlx2*, *Sema4f*, *Kit*, *Kitl*, *Inpp5d*, *Igfbp3*, *Prdm16*, *Sfn*, *Irf6* and *Clu* were identified as miRNA-mRNA crosstalk pairs.

Conclusion: These genes and signaling pathways might control SKPs proliferation and SKPs differentiation into SFBs during the process of SKPs differentiation, which might promote the application of SKPs in the clinical treatment of skin-related diseases by regulating SKPs proliferation and SKPs differentiation.

Keywords: Skin derived precursors, Fibroblasts, Stem cell, RNA sequencing, Transcriptome analysis, microRNA.

1. INTRODUCTION

In recent years, research on stem cells has received extensive attention. Studying the characteristics and regulation of stem cells is conducive to the development of treatments for diseases spanning multiple clinical fields. Studying skin stem cells has practical significance because skin samples are easily obtainable. Skin-Derived Precursors (SKPs) from the dermis display the characteristics of stem cells, such as self-renewal and multilineage differentiation [1-5]. SKPs transplanted into the dermis display a Fibroblast (FB)-like

morphology and express FB markers such as type I collagen, vimentin and fibronectin [6, 7]. As the main cell type in the dermis, FBs play a crucial role in maintaining normal skin morphology and function. Because SKPs can differentiate into FBs, SKPs may play important roles in tissue repair, the prevention of skin aging and the treatment of atrophic skin diseases by supplementing the lost FBs. In fact, our previous studies have demonstrated that SKPs are capable of exerting anti-photoaging effects on the skin [8].

Complex mutual interactions exist between the endogenous genetic mechanisms and the environment of the stem cells. The genetic mechanisms of stem cells are affected by specific factors that determine whether stem cells undergo self-renewal, quiescence, proliferation, differentiation or apoptosis. Small RNAs, including microRNAs (miRNAs),

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are special molecules in organisms that silence gene expression and play important roles in the regulation of cell growth, gene transcription and translation. miRNAs regulate gene expression and cellular phenotype through a variety of mechanisms, including messenger RNA (mRNA) degradation, translation inhibition and heterochromatin formation.

Previously, our research group reported the comparison of the mRNAs in mouse SKPs and SKP-derived fibroblast (SFBs) by RNA sequencing (RNA-Seq) [9]. Here, we used RNA-Seq to find the differentially expressed miRNAs between mouse SKPs and SFBs. Moreover, based on the results of RNA-Seq for miRNAs and mRNAs, we conducted a joint analysis of the differentially expressed miRNAs and their corresponding target genes for the first time. The present study lays the foundation for further investigation of the molecular mechanisms during the differentiation of SKPs toward FBs. We expect to identify crucial factors and pathways that might enhance the proliferation capacity of SKPs and promote SKP differentiation into FBs, making it possible to take advantage of the potential application of SKPs in the treatment of skin-related diseases.

2. MATERIALS AND METHODS

2.1. Cell Preparation and Immunofluorescence Staining

Skin tissues were collected from the back of 12 3-day-old neonatal BALB/c mice for cell isolation. 3 replicates for each cell type (SKPs and SFBs) group were used for the studies of RNA-seq. The mice were purchased from the Center of Experimental Animals, Sichuan University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University (2013006A). The protocols for isolating SKPs and FBs, inducing SKPs to differentiate into SFBs, immunofluorescence staining and primer information were described in Supplementary Materials (S1 and S2).

2.2. RNA-Seq

2.2.1. Library Preparation

The complementary DNA (cDNA) library was prepared at BGI-Shenzhen. The total RNAs from SKPs and SFBs were first treated with DNase I to degrade contaminating DNA. The resulting fragments were enriched by PCR amplification. The library products were used for sequencing using an Illumina HiSeq 2000. SKPs were considered the control and SFBs the treatment. The 49-nt tags from sequencing went through data-cleaning analysis first, which included removing the low-quality tags and the 5' adaptor contaminants to obtain credible, clean tags.

