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A preliminary study of differentially expressed genes in expanded skin and normal skin: implications for adult skin regeneration

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Abstract In adults, severely damaged skin heals by scar formation and cannot regenerate to the original skin structure. However, tissue expansion is an exception, as normal skin regenerates under the mechanical stretch resulting from tissue expansion. This technique has been used clinically for defect repair and organ reconstruction for decades. However, the phenomenon of adult skin regeneration during tissue expansion has caused little attention, and the mechanism of skin regeneration during tissue expansion has not been fully understood. In this study, microarray analysis was performed on expanded human skin and normal human skin. Significant difference was observed in 77 genes, which suggest a network of several integrated cascades, including cytokines, extracellular, cytoskeletal, transmembrane molecular systems, ion or ion channels, protein kinases and transcriptional systems, is involved in the skin regeneration during expansion. Among these, the significant expression of some regeneration related genes, such as HOXA5, HOXB2 and AP1, was the first report in tissue expansion. Data in this study suggest a list of candidate genes, which may help to elucidate the fundamental mechanism of skin regeneration during tissue expansion and which may have implications for postnatal skin regeneration and therapeutic interventions in wound healing.

Keywords Skin regeneration · Tissue expansion · Mechanical stretch · Gene expression

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Introduction

Although scarless wound healing can be observed in early gestational stage, postnatal skin cannot achieve complete regeneration when deeply injured [10]. However, conventional tissue expansion is an exception,because normal skin regenerates during the process of tissue expansion [8].

Tissue expansion was introduced clinically in 1957 for the reconstruction of a partially avulsed ear [28]. By implanting a silicon sac subcutaneously and regularly injecting sodium chloride into it, new skin forms under the mechanical stretch, providing a supply of tissue similar in color, structure, and adnexal distribution to the lost tissues for defect repair [1, 8, 28]. This technique has been used clinically for many years; however, the phenomenon of skin regeneration during expansion has received little attention, and the molecular mechanism of skin regeneration during expansion is not well understood.

In 1986, Austad et al. [2] initially discovered mitosis increased after tissue expansion. Subsequently, several studies have determined that an increase in tissue surface area expansion is the result of new regenerated tissue rather than "mechanical creep" under stretch [19, 26]. Studies have been performed to explore the mechanism of skin regeneration during tissue expansion. These published research studies mostly focused on animals, cultured cells, other stretched tissues, or focused on specific genes [15, 17, 31]. However, no other studies have systematically

disclosed this kind of network, especially that involving in human skin regeneration during tissue expansion.

In this study, microarray analysis was performed on the expanded skins in order to identify genes with potential regenerative functions, and the nearby normal skin from the same patient was used as controls.

Materials and methods

Patient information and sample collection

Expanded skin and adjacent normal skin were collected from the same patient who had undergone plastic and reconstructive surgery using tissue expansion technique. Six samples from three patients were included in this study, in the 20–40 age range. The specimens were collected during the second-stage surgery when the expanders were removed. The information of patients was listed in Table 1. All the patients were under tissue expansion for 3 months with an injection of sodium chloride each week during this period, and with a 1-week break when there was enough skin tissue for repair and reconstruction. There were no complications during the expansion process. All samples were provided by our hospital with the patient's informed consent and approval of the hospital's Ethical Committee. All the patients were healthy and none of the patients were smokers. Part of the specimens (1×1 cm) were fixed for histological examination while the rest of tissues were homogenized in Trizol (Invitrogen), and fast-frozen in liquid nitrogen for storage until RNA extraction.

Histological examination

For HE and immunohistochemistry assays, tissues were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. 6-μm sections were stained with hematoxylin and eosin (H&E) for conventional morphological evaluation, and with anti-Ki67 (Dako) for detection of proliferating cells.

Microarray process

Genome-wide expression profile analysis by Illumina Bead Array HumanRef-6 BeadChip was provided by United Gene Holdings, Ltd., Shanghai, China. Microarray sequences included 48,000 full-length and partial complementary DNAs representing known, novel, control genes, and spliceosome from NCBI RefSeq, UniGene, RefSeq Gnomon, Genome-Annotation RefSeq. The microarray assay was performed in accordance with the manufacturer's protocol.

