Differential miRNA expression profiles in human keratinocytes in response to protein kinase C inhibitor

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Abstract. Aberrant expression of microRNAs (miRNAs) is widely accepted to be involved in keratinocyte differentiation and to be dependent on activation of the protein kinase C (PKC) pathway. However, the miRNA profiles and biological characteristics of keratinocytes induced by specific inhibitors of PKC have yet to be elucidated. The present study aimed to explore the differential miRNA expression profiles in keratinocytes treated with the PKC inhibitor GF109203X, by conducting a bioinformatics analysis. Parts of the GF109203X-induced keratinocytes formed distinct clones after 2 days of culture, and the expression of intergrin β1, cytokeratin (CK)19 and CK14 were positive, whereas CK10 expression was negative. A total of 79 miRNAs were differentially expressed in keratinocytes treated with GF109203X, among which 45 miRNAs were upregulated and 34 were downregulated. The significantly upregulated microRNAs included hsa-miR-1-3p and miR-181c-5p, whereas hsa-miR-31-5p and hsa-let-7c-3p were significantly downregulated. In addition, the results of reverse transcription-quantitative polymerase chain reaction exhibited consistency with the microarray results. An enrichment analysis demonstrated that certain target genes of the differentially expressed miRNAs serve an important role in cell proliferation and differentiation, cell cycle progression and apoptosis, etc. These results revealed that GF109203X induced the differential expression of certain miRNAs when keratinocytes began showing the characteristics of epidermal-like stem cells, which may provide a novel approach for wound healing and regeneration of skin tissues.

Introduction

MicroRNAs (miRNAs) are short noncoding RNA molecules, usually between 22 and 23 nucleotides in length, which regulate the expression of protein-coding genes at the post-transcriptional level by interfering with the translation of mRNAs or by inducing their degradation (1). In humans, miRNAs have been proposed to regulate ~60% of all protein-coding genes and fulfill regulatory functions, as established by their involvement in numerous processes and diseases (2,3). In addition, previous studies have reported that miRNAs are involved in the self-renewal and cell-fate decisions of stem cells, control of the cell cycle, and maintenance of the balance of keratinocyte proliferation, differentiation and apoptosis, whereas their aberrant expression can lead to disease development (4,5). For example, our previous study (6) observed iRNA expression profiles of epidermal cells at various stages of differentiation; concluding that the expression of 191 miRNAs was significantly altered, the target genes of which are closely correlated with cell proliferation, differentiation, apoptosis and migration. Furthermore, Liu et al (7) detected a significant differential miRNA expression profile in cutaneous wounds between diabetic rats and normal rats, which may be closely associated with the mechanisms underlying diabetic wound healing. Sonkoly et al (8) reported that upregulation of miR-203 in human keratinocytes may be required for their differentiation, which is dependent on activation of the protein kinase C (PKC)/activator protein-1 (AP-1) pathway. Conversely, pretreatment with the specific PKC inhibitor, GF109203X, not only suppressed 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced miR-203 expression, but also suppressed it to below the basal level. A downstream target for PKC action in keratinocytes is AP-1, which is a transcription factor that consists of homodimers or heterodimers of the Jun and Fos families of nuclear proteins, and serves essential roles in the regulation of keratinocyte growth and differentiation (8). miRNAs regulate keratinocyte differentiation by activating the PKC signaling pathway; however, to the best of our knowledge, there are currently no reports on the differential miRNA expression profiles of keratinocytes following treatment with the specific PKC inhibitor, GF109203X.