2.2.2. Mapping Tags to the Reference Genome

The small RNA tags were mapped to the genome by SOAP [10] to analyze their expression and distribution in the genome. The tags were annotated as miRNAs, ribosomal RNAs (rRNAs), small conditional RNAs (scRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNA) and transfer RNAs (tRNAs) from GenBank and Rfam. The small RNA tags were aligned to exons and introns of mRNAs to find the degraded fragments of mRNAs in the small RNA tags. Finally, all alignments and annotations were summarized.

2.2.3. Differential Expression Analysis of Known miRNAs

To identify miRNAs that were differentially expressed between SKPs and SFBs, the expression of known miRNAs was compared. P-value was used corresponding to a differential gene expression test at statistically significant levels, and “FDR (False Discovery Rate) ≤ 0.001 and the absolute value of $\log_2 \text{Ratio} \geq 1$ ” were used to identify differential expressed miRNAs as the threshold, which have been described previously in detail [9].

The results are displayed in scatter plots.

2.3. Real-time Quantitative Reverse Transcription PCR (qRT-PCR)

Differentially expressed miRNAs related to cell proliferation and cell differentiation were selected based on the results of the RNA-Seq. A total of 19 candidate miRNAs were identified. Validation was performed using qRT-PCR. Pearson's correlation coefficient between qRT-PCR data and RNA-Seq data was calculated to validate the RNA-Seq experiments. Student's T-test was used to compare the expression levels between SKPs and SFBs, and $P < 0.01$ was considered statistically significant.

For qRT-PCR, total RNA was isolated and reverse-transcribed into cDNA using a reverse transcription kit (TruScript First Strand cDNA Synthesis Kit). qRT-PCR was performed using the StepOnePlus Real-Time PCR system. Each group (containing three biological samples) was repeatedly analyzed using the two techniques. U6 was selected as the internal reference gene. The fold change in expression between FBs and SKPs was expressed as the $\log_2$ ratio of the normalized expression levels in FBs to those in SKPs ($\log_2 \text{FBs/SKPs}$) and reported as the mean $\pm$ stand error, which was compared with the sequencing results. SFBs and PFBs were compared using Student's T-test, and $P < 0.01$ indicated that the difference was statistically significant.

2.4. Analysis of miRNA-mRNA Regulatory Network

If a differentially expressed miRNA showed a fold-change greater than 2 and satisfied the requirement of $P < 0.05$ in RNA-Seq, its target genes were predicted using three software programs, TargetScan, MiRanda and PicTar. The final candidate miRNA target genes were those in the intersection of the differentially expressed genes from RNA-Seq and the predicted target genes of the differentially expressed miRNAs. Then, the miRNA-mRNA regulatory networks and pairs were constructed.

2.5. GO and KEGG Pathway Enrichment Analysis

Gene Ontology (GO) is an international standardized classification system for gene function, which supplies a consistent vocabulary to comprehensively describe the properties of genes and gene products. There are three ontologies in GO: molecular function, cellular component and biological process. For KEGG pathway annotation, which is the major public pathway-related database, the calculating formula is the same as that used in GO analysis. GO and KEGG enrichment analysis were used on the final candidate target genes as described previously [9].

3. RESULTS

3.1. Differentiation of SKPs into Fibroblasts

Cells were successfully isolated from mouse dorsal skin and cultured. They formed suspended spheres and exhibited clonal growth (Fig. 1A). Under the conditions that induce SKPs to differentiate toward SFBs, the cells appeared to be spindle shaped (Fig. 1B), similar to the characteristic morphology of primary-culture FBs (PFBs) (Fig. 1C).

The cells were evaluated by immunofluorescence staining for specific SKP markers. The cultured cells stained positive for sex determining region Y (SRY)-box 2 (Sox2) (Fig. 2A and B), nestin (Fig. 2D and E), vimentin (Fig. 2F and G) and fibronectin (Fig. 2J). The differentiated cells were positive for vimentin (Fig. 2H), fibronectin (Fig. 2K) and type I collagen (Fig. 2N), consistent with the results of the immunofluorescence staining of PFBs (Fig. 2I-O).

3.2. Quality of the Samples by RNA-Seq

Among the annotation results, the total number of rRNAs annotated may serve as a standard for sample quality control. Under normal circumstances, the total rRNA ratios in high-quality animal samples should be lower than 40%. Our two samples both had total rRNA ratios of less than 40%, indicating high sample quality (Fig. 3).