RNA extraction and amplification

Total RNA was extracted from skin specimens with Tryzol (Invitrogen). The RNA qualified by OD260/OD280 value between 1.8 and 2.0, and clear straps after polyacrylamide gel electrophoresis. The RNA sample was then dried and stored at $-20°C$.

Anti-sense RNA synthesis, amplification and purification

The synthesis, amplification, and purification of anti-sense RNA were performed with Ambion Illumina RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) according to the instruction within the kit. Specifically, first reverse transcription was carried out to synthesize first-strand cDNA and then the second strand. After purification, cDNA was transcribed in vitro into cRNA for hybridization.

Hybridization

After labeled with biotin-16-UTP (Perkin Elmer Life and Analytical Sciences, MA, USA), cRNA samples was hybridized to the Illumina Sentrix Human-6 Expression Bead Chip at 58°C for 18 h, according to the Manufacturer's instructions (Illumina, Inc., CA, USA). The Bead Chips were washed and then stained with cyanine3-streptavidin (Amersham Biosciences, Buckinghamshire, UK) for detection.

Detection and data analysis

Arrays were scanned with an Illumina Bead array Reader. Data processing and analysis were performed using Illumina BeadStudio software. Additional information on data

Table 1 Patient information

Patient	Age (years)	Sex	Ethnicity	Site	Sample	Expander specification (ml)	Inflation volume (ml)
Case 1	20	Female	Asian	Face	Normal skin and expanded skin	50	129
Case 2	40	Male	Asian	Forehead	Normal skin and expanded skin	100	270
Case 3	23	Female	Asian	Forehead	Normal skin and expanded skin	80	210

processing and analysis is available from the Web site: http://www.illumina.com/. In order to minimize the effects of variation arising from non-biological factors, Cubic Spline normalization algorithms were used to transform sample signals as previously described [40]. The statistical significance of the expression of each gene in the normal and expanded skin samples was compared with each other by paired t test followed by multiple testing correction to determine the false discovery rate (FDR) as described by Benjamini and Hochberg [3]. A FDR-adjusted p value of <0.05 was considered statistically. The functional calcification of the differentially expressed genes identified by t test was listed in Table 3 and the clustering analysis results were shown in Fig. 3. Hierarchical Clustering analysis displays similarity of gene expression among the cohorts. Genes represented by rows were clustered according to their similarities in expression patterns for each tissue [11]. The fold change at in transcript level was also calculated. Among all the differential expressed genes, genes with more than twofold difference between the expanded skin and the normal skin were listed in Table 4.

Reverse transcription polymerase chain reaction

The transcript level of the selected gene was confirmed by Reverse transcription polymerase chain reaction (RT-PCR). The primers for the gene were designed using the software Primer Premier 5 (Premier Biosoft International, CA, USA) and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co, Ltd. The primer sequences are shown in the Table 2. RT-PCR was performed in accordance with the standard protocol. Specifically, total RNA was extracted from specimens with Tryzol (Invitrogen) and it was reverse transcribed into cDNA with an RT-PCR kit (TaKaRa, Shiga, Japan). Subsequently, 2 μl of each reaction product was amplified in 50 μl of a PCR mixture. Then 26 cycles were performed with GeneAmp PCR System2400 (ABI, USA) at 95°C for 5 min, 95°C for 30 s, 60°C for 40 s, 72°C for 1 min, and 72°C for 10 min at the end of the procedure. β-actin was used as an internal control. The sense and antisense primers used for these analyses were as presented in Table 2. The amplified products were separated on 2% agarose gel and they were visualized with ethidium bromide (Sigma).