PKC was initially discovered in 1977 as a proteolytically activated protein kinase. Later, it was verified as a...
Ca²⁺-activated, phospholipid-dependent Ser/Thr kinase, firmly associated with signal transduction (9). PKC family isoforms are divided into three subgroups: The calcium- and phorbol ester-dependent ‘classical/ conventional’ subgroup (PKCo, βI, βII, γ), the calcium-independent ‘novel’ subgroup (PKC ξ, δ, ε, η, θ) and the calcium- and phorbol ester-independent ‘atypical’ subgroup (PKC ζ, ι, λ) (9). The rapid activation of PKC enzymes forms part of the signal transduction pathways elicited by numerous hormones, and their phosphorylation of target proteins leads to various cellular responses, including cell proliferation, differentiation and apoptosis (10). In keratinocytes, several cellular functions are also mediated by signaling via PKC, including translocation of the desmoyokin/AHNAK protein, inhibition of proliferation, and differentiation (11). GF109203X is a specific inhibitor of PKC, which competes at the ATP-binding site and regulates the development of keratinocytes. Le Panse et al (12) indicated that GF109203X inhibited c-Fos and c-Jun mRNA expression; in keratinocytes these proto-oncogenes are involved in the cellular differentiation process rather than in cellular proliferation. In addition, it has been verified that GF109203X effectively inhibits granular cell differentiation marker expression when used at 1 and 5 µM concentrations; however, it does not alter keratin (K)1 or K14 expression (13). GF109203X has also been reported to block TPA-induced tumor susceptibility gene 101 protein and K10 upregulation during early keratinocyte differentiation (14). Furthermore, keratinocyte differentiation is preceded by a commitment to irreversible cell cycle withdrawal, and GF109203X may induce marked protection from loss of growth potential in human keratinocytes (15). GF109203X may also suppress the ultraviolet B-induced reduction of cell survival, caspase-9 activation, downregulation of human inhibitor of apoptosis protein-1, X-linked inhibitor of apoptosis protein and c-Jun kinase PKB (but not myeloid cell leukemia-1), and upregulation of glucose-regulated protein 78 in HaCaT cells (16). Overall, these data indicated that GF109203X may have influence on keratinocyte differentiation. However, the miRNA profiles and biological characteristics of keratinocytes induced by specific PKC inhibitors have yet to be elucidated.

The present study aimed to explore the differential miRNA expression profile and biological characteristics of keratinocytes treated with the specific PKC inhibitor, GF109203X. The findings of the present study may provide a novel approach for wound healing and regeneration of skin tissues.

Materials and methods

Sample collection. Prepuce samples were obtained from 5 male patients (age, 16-30 years) who were healthy patients except their prepuce was too long and underwent circumcision at the Department of Urology Surgery, The First Affiliated Hospital of Nanchang University (Nanchang, China) between March 2014 and April 2014. The present study was conducted in accordance with the Declaration of Helsinki, with approval obtained from the Nanchang University Ethics Committee. Written informed consent was obtained from all participants.

Cell culture and identification. The epidermis was digested with trypsin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 4°C in the dark for 8 h. Rapid adhesion to collagen IV (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to isolate human differentiated keratinocytes from epidermal stem cells, as previously described (6). The differentiated keratinocytes were cultured in vitro in keratinocyte serum-free medium supplemented with 10 µg/ml epidermal growth factor and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified chamber with 5% CO₂ for 2 days, and were then divided into two groups. In the experimental group (EXP), the primary keratinocytes were treated with GF109203X (Selleck Chemicals, LLC, Houston, TX, USA) for 2 days, at a final concentration of 10 µM. In the control group (CON), the primary keratinocytes were treated with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) for 2 days, at a final concentration of 10 µM. The cellular morphology of the two groups was observed under an inverted phase contrast microscope (CTR6000; Leica Microsystems GmbH, Wetzlar, Germany). Immunostaining of integrin β1 (catalog no. AW5254), cytokeratin (CK)19 (catalog no. AM8477b), CK1 (catalog no. AP9695c) and CK10 (catalog no. AP6704c; Abgent Inc., San Diego, CA, USA) was used for cell identification, which was performed according to the manufacturer's protocols.

Extraction of total RNA. Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and purified with the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA quality and quantity were measured using a NanoDrop spectrophotometer (ND-1000; NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and RNA integrity was determined by electrophoresis on a denaturing agarose gel, which was prepared in house. On the denaturing gel, the 28S and 18S ribosomal RNA bands were visible, which suggested that the extracted total RNA was complete, RNA degradation and contamination were low and the extracted total RNA exhibited high levels of purity.