3.3. qRT-PCR for miRNA Validation

Nineteen differentially expressed miRNAs in SKPs and SFBs were selected to verify the RNA-Seq data by qRT-PCR. No significant differences in the expression of these candidate miRNAs were noted between SFBs and PFBs, except for miR-342-5p and miR-3064-5p (Table 1). However, these two miRNAs were both downregulated in SFBs and PFBs compared with SKPs, which was consistent with the results from RNA-Seq. The Pearson correlation coefficient between qRT-PCR data and RNA-Seq data was 0.991, which indicates that the RNA-Seq data were highly correlated with the qRT-PCR data.

3.4. Integrated Analysis of miRNAs and mRNAs in SKPs and SFBs

During the differentiation of SKPs into SFBs, the expression of 155 miRNAs was elevated, while the expression of 52 miRNAs was reduced (Fig. 4) (all the differentially ex-

pressed miRNAs were shown in Supplementary Material S3). The intersection of the differentially expressed mRNAs from RNA-Seq and the predicted target genes of the differentially expressed miRNAs was determined (the predicted target genes of the differentially expressed miRNAs were shown in Supplementary Material S4 and S5). A total of 835 genes were identified as the final candidate target genes. GO functional enrichment analysis showed that the candidate upregulated and downregulated target genes were significantly enriched in 97 cellular functions, including cell differentiation and cell proliferation (Fig. 5). KEGG pathway analysis revealed that target genes were most significantly enriched in the cytokine-cytokine receptor interaction, cancer pathways and axon guidance (Supplementary Table S1). The miRNA-mRNA regulatory networks (Fig. 6) and 507 miRNA-mRNA pairs were constructed (Supplementary Table S2). Table 2 presents the top upregulated and downregulated target genes that were related to cell proliferation and differentiation process in SKP differentiation along with their respective negatively correlated miRNAs and the potentially involved signaling pathways.

4. DISCUSSION

RNA-Seq is a next-generation sequencing-based approach for transcriptome analysis and is characterized by high sensitivity and wide coverage. Such characteristics are why we employed RNA-Seq to compare the transcriptomes between SKPs and SFBs. The results of RNA-Seq were highly correlated with the results of the qRT-PCR for both mRNA [9] and miRNA, indicating that the results of the RNA-Seq were reliable.

To understand the differentiation of SKPs into SFBs, we investigated the differentially expressed miRNAs and mRNAs related to the functional group of cell proliferation and differentiation between SKPs and SFBs. GO functional enrichment revealed that the final miRNA target genes were significantly enriched in multiple cellular functions, indicating that complex molecular mechanisms are involved in the differentiation of SKPs toward SFBs. KEGG pathway analysis revealed that target genes were most significantly enriched in the cytokine-cytokine receptor interaction, cancer pathways and axon guidance pathways. These data suggest that these signaling pathways may play an important role in SKP differentiation and the regulation of these signaling pathways may activate SKP differentiation.

Fig. (1). Morphology of SKPs and FBs. (A) SKPs displayed a sphere-like structure in culture. (B) SFBs grew from the SKP spheres and had the same morphology as PFBs. (C) PFBs were typically spindle-shaped. Scale bars, 500 μ m.

Fig. (2). Identification of SKPs and SFBs by immunofluorescence staining. SKPs expressed SOX2 (A), nestin (D), vimentin (G) and fibronectin (J) but did not express collagen 1 (M). SFBs expressed vimentin (H), fibronectin (K) and collagen 1 (N) but did not express SOX2 (B) or nestin (E). The same results as for SFBs were observed with PFBs (C, F, I, L and O). Scale bars, 200 μ m (A, D), 100 μ m (B, C, E, F, G, J, M) and 50 μ m (H, I, K, L, N, O).

A relational network of miRNAs and the candidate target genes was established. The integrated analysis showed that a gene might be targeted and regulated by multiple miRNAs, while one miRNA might be involved in the regulation of

multiple target genes. We further identified the potential crosstalk of miRNA-mRNA that regulates SKP proliferation and differentiation toward FBs.