Results

Histological findings

The expanded skin was histological similar to normal skin, with normal skin structures, including skin appendages as shown by HE staining. However, there were some

Table 2 RT-PCR primers

Primer	Sequence
HOXA5	Sense GCACCCACATCAGCAGCAGA
HOXA5	Anti-sense TCGGAGAGGCAAAGAGCATG
HOXB2	Sense CCAAGAAACCCAGCCAATCC
HOXB2	Anti-sense GCGTGTTGGTGTAAGCCGTG
DKK2	Sense ATTCGTCCTGCTGCCTGCTC
DKK2	Anti-sense GACACACCATGCAGGCCGAT
CNN1	Sense AGAAGTATGACCACCAGCGG
CNN1	Anti-sense TGGACTGCACCTGTGTATGG
AP-1	Sense GACCTTCTATGACGATGCCC
AP-1	Anti-sense CCCTCCTGCTCATCTGTCAC
GADD45β	Sense AGAAGATGCAGACGGTGACC
GADD45β	Anti-sense AGAAGGACTGGATGAGCGTG
FOXA1	Sense TTGAAGACTCCAGCCTCCTC
FOXA1	Anti-sense GTGCCTGTTCGTATGCCTTG
IL-6	Sense CCACACAGACAGCCACTCAC
IL-6	Anti-sense TGGCATTTGTGGTTGGGTCA
GAPDH	Sence CGGGAAATCGTGCGTGAC
GAPDH	Anti-sense TGGAAGGTGGACAGCGAGG

differences between normal skin and the expanded skin. For example, there were more cells with deeply stained nuclear in the expanded skin than in normal skin, suggesting more active cell proliferation in the epidermis of the expanded skin (Fig. 1) Ki67 Immunohistochemical staining elucidated enhanced cell proliferation in the expanded skin. This kind of enhanced cell proliferation was largely located and well distributed in the epidermis of the expanded skin (Fig. 2).

Identification of differentially expressed genes

A total of 48,000 human genes were analyzed by microarray; 77 of them showed differential expression in expanded skin compared with the normal skin. Among those, 20 genes were down-regulated, and 57 genes were up-regulated. The overview of differently expressed genes is shown in Fig. 3. Among those, 48 genes had at least twofold increases at transcript level. Such genes were further classified by their function in Table 3 (genes with unclear functions were not included). Gene expression for most growth factors and their receptors was not statistically different between the expanded skin and the normal skin; however, the expression of some cytokines such as IL-6 was higher in the expanded skin than the normal skin. Genes related to signaling pathways such as GADD45β and DKK2 were also higher expressed in the expanded skin. Other genes higher expressed in the expanded skin mostly related to extracellular, cytoskeletal, transmembrane molecular, ion or ion channels, and protein kinases (Table 4).

Fig. 1 HE staining showed that the expanded skin was histological similar to normal skin with normal skin structures and skin appendages. However, there were more the cells with deeply stained nuclear in the expanded skin than in normal skin (**a** Normal skin, **b** expanded skin, 100×)
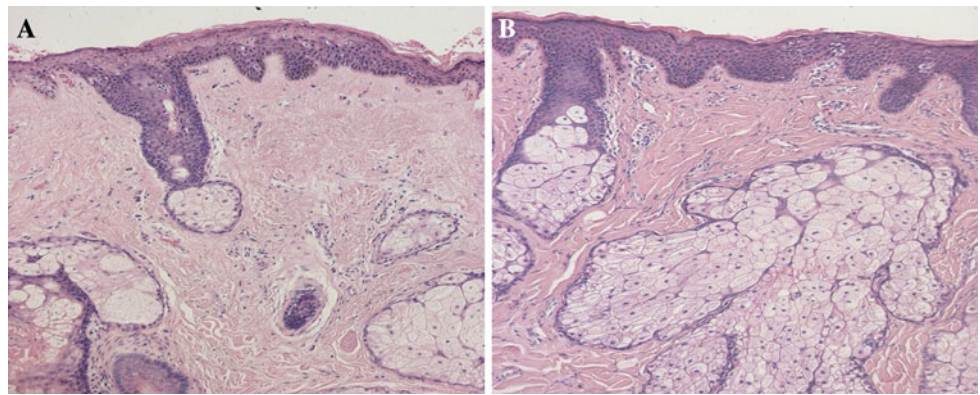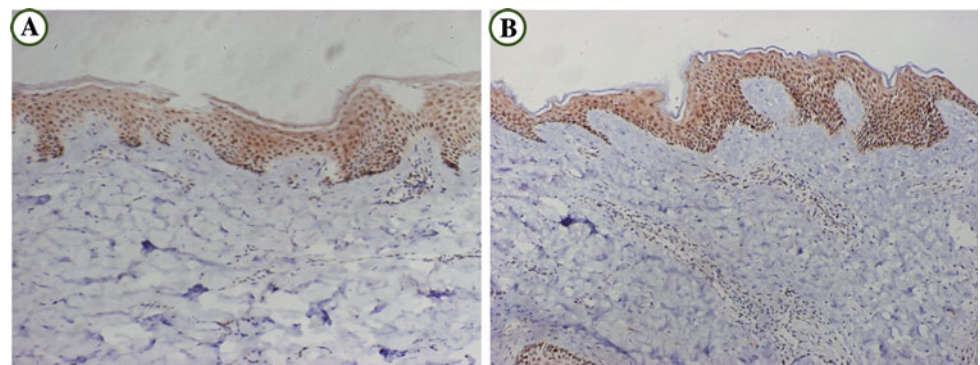