miRNA labeling and array hybridization. After quality control, miRNA was labeled with the miRCURY™ Power Labeling kit (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's protocol. Briefly, 5 µl calf intestine phosphatase (CIP) reaction solution (1 µl total RNA, 0.5 µl CIP buffer, 0.5 µl CIP and 3 µl ddH₂O) was incubated at 37°C for 30 min, and then at 95°C for 5 min to terminate the reaction. Subsequently, 3.0 µl labeling buffer, 1.5 µl fluorescent label (Hy3™), 2.0 µl DMSO and 2.0 µl labeling enzyme were added to the mixture. The system was incubated at 16°C for 1 h, and subsequently at 65°C for 15 min to terminate the labeling reaction. The Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array (v.18.0) (Exiqon A/S) according to the manufacturer’s protocol. Briefly, 25 µl Hy3™-labeled samples were mixed with 25 µl hybridization buffer and were denatured for 2 min at 95°C, after which the samples were incubated on ice for 2 min and hybridized to the microarray for 16-20 h at 56°C in a 12-Bay Hybridization system (Roche Nimblegen, Inc., Madison, WI, USA). Following hybridization, the slides were obtained and washed several times using a wash buffer kit (Exiqon A/S). Finally, the slides were scanned using the Axon GenePix 4000B Microarray Scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA).
Expression levels were calculated using the comparative method. The sequences are presented in Table I. Primers, which were synthesized by Bioligo Life Technology (Shanghai, China) and the annealing temperature and product length are as follows:

| miRNA     | Sequences                        | Annealing temperature (°C) | Product length (bp) |
|-----------|----------------------------------|---------------------------|---------------------|
| U6        | F:5’GCTTCGGCAGCATACTAAAAT3’      | 60                        | 89                  |
|           | R:5’CGCTTCCAGAATTTGCCGTGCTAT3’   |                           |                     |
| hsa-miR-1-3p | GSP:5’GGGGCTGGAATGAAAGAAGT3’    | 60                        | 65                  |
|           | R:5’GTGCGGTGTCGAGTGGAGTGC3’      |                           |                     |
| hsa-miR-31-5p | F:5’GGAGGGAAGATTGCGTGC3’      | 60                        | 64                  |
|           | R:5’CAGTGCGGTGTCGAGTGT3’        |                           |                     |

**Data processing and analysis.** Scanned images were then imported into GenePix Pro 6.0 software (Axon Instruments; Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in all samples were chosen for calculating the normalization factor. Expressed data were normalized using the median normalization. Following normalization, significant differentially expressed miRNAs between the two groups were identified through fold change and P-value (fold change >2 and P<0.05). Differential miRNA expression between the two cell groups was analyzed using a Student’s t-test. Finally, hierarchical clustering was performed to detect distinguishable miRNA expression profiling among samples.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and miRNA target prediction.** According to the microarray results, the expression levels of hsa-miR-1-3p were upregulated and the expression levels of hsa-miR-31-5p were downregulated in the experimental group compared with the control group, exhibiting strong original signals and clear differences. Therefore, both of these miRNAs were selected for RT-qPCR verification. In RT-qPCR, small nuclear (sn)RNA U6 was used as an endogenous control. Firstly, cDNA was synthesized using a Gene Amp PCR system 9700 (Applied Biosystems, Thermo Fisher Scientific, Inc.). RT was performed in a 20 µl reaction containing 200 ng total RNA, 0.3 µl 1 µM RT primer, 2 µl 2.5 mM dNTP (HyTest Ltd, Turku, Finland), 2 µl 10x RT buffer (Epicentre; Illumina, Inc., San Diego, CA, USA), 1 µl 50 U/µl RT enzyme (Epicentre; Illumina, Inc.), 0.3 µl 40 U/µl RNase inhibitor (Epicentre; Illumina, Inc.), 20 µl nuclease free water and 0.2 µl MMLV High Performance Reverse Transcriptase (Epicentre; Illumina, Inc.). The stem-loop RT reaction was performed at 16°C for 30 min, followed by 42°C for 30 min and 85°C for 5 min. A total of 2 µl RT reaction was then used with 1 µl specific primers for each of the hsa-miR-1-3p and hsa-miR-31-5p in triplicate wells for PCR on an Applied Biosystems ViiA 7 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.). The thermal cycling parameters were as follows: An initial pre-denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 60 sec, followed by 95°C for 10 sec, 60°C for 60 sec and 95°C for 15 sec. The primers, which were synthesized by Bioligo Life Technology (Shanghai, China) and the sequences are presented in Table I. Expression levels were calculated using the comparative quantitative cycle (Cq) method (17). RT-qPCR was performed in triplicate for each treatment group. To demonstrate the function of differential miRNAs, target gene prediction and functional analysis were conducted. The following websites: http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/ and http://www.targetscan.org/ were used to predict target genes of the differentially expressed miRNAs. In addition, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to identify the roles of these target genes in biological pathways or GO terms, which were accessed from the databases of http://www.geneontology.org/ and http://www.genome.jp/kegg/, respectively.