A Pie chart for annotation_SKP-miRNA-total

B Pie chart for annotation_FB-miRNA-total

Fig. (3). Annotation of small RNAs. (A) Annotation of small RNAs in SKPs. (B) Annotation of small RNAs in SFBs.**Table 1.** Validation in RNA-Seq results and comparison of miRNA expression in SFBs and PFBs by qRT-PCR. Fold changes are shown in (SFB or PFB miRNA expression level)/(SKP miRNA expression level) and are presented as the Mean $\pm$ SE.

-		RNA-Seq	qRT-PCR		
Accession Number	Category	SFBs/SKPs	SFBs/SKPs	PFBs/SKPs	-
-	-	Fold Change	Fold Change (Mean $\pm$ SE)	Fold Change (Mean $\pm$ SE)	P value
-	Upregulated	-			
MIMAT0000217	mmu-miR-188-5p	4.4	5.47 $\pm$ 0.37	5.22 $\pm$ 0.47	0.7
MIMAT0000225	mmu-miR-195a-5p	7.07	7.38 $\pm$ 0.82	7.71 $\pm$ 0.74	0.78
MI0014696	mmu-miR-3470a	12.8	14.03 $\pm$ 1.71	14.14 $\pm$ 1.47	0.96
MI0014697	mmu-miR-3470b	8.13	7.63 $\pm$ 0.42	7.88 $\pm$ 0.51	0.72
MIMAT0004853	mmu-miR-874-3p	27	31.4 $\pm$ 5.6	31.9 $\pm$ 6.3	0.95
MIMAT0000571	mmu-miR-331-3p	3.16	3 $\pm$ 0.07	2.96 $\pm$ 0.13	0.79
MIMAT0017206	mmu-miR-486-3p	3.11	3 $\pm$ 0.3	3.3 $\pm$ 0.38	0.57
MIMAT0000669	mmu-miR-221-3p	2.69	2.91 $\pm$ 0.08	2.80 $\pm$ 0.25	0.3
-	Downregulated	-			
MI0018023	mmu-miR-5114	3.33	3.19 $\pm$ 0.2	3.41 $\pm$ 0.06	0.35
MIMAT0004653	mmu-miR-342-5p	2.79	4.06 $\pm$ 0.19	2.73 $\pm$ 0.13	0.005 *
MIMAT0000542	mmu-miR-34a-5p	2.92	2.69 $\pm$ 0.23	3.05 $\pm$ 0.25	0.35
MIMAT0014834	mmu-miR-3064-5p	3.33	5.59 $\pm$ 0.25	3.52 $\pm$ 0.32	0.007 *
MIMAT0017030	mmu-miR-328-5p	3.01	2.79 $\pm$ 0.14	2.86 $\pm$ 0.18	0.78
MIMAT0000209	mmu-miR-129-5p	5.11	4.06 $\pm$ 0.21	4.49 $\pm$ 0.51	0.48
MIMAT0004893	mmu-miR-574-5p	5.3	6.01 $\pm$ 0.98	6.95 $\pm$ 0.93	0.49
MIMAT0000247	mmu-miR-143-3p	4.18	4.56 $\pm$ 0.71	4.46 $\pm$ 0.53	0.91
MIMAT0003898	mmu-miR-760-3p	4.18	4.53 $\pm$ 0.14	4.73 $\pm$ 0.25	0.52
MIMAT0004837	mmu-miR-615-5p	5.8	4.98 $\pm$ 0.29	5.23 $\pm$ 0.40	0.64
MIMAT0004861	mmu-miR-877-5p	5.08	5.47 $\pm$ 0.42	6.04 $\pm$ 0.55	0.45

*represents statistically significant.

Scatter plot (control:x | treatment:y)

Fig. (4). Scatter plot of differentially expressed miRNAs between SKPs and SFBs according to RNA-Seq.