Fig. 2 Ki67 immunohistochemical staining of normal skin (**a**) and the expanded skin (**b**). Enhanced cell proliferation was observed in the expanded skin. Ki67 positive cells (nuclear stained brown) spread all over the epidermis of the expanded skin (100×)

The most significantly up-regulated genes were HOXA5 and HOXB2, which were 24.33 times and 4.13 times higher in the expanded skin than normal skin, respectively. Such results have been verified by RT-PCR (Fig. 4).

Discussion

It is difficult to repair, replace or regenerate injured skin tissue. In fact, most of the repair after a wound is incomplete with a loss of the original structure or function to various degrees, despite the presence of the components which are necessary to complete skin regeneration [25]. Complete regeneration can be only observed in the skin of an early gestational fetus, an ability that is thought to be lost with maturity [27].

However, tissue expansion provides an example of postnatal skin regeneration. During tissue expansion, cellular proliferation increased, more collagen synthesized, growth factors secreted, and vascularization was intensified [2, 7, 19]. New skin regenerates under the mechanical stretch generating during expansion and the regenerated new skin is histologically similar to the normal skin [8, 19, 26, 36, 39]. Such findings were also observed in our histological results. However, an enhance cell proliferation was observed in the epidermis of the expanded skin, which is different from the previous finding that the cell proliferation returned to baseline over 2–5 days [2]. It may due to a lag between the skin growth and tissue expansion or due to the gravity of the tissue expander. The mechanical stretch still exists even though the expansion has been stopped, which therefore provide stimulus for such skin growth. Since the expanded skin is generally histologically similar to the normal skin, the differentially expressed genes may be closely related to the cell proliferation in the expanded skin and may be the potential reason for the skin regeneration during expansion.

The mechanism of skin regeneration during skin expansion has been described in previous studies using tissues and cultured cells, as follows: Mechanical stretch deforms extracellular matrix and this mechanical signal is transmitted into cells by adhesion complexes within membrane. Then, a complex network is initiated including growth factors, extracellular, cytoskeletal, transmembrane molecular systems, ion channels, protein kinases, and transcriptional systems [16, 34, 37].