**Results**

**Biological characteristics of the cells.** Non-adherent cells were irregular in shape, size and distribution after culturing for 2 days, and were loosely attached to the plate wells with no clones detected under an inverted microscope. These results suggested that the characteristics of non-adherent cells were in accordance with terminally differentiating dermal keratinocytes. In the experimental group, parts of the keratinocytes induced by GF109203X attached to the well and formed clones, and the expression of CK19, CK14 and integrin β1 was positive, whereas CK10 expression was negative, which is in agreement with the characteristics of epidermal-like stem cells (data not shown). However, in the control group, the number of cells was significantly decreased with no clones detected, and the expression of CK10 was positive, whereas the expression of CK19, CK14 and integrin β1 was negative, which is in accordance with the characteristics of terminally differentiating epidermal keratinocytes.

**Extraction and qualification of total RNA.** The A260/A280 ratio of RNA is a method used to detect RNA purity; samples ~2.0 are considered to represent pure RNA. A ratio <1.8 indicates sample contamination. A ratio ≥2.0 indicates RNA hydrolysis. Therefore, a ratio range between 1.8 and 2.1 is considered acceptable. In addition, the A260/A230 ratio should be >1.8 for pure RNA. As demonstrated in Table II, the extracted RNAs conformed to the quality standards and therefore qualified for the subsequent miRNA experiments. On the denaturing electrophoresis gel (Fig. 1), the 18S and 28S rRNA bands were clearly visible in the RNA samples, suggesting good integrity.
The miRNA expression variations and patterns between the two groups are presented in Fig. 1B and C. According to data processing and analysis, a total of 45 miRNAs were upregulated, whereas 34 miRNAs were downregulated in the experiment group compared with expression in the control group (Table III). The miRNAs with the greatest upregulation and downregulation were hsa-miR-1-3p (5.0265-fold) and hsa-miR-31-5p (13.9011-fold), respectively.

**Verification of the microarray data by RT-qPCR.** In order to verify the microarray results, RT-qPCR assays were performed on selected miRNAs (hsa-miR-1-3p and hsa-miR-31-5p) in the EXP and CON groups. Amplification and dissociation curve charts for hsa-miR-1-3p, hsa-miR-31-5p and snRNA U6 were generated (Fig. 2). The 2^ΔΔCq value of the miRNAs was calculated according to the relative quantitative method. The 2^ΔΔCq analysis revealed an upregulation of hsa-miR-1-3p (1.724) and downregulation of hsa-miR-31-5p (0.458), which is consistent.
with the microarray results, thus suggesting that the microarray data were reliable.

**Prediction of target genes.** To demonstrate the function of differential miRNAs, target gene prediction and functional analysis were conducted. Databases of identified target genes can be accessed to compile potential targets for differential miRNAs, due to the development of numerous computational algorithms (18). The present study obtained all the target genes of the 79 differentially expressed miRNAs according to three public databases. Subsequently, GO and KEGG analysis were used to identify the biological functions of these target genes.

The most enriched GO terms of the three ontologies are listed in Tables IV-VI. Transcription process, apoptosis process and cell proliferation process were among the most significantly enriched in terms of biological process; the cellular component GO analysis demonstrated that the target genes were associated with the nucleus, cytoplasm and cytosol; and protein binding, DNA binding, ATP binding