MiR-486-3p expression is reduced in psoriatic skin lesions, and this decrease promotes the expression of K17 in keratinocytes and accelerates the proliferation of keratinocytes, thereby contributing to the formation of psoriatic skin lesions [11]. Overexpression of miR-486-3p represses cell proliferation and metastasis in cervical cancer [12]. *Wnt4* represents an mRNA with increased expression, whereas its predicted correlated miR-486-3p was downregulated. *Wnt4* expression increases during the differentiation of adipose-derived mesenchymal stem cells (MSCs) and bone marrow-derived MSCs [13]. *Wnt4* expression is also increased during adipocyte differentiation, where it activates β -catenin and promotes the differentiation of adipocytes [14]. As *Wnt4* is a predicted target gene of miR-486-3p, the reduction in miR-486-3p expression may promote SKP proliferation and differentiation into SFBs through targeted regulation of *Wnt4* or the Wnt signaling pathway during the differentiation of SKPs to SFBs.

The distal-less homeobox2 (*Dlx2*) gene plays an important role in organ formation and development by promoting the differentiation of retinal ganglion cells (RGCs) [15] and stimulating osteogenic differentiation through the regulation of osteogenesis-related genes [16]. Semaphorin 4f (*Sema4f*) plays key roles in neurodevelopment and adult brain remodeling. An *in vitro* study demonstrated that the endogenous *Sema4f* expressed by brain cells regulates the migration of oligodendrocyte precursors and promotes the differentiation of oligodendrocyte precursors [17]. Our sequencing results

showed that miR-504-5p expression was decreased during the differentiation process. Both *Dlx2* and *Sema4f* were the predicted target genes of miR-504-5p. *Dlx2* is involved in negative regulation of the Notch signaling pathway, whereas *Sema4f* is involved in the Axon guidance pathway. The above results indicate that the miR-504-5p expression decrease may promote the differentiation of SKPs into SFBs through simultaneous targeting of *Sema4f* and *Dlx2* by activating axon guidance and inhibiting the Notch signaling pathway.

Considering the group of miRNAs whose expression was elevated and whose predicted target genes were reduced, the target gene that was the most downregulated during the differentiation process was *Kit*. The protein KIT is a member of the receptor tyrosine kinase family. Activation of KIT expression inhibits the differentiation of olfactory epithelial stem cells toward olfactory epithelial cells [18]. KIT ligand (KITL, also known as stem cell factor) can suppress the differentiation of stem Leydig cells by inhibiting the expression of steroidogenic enzymes [19]. miR-149-3p, which was overexpressed, promotes a visceral-like switch during adipocyte differentiation [20]. The above results suggest that miR-149-3p may promote SKP differentiation and proliferation in the process of SKP differentiation into SFBs by negatively regulating the predicted target gene *Kit/Kitl* through the involved cytokine-cytokine receptor interaction signaling pathway.

Fig. (5). GO functional classification of the candidate target differentially expressed genes (DEGs). **(A)** GO annotations of the downregulated genes whose predicted targeting miRNAs were upregulated. **(B)** GO annotations of the upregulated genes whose predicted targeting miRNAs were downregulated.

Fig. (6). The network of miRNAs and target genes. **(A)** The network of upregulated miRNAs with downregulated target genes. **(B)** The network of downregulated miRNAs with upregulated target genes.

Table 2. Most upregulated and downregulated target genes related to cell proliferation/differentiation with possible signaling pathways and their negatively correlated miRNAs.