Similar results were also observed in our study. For example, the over-expression of CNN1, ACTG2, MYH11, and C6orf32 identified the role of cytoskeleton in this process. In addition the over-expression of LOX, SMOC2, PLEK, MMP7, ADH1A, KCNMB1, HAK, and LOX showed metal ion channels and protein kinases contributed to mechanical stretch initiated skin regeneration as well.
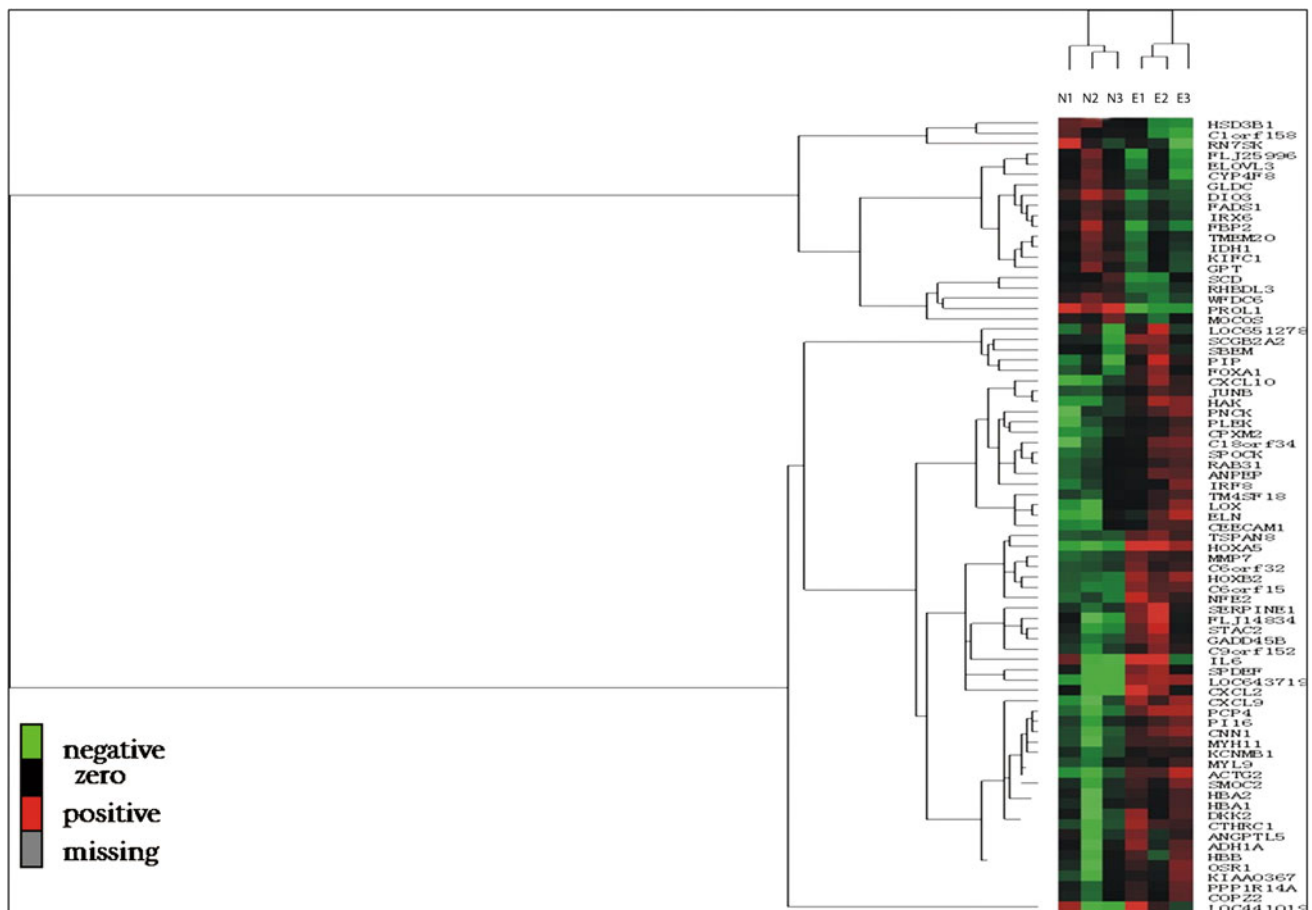
Fig. 3 Hierarchical clustering analysis for differential gene expression profiles in normal skin and the expanded skin ($N = 6$). 77 genes showed differential expression ($p < 0.05$). Genes represented by *rows* were clustered according to their similarities in expression patterns for each tissue. The shade of *red* and *green* indicates up- or down-regulation of a given gene according to the color scheme shown below (E1, N1, E2, N2, E3, and N3 stand for the expanded skin and the normal skin from case 1, case 2, and case 3)

Table 3 Functional classification of variably expressed genes

Class	No. of upregulated genes	No. of downregulated genes
Extracellular matrix and cell adhesion	9	0
Cytoskeleton organization and biogenesis	4	1
Transmembrane molecules	2	1
Cytokine and chemokine	4	0
Ion channel	1	0
Signal transduction	4	0
Transcription factor activity	8	1
Cell proliferation	2	1
Metabolic process	2	11
Other	21	5