| miRNA probe ID | miRNA   | EXP/CON |
|----------------|---------|---------|
|                |         |         |
| 10916          | hsa-miR-1-3p | 5.026537 |
| 42660          | hsa-miR-144-5p | 3.747800 |
| 145844         | hsa-miR-374a-5p | 3.715758 |
| 148228         | hsa-miR-378e  | 3.138800 |
| 168635         | hsa-miR-378c  | 2.817634 |
| 147755         | hsa-miR-483-5p | 2.683051 |
| 46944          | hsa-miR-1297  | 2.674356 |
| 147851         | hsa-miR-3201  | 2.632528 |
| 147604         | hsa-miR-4285  | 2.624796 |
| 29577          | hsa-miR-374a-3p | 2.570440 |
| 168935         | hsa-miR-4687-3p | 2.562501 |
| 146072         | hsa-miR-1469  | 2.484515 |
| 168944         | hsa-miR-4707-5p | 2.460301 |
| 17752          | hsa-miR-4285  | 2.456365 |
| 11053          | hsa-miR-32-5p  | 2.405171 |
| 33596          | hsa-miR-126-5p | 2.382427 |
| 147926         | hsa-miR-4329  | 2.377924 |
| 27536          | hsa-miR-190a-5p | 2.370629 |
| 42782          | hsa-miR-31-5p  | 2.338371 |
| 42640          | hsa-miR-20b-5p  | 2.337649 |
| 11004          | hsa-miR-203a-3p | 2.295231 |
| 169221         | hsa-miR-4748  | 2.294769 |
| 169230         | hsa-miR-4747-3p | 2.281731 |
| 17503          | hsa-miR-590-5p  | 2.268278 |
| 147840         | hsa-miR-1973  | 2.262449 |
| 4040           | hsa-miR-9-5p   | 2.239972 |
| 169395         | hsa-miR-4484  | 2.239785 |
| 148620         | hsa-miR-454-3p  | 2.196783 |
| 42800          | hsa-miR-582-5p  | 2.188416 |
| 17315          | hsa-miR-4707-5p | 2.175560 |
| 169399         | hsa-miR-4750-5p | 2.144243 |
| 10923          | hsa-miR-107    | 2.143846 |
| 169183         | hsa-miR-4644  | 2.128833 |
| 169170         | hsa-miR-4472  | 2.104725 |
| 146089         | hsa-miR-454-3p  | 2.094303 |
| 168696         | hsa-miR-4739  | 2.094056 |
| 168893         | hsa-miR-4505  | 2.093298 |
| 169272         | hsa-miR-4419b  | 2.080859 |
| 42496          | hsa-miR-181c-5p | 2.041769 |
| 169110         | hsa-miR-4497  | 2.040852 |
| 168670         | hsa-miR-4694-5p | 2.031255 |
| 146086         | hsa-miR-30a-5p  | 2.030778 |
| 148509         | hsa-miR-328-5p  | 2.028205 |
| 169375         | Has-miR-660-3p  | 2.005712 |

| miRNA probe ID | miRNA   | EXP/CON |
|----------------|---------|---------|
| 11052          | hsa-miR-31-5p | 13.901180 |
| 42668          | hsa-miR-144-5p | 7.137004 |
| 42959          | hsa-miR-514a-3p | 6.094606 |
| 169159         | hsa-miR-5421  | 4.968959 |
| 147809         | hsa-miR-514b-3p | 4.793510 |

miR/miRNA, microRNA; EXP, experimental; CON, control.
and transcription factor binding were significantly enriched in terms of molecular function. Taken together, these results suggested that the target genes of differentially expressed miRNAs may be involved in cell proliferation, division,
mitosis, apoptosis and differentiation. Finally, KEGG analysis indicated that 77 pathways were associated with the target genes of differentially expressed miRNAs; 15 significantly enriched pathways are presented in Table VII, including the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, protein processing in endoplasmic reticulum, focal adhesion and mammalian target of rapamycin (mTOR) signaling, which are associated with cell growth, differentiation, apoptosis and migration.

Discussion

In the present study, following incubation with GF109203X, some of the surviving keratinocytes reverted from a differentiated to a dedifferentiated state, as evidenced by the high colony-forming efficiency and expression of biological markers of keratinocyte stem cells, including integrin β1, CK19 and CK14. However, in the CON group these alterations were not detected. These findings suggested that terminally differentiating epidermal keratinocytes may acquire some stem cell characteristics by modulation with GF109203X treatment. Therefore, dedifferentiation of human terminally differentiating keratinocytes may be induced by GF109203X in vitro. Mature cell dedifferentiation is a popular phenomenon, in which terminally differentiating epidermal cells can revert to their ancestor cells by dedifferentiation; i.e., epidermal cells can revert from the ‘old’ differentiated state to the not fully differentiated ‘young’ state, or even the ‘naive’ state with the characteristics of epidermal stem cells. Previous studies have confirmed that keratinocytes can be dedifferentiated into their progenitor cells, and have identified that dedifferentiated young epidermal cells may be used to treat severe wounds (19,20). In addition, Sun et al (21) demonstrated