Signaling Pathway	Upregulated Target Gene (Gene ID)	Accession Number	Downregulated miRNA(s)
Notch signaling pathway	<i>Dlx2</i>	MIMAT0004889	mmu-miR-504-5p
Wnt signaling pathway	<i>Wnt4</i>	MI0014696	mmu-miR-3470a
Pathways in cancer	-	MI0014697	mmu-miR-3470b
-	-	MI0022357	mmu-miR-3473e
-	-	MIMAT0017206	mmu-miR-486-3p
Axon guidance	<i>Sema4f</i>	MI0022357	mmu-miR-3473e
-	-	MIMAT0004889	mmu-miR-504-5p
-	Downregulate Target Gene (Gene ID)	-	Upregulated miRNA(s)
Cytokine-cytokine receptor interaction	<i>Kit</i>	MIMAT0016990	mmu-miR-149-3p
Pathways in cancer	-	MIMAT0009408	mmu-miR-1943-5p
-	-	MIMAT0004864	mmu-miR-297a-3p
-	-	MIMAT0004827	mmu-miR-297b-3p
-	-	MIMAT0004866	mmu-miR-297c-3p
-	-	MIMAT0004882	mmu-miR-466f-3p
-	-	MIMAT0003898	mmu-miR-760-3p
-	-	MIMAT0017278	mmu-miR-92b-5p
Cytokine-cytokine receptor interaction	<i>Kitl</i>	MIMAT0000146	mmu-miR-134-5p
Pathways in cancer	-	MIMAT0009392	mmu-miR-1929-5p
-	-	MIMAT0000542	mmu-miR-34a-5p
-	-	MIMAT0004882	mmu-miR-446f-3p
-	-	MIMAT0003482	mmu-miR-499-5p
-	-	MI0018023	mmu-miR-5114
-	-	MIMAT0009421	mmu-miR-669o-5p
Insulin signaling pathway	<i>Inpp5d</i>	MIMAT0005858	mmu-miR-1197-3p
-	-	MIMAT0019136	mmu-miR-1306-5p
-	-	MIMAT0000661	mmu-miR-214-3p
-	-	MIMAT0000538	mmu-miR-31-5p
-	-	MI0003491	mmu-miR-484
p53 signaling pathway	<i>Igfbp3</i>	MIMAT0017030	mmu-miR-328-5p
-	-	MI0003491	mmu-miR-484
Transforming growth factor- β signaling pathway	<i>Prdm16</i>	MIMAT0009457	mmu-miR-1839-3p
-	-	MIMAT0017053	mmu-miR-212-5p
-	-	MIMAT0014852	mmu-miR-3072-5p

(Table 2) contd....

-	Downregulate Target Gene (Gene ID)	-	Upregulated miRNA(s)
-	-	MIMAT0017030	mmu-miR-328-5p
-	-	MIMAT0017179	mmu-miR-365-2-5p
-	-	MIMAT0004882	mmu-miR-466f-3p
-	-	MIMAT0004885	mmu-miR-467c-5p
-	-	MIMAT0003783	mmu-miR-615-3p
-	-	MIMAT0000541	mmu-miR-96-5p
p53 signaling pathway	<i>Sfn</i>	MIMAT0016990	mmu-miR-149-3p
-	<i>Irf6</i>	MIMAT0005843	mmu-miR-1188-5p
-	-	MIMAT0004861	mmu-miR-877-5p
Intrinsic apoptotic signaling pathway	<i>Clu</i>	MIMAT0004629	mmu-miR-22-5p

Inositol polyphosphate-5-phosphatase D (INPP5D) is also known as *Ship1*. *Ship1*-deficient hematopoietic stem cells exhibit a strong tendency toward myeloid differentiation [21]. Decreased expression of miR-31-5p in cartilage-derived mesenchymal stem cells inhibits osteogenic differentiation, resulting in the occurrence of osteoarthritis [22]. Our study showed that miR-31-5p expression was elevated in the process of SKP differentiation toward SFBs, and this miRNA might promote the process by negatively regulating its predicted target gene *Ship1*. Hypoxia inhibits the osteogenic differentiation of MSCs by promoting the expression of insulin-like growth factor binding protein 3 (IGFBP3) [23]. Overexpression of IGFBP3 reduces the proliferation of human adipose-derived stem cells, suggesting that inhibition of IGFBP3 expression may promote the proliferation of SKPs and stimulate the differentiation of SKPs toward SFBs. miR-484 is expressed during active cortical neurogenesis. Overexpression of miR-484 promotes the differentiation of neural progenitors [24]. The expression of miR-484 was elevated during cell differentiation in our study, indicating that miR-484 might promote cell differentiation by downregulating its target genes *Ship1* and *Igfbp3*. The expression of PR-domain containing 16 (*Prdm16*) is decreased in the process of neural stem cell differentiation into ependymal cells [25]. Inhibition of *Prdm16* expression plays a key role in the development of multipolar cells at the ventricular periphery. *Prdm16* plays an important role in dynamic cellular redox changes in the developing neocortex during neural differentiation [26]. *Prdm16* is involved in the negative regulation of transforming growth factor- β (TGF- β) signaling, whereas *Igfbp3* is involved in the P53 signaling pathway. These genes were both predicted target genes of miR-328-5p, whose expression was increased during SKP differentiation. These findings indicate that miR-328-5p may promote SKP differentiation by negative regulation of *Prdm16* and *Igfbp3* through activating TGF- β and inhibiting the p53 signaling pathway.