Despite of these similarities, there was some difference observed in this study. For example, the expression of growth factors was not statistically different between the normal skin and the expanded skin. Moreover, different from the reported role of protein kinase C-dependent pathway in the mechanical initiated tissue growth [34], the up-regulation of certain genes in this study exhibited some other signaling pathways clues related to skin regeneration during skin expansion. More specifically, the up-regulation of DKK2 suggested a down regulated WNT signaling during skin expansion [21]. The up-regulation of GADD45β, a known protein which activate the p38/JNK pathway

Table 4 Differential expression of the genes with at least two folds increase between the expanded skin and the unexpanded normal skin

Gene symbol	Gene name	Ratio
Cytokines		
CXCL2	Chemokine (C-X-C motif) ligand 2	15.88
IL6	Interleukin 6 (interferon, beta 2)	10.03
CXCL9	Chemokine (C-X-C motif) ligand 9	5.10
CXCL10	Chemokine (C-X-C motif) ligand 10	4.36
CNN1	Calponin 1	5.30
Extracellular Matrix		
ELN	Elastin	5.63
PIP	Prolactin-induced protein	5.36
CTHRC1	Collagen triple helix repeat containing 1	4.58
LOX	Lysyl oxidase	3.45
SMOC2	SPARC related modular calcium binding 2	2.69
LOC651278	Similar to serine hydrolase-like 2	2.37
SERPINE1	Serpin peptidase inhibitor	4.38
MMP7	Matrix metallopeptidase 7	2.31
C6orf15	Chromosome 6 open reading frame 15	3.71
Cystoskeleton		
ACTG2	Actin	7.01
MYH11	Myosin	3.27
C6orf32	Family with sequence similarity 65	2.40
Signal Transduction		
STAC2	SH3 and cysteine rich domain 2	4.28
DKK2	Dickkopf homolog 2	3.08
GADD45B	Growth arrest and DNA-damage-inducible, beta	3.01
TSPAN8	Tetraspanin 8	2.57
PLEK	Pleckstrin	2.42
ANGPTL5	Angiopoietin-like 5	2.57
Transcription factor activity		
HOXA5	Homeobox A5	24.33
SPDEF	SAM pointed domain containing ets transcription factor	6.81
HOXB2	Homeobox B2	4.13
NFE2	Nuclear factor (erythroid-derived 2)	3.88
FOXA1	Forkhead box A1	2.25
JUNB	Jun B proto-oncogene	2.09
Ion channel and metal ion binding		
SMOC2	SPARC related modular calcium binding 2	2.69
PLEK	Pleckstrin	2.42
ADH1A	Alcohol dehydrogenase 1A (class I)	2.13
KCNMB1	Potassium large conductance calcium-activated channel, subfamily M	2.08
Protein kinases		
HAK	Alpha-kinase 2	4.31
LOX	Lysyl oxidase	3.45
PNCK	Pregnancy up-regulated non-ubiquitously expressed CaM kinase	3.23

suggested the involvement of p38/JNK pathway in this process [29]. Furthermore, the expression of PAI-1, FOXA1, AP-1, Actin and CNN1 showed that signaling pathways related to cell proliferation also played a role [4, 12, 13, 30, 38], which was consistent with the histological findings that demonstrate cell proliferation was
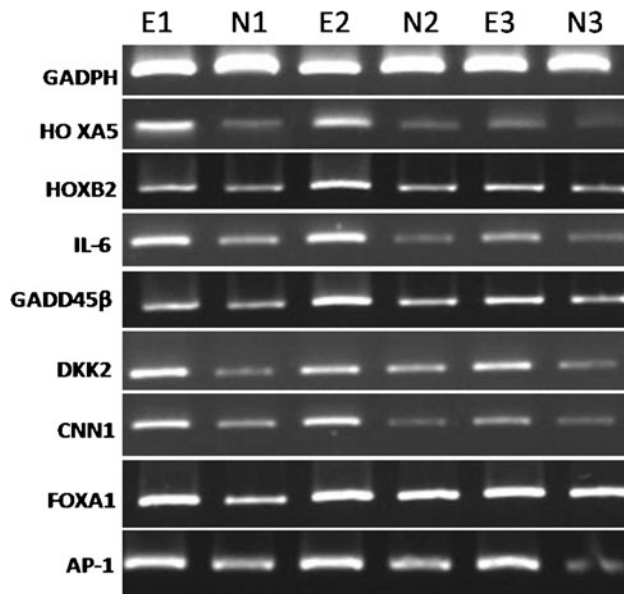
Fig. 4 RT-PCR results for the verification of the microarray results of selected genes. E1, N1, E2, N2, E3, and N3 stand for the expanded skin and the normal skin from case 1, case 2, and case 3