| Term ID | Term name | Hit number |
|---------|-----------|------------|
| GO:0006351 | Transcription, DNA-dependent | 514 |
| GO:0006355 | Regulation of transcription, DNA-dependent | 328 |
| GO:0045944 | Positive regulation of transcription from RNA polymerase II promoter | 259 |
| GO:0007165 | Signal transduction | 243 |
| GO:0006915 | Apoptotic process | 216 |
| GO:0045893 | Positive regulation of transcription, DNA-dependent | 200 |
| GO:00010122 | Negative regulation of transcription from RNA polymerase II promoter | 198 |
| GO:0010467 | Gene expression | 181 |
| GO:0045892 | Negative regulation of transcription, DNA-dependent | 161 |
| GO:0043066 | Negative regulation of apoptotic process | 143 |
| GO:0008285 | Negative regulation of cell proliferation | 123 |
| GO:0006366 | Transcription from RNA polymerase II promoter | 112 |
| GO:0008284 | Positive regulation of cell proliferation | 105 |
| GO:0008283 | Cell proliferation | 99 |
| GO:0051301 | Cell division | 94 |
| GO:0007049 | Cell cycle | 85 |
| GO:0006357 | Regulation of transcription from RNA polymerase II promoter | 79 |
| GO:0006367 | Transcription initiation from RNA polymerase II promoter | 73 |
| GO:0016055 | Wnt receptor signaling pathway | 65 |
| GO:0001525 | Angiogenesis | 65 |
| GO:0043065 | Positive regulation of apoptotic process | 64 |
| GO:0007173 | Epidermal growth factor receptor signaling pathway | 62 |
| GO:0009617 | Induction of apoptosis | 60 |
| GO:0007050 | Cell cycle arrest | 58 |
| GO:0007067 | Mitosis | 58 |
| GO:0000082 | G1/S transition of mitotic cell cycle | 47 |
| GO:0007243 | Intracellular protein kinase cascade | 43 |
| GO:0007219 | Notch signaling pathway | 43 |
| GO:0006260 | DNA replication | 42 |
| GO:0030335 | Positive regulation of cell migration | 41 |
| GO:0016477 | Cell migration | 40 |
| GO:0019827 | Stem cell maintenance | 40 |

GO, Gene Ontology.
that dedifferentiation of human terminally differentiating keratinocytes into their precursor cells may be induced by basic fibroblast growth factor. Another study indicated that dedifferentiated epidermal cells are able to form clones and generate a complete epithelium following migration to cutaneous wounds (22). Zhao et al (23) demonstrated that LiCl and glycogen synthase kinase-3β inhibitor-induced cells are able to regenerate skin, in a manner equivalent to that of epidermal stem cells. These findings suggested that dedifferentiation is a promising method for the production of abundant epidermal stem cells, which may be used to bioengineer skin equivalents and as stem cell-based therapies in cutaneous repair and regeneration. It is well known that poor wound healing after trauma, surgery, acute illness or chronic disease conditions affects millions of people worldwide each year (24), and the cost of non-healing wounds is a great burden to health care systems (25). The efficacy of conventional approaches to treating cutaneous wounds is limited; dressings, periodic debridement, eliminating causative factors and innovations in surgical autologous grafting techniques are inherently limited to the size of available donor sites and are insufficient for global burn injuries (26). Therefore, the present study offers a potential novel strategy for the treatment of cutaneous wounds.

Furthermore, dedifferentiated cells are readily available in large quantities with the use of simple methods, and are considered moral and ethical alternatives for disease therapy, with no risk of genetic incompatibility or tissue rejection.