The clusterin (*Clu*) gene was the most downregulated in the process of SKP differentiation. Secretory clusterin (sCLU) is a multifunctional glycoprotein widely expressed in a variety of tissues. Decreased sCLU expression has been associated with the occurrence of erosive joint diseases and the

disease activity. Treatment of mouse bone marrow-derived macrophages and osteoclast precursor cells with sCLU can inhibit the proliferation of both cells [27]. In our RNA-Seq results, miR-22-5p was the only upregulated miRNA that might regulate *Clu* expression, suggesting that miR-22-5p might promote SKP proliferation in the process of SKP differentiation by inhibiting *Clu* expression through inhibiting the intrinsic apoptotic signaling pathway, in which *Clu* is possibly involved.

These results indicate that the miRNA-mRNA pairs miR-486-3p-*Wnt4*, miR-504-5p-*Dlx2/Sema4f*, miR-149-3p-*Kit/Kitl*, miR-31-5p-*Inpp5d*, miR-484-*Inpp5d/Igfbp3*, miR-328-5p-*Igfbp3/Prdm16* and miR-22-5p-*Clu* may play an important role in SKP proliferation and SKP differentiation into SFBs during the process of SKP differentiation. Our speculations are that silencing of miR-486-3p and miR-504-5p expression or overexpression of miR-149-3p, miR-31-5p, miR-484, miR-328-5p and miR-22-5p might promote SKP proliferation and activate SKPs to differentiate by regulating their target genes. The most upregulated target genes are involved in the Wnt, Notch and axon guidance signaling pathways, whereas the most downregulated target genes are involved in the cytokine-cytokine receptor interaction, TGF- β , p53 and apoptotic signaling pathway. These data suggest that these signaling pathways may play an important role in maintaining SKP self-stabilization. Activation of the Wnt signaling pathway, TGF- β signaling pathway and the axon guidance signaling pathway and inhibition of the Notch signaling pathway, cytokine-cytokine receptor interaction, p53 signaling pathway and apoptotic signaling pathway may promote SKP proliferation and activate SKPs to differentiate. However, these hypothesis should be validated by future experiments both *in vitro* and *in vivo*.

CONCLUSION

We performed RNA-Seq to identify the miRNAs and mRNAs that were differentially expressed during the differentiation of SKPs toward SFBs. The differentially expressed miRNAs and mRNAs were subjected to joint analysis. The results indicate that the upregulated genes (*Wnt4*, *Dlx2* and *Sema4f*) and downregulated genes (*Kit*, *Kitl*, *Inpp5d*, *Igfbp3*,

Prdm16, *Sfn*, *Irf6* and *Clu*) might play important roles in the process of SKP differentiation. The miRNAs that were down-regulated (miR-486-3p and miR-504-5p) and the miRNAs that were upregulated (miR-149-3p, miR-31-5p, miR-484, miR-328-5p and miR-22-5p) might regulate one or more genes mentioned above through the Wnt, TGF- β , axon guidance, Notch, cytokine-cytokine receptor interaction, p53 and apoptotic signaling pathways, thereby controlling SKP proliferation and differentiation in the process of SKP differentiation into SFBs. The results of the present study may be useful for investigating the specific molecular mechanisms in SKP differentiation into SFBs. Regulation of miRNA-mRNA crosstalk and related signaling pathways may promote SKP proliferation and activate SKPs to differentiate. Further exploration and verification based on the present study would make it possible to optimize and broaden the application of stem cells to skin regeneration therapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University (2013006A), Chengdu, China.

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. The reported experiments were in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (<http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf>) published by the National Academy of Sciences, The National Academies Press, Washington DC, USA.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (grant number 81673084) and 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University, China.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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