sustained even though the expansion was suspended for 1 week.

However, more important information may come from the expression of some transcription factors such as HOXA5, HOXB2, FOXA1, and AP-1. In this study, HOXA5, which was never reported in the area of skin regeneration and wound healing, increased significantly after skin expansion and was the most obvious up-regulated amongst all the genes. HOX genes are a highly conserved group of homeobox-containing genes, first discovered in Drosophila. In mammals, the 39 HOX genes, which encode a class of transcription factors are found in clusters named A, B, C, and D on four separate chromosomes. Expression of these proteins is spatially and temporally regulated during embryonic development [23]. HOXA5, a member of the HOX gene family, has been implicated in gene expression regulation, cell fate determination, and is critical for morphogenesis during embryogenesis.

Previous studies have demonstrated HOXA5's role in controlling the proliferation, differentiation, and apoptosis of epidermal cells [9, 14, 22]. Other studies showed that HOXA5 plays a role in ECM deposition and in the release of some growth factors [6, 24] and in neovascularization as proven in some carcinoma studies [5]. In addition, the fact that disordered expression of HOXA5 may lead to some epithelial carcinomas further supports its role in skin homeostasis and skin regeneration [32]. Recently, HOX gene family has become increasingly implicated in skin development, embryonic scarless wound repair, and their role in the regeneration of tissues that are more complicated than skin, such as the tail fin of

zebrafish and the limbs of axolotl, has also been demonstrated [18, 33, 35].

Thus, HOXA5 may play an important role in adult skin regeneration during tissue expansion. Moreover, the expression of HOXB2 was also up-regulated. Whether HOXA5, in combination with HOXB2, contributes to complete skin regeneration has yet to be determined. However, it can be stated that activating a transient and simultaneous expression of other upstream and downstream genes of the same HOX cluster as well as genes from other clusters is one pattern of HOX genes' role [20].

FOXA1 which is widely expressed in proliferating cells was also over-expressed during skin expansion. Similar to HOX genes, FOXA1 has also been reported to play a role in hair follicle regeneration, and its role in hair follicle regeneration may have some relationship with HOX gene family according to the existed literature [41]. Together with FOXA1, AP-1 was up-regulated as well. Studies of other tissues showed that AP-1 could mediate mechanical responsive genes expression and contribute to the mechanical stretch initiated cell proliferation [30].

Due to the ethical problem and the limited source of normal skin, it is difficult to acquire large quantities of samples for the assay. Despite limited number of samples, various measures have been taken to make the result dependable. For example, each pair of the expanded skin and its normal control was collected from the same anatomical site from the same patient and the sampling location among the three patients is similar, which can minimize confounding factors caused by anatomy and individual variation. Moreover, in the process of data acquiring and analysis, multiple detections and corrections were adopted to increase the accuracy. With the preliminary findings of this study, it is still hard to tell the relationship between gene expression and expansion volume and the specific function of certain genes as well as their relation with other genes during expansion. Further study is still needed to find answers to these questions.

In summary, this study first elucidated the gene expression pattern in the expanded skin, suggesting a list of target genes which may be closely related to adult skin regeneration during skin expansion. Among those genes, HOXA5 and HOXB2 were obviously up-regulated. So far, their actual role in adult skin regeneration remains largely unknown. Still, the new information regarding HOX genes in skin regeneration during skin expansion may have some implications for identifying the fundamental mechanism of skin regeneration, and it may lead to the development of new wound healing therapies.

Conflict of interest The authors have declared that no conflict of interest exists.

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