The present study used microarray hybridization to comparably observe the expression of miRNAs between EXP and CON groups. The results detected 45 upregulated miRNAs and 34 downregulated miRNAs when keratinocytes began exhibiting the characteristics of epidermal-like stem cells. In the present study, hsa-miR-1-3p was the most significantly upregulated miRNA and hsa-miR-31-5p was the most significantly downregulated miRNA. Hsa-miR-1-3p is also known as miR-1, which is significantly positively correlated with expression of the proliferation marker Ki67, and is involved in proliferation (27). A previous study demonstrated that inhibition of PKC prevented the upregulation of miR-1 induced by constitutively active Gαi2, demonstrating a role for PKC in the regulation of muscle-specific miRNA (28). In the present study, miR-1 was upregulated in keratinocytes treated with the PKC inhibitor GF109203X, which may also serve an important role in proliferation. Hsa-miR-31-5p is also known as miR-31, which has been implicated as a key regulator of keratinocyte differentiation and proliferation. Peng et al (29)

Table V. Enriched terms in GO molecular functions.

| Term ID       | Term name                                                                 | Hit number |
|---------------|---------------------------------------------------------------------------|------------|
| GO:0005515    | Protein binding                                                           | 1,554      |
| GO:0003677    | DNA binding                                                               | 428        |
| GO:0005524    | ATP binding                                                               | 413        |
| GO:0003700    | Sequence-specific DNA binding transcription factor activity                | 295        |
| GO:0043565    | Sequence-specific DNA binding                                             | 166        |
| GO:0003723    | RNA binding                                                               | 142        |
| GO:0003682    | Chromatin binding                                                         | 126        |
| GO:0008134    | Transcription factor binding                                              | 111        |
| GO:0042802    | Identical protein binding                                                 | 109        |
| GO:0019901    | Protein kinase binding                                                    | 104        |
| GO:0005525    | GTP binding                                                               | 98         |
| GO:0003713    | Transcription coactivator activity                                        | 91         |
| GO:0003714    | Transcription corepressor activity                                        | 70         |
| GO:0004672    | Protein kinase activity                                                   | 70         |
| GO:0019899    | Enzyme binding                                                            | 67         |
| GO:0003924    | GTPase activity                                                           | 67         |
| GO:0044212    | Transcription regulatory region DNA binding                               | 63         |
| GO:0019904    | Protein domain specific binding                                            | 58         |
| GO:0008022    | Protein C-terminus binding                                                | 51         |
| GO:0003705    | RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity | 39         |
| GO:0005096    | GTPase activator activity                                                 | 37         |
| GO:0004725    | Protein tyrosine phosphatase activity                                     | 36         |
| GO:0001077    | RNA polymerase II core promoter proximal region                           | 32         |
|               | Sequence-specific DNA binding transcription factor                        |            |
|               | Activity involved in positive regulation of transcription                 |            |

GO, Gene Ontology.
indicated that miR-31 is an endogenous negative regulator of factor inhibiting hypoxia-inducible factor-1 expression, which results in keratinocyte differentiation by enhancing Notch signaling; this finding is in accordance with the results of the present study. Furthermore, nuclear factor-κB-induced miR-31 promotes keratinocyte proliferation by suppressing protein phosphatase 6 in psoriasis (30). Recently, in human metastatic cutaneous squamous cells, the increased expression of miR-31 was revealed to promote migration, invasion and colony forming ability (31). Taken together, these findings suggested that miR-31 is a multifunctional miRNA that serves important roles in physiological and pathological conditions of epidermal keratinocytes; however, the molecular mechanisms of PKC and miR-31 remain poorly characterized and require further study. In addition, the present study demonstrated that miR-181c-5p and miR-374a are predominantly expressed in GF109203X-induced keratinocytes, which is in accordance with our previous observation that these miRNAs were upregulated in native keratinocyte stem cells (6). Hsa-miR-181c-5p has functional relevance in the maintenance of stemness, which may regulate cell proliferation and cell cycle progression via the Notch signaling pathway and bone morphogenetic protein pathway in cancer stem cells (32). In addition, miR-374a has been reported to promote the proliferation of osteosarcoma cells by targeting Axin2 (33). Overall, these data indicated that these miRNAs may promote proliferation and maintain the undifferentiated state when keratinocytes were induced to re-express the biological characteristics of epidermal-like stem cells by GF109203X.

The enrichment analysis of the differentially expressed miRNAs demonstrated that hsa-miR-181c-5p, hsa-miR-378c and hsa-miR-20b-5p are involved in numerous KEGG pathways that regulate cell proliferation, differentiation and motility, which may serve important roles in dedifferentiation. Hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-590-5p, hsa-let-7f-5p and hsa-miR-378c are involved in the MAPK signaling pathway, which has been reported to be involved in cell proliferation, differentiation, inflammation and tumor growth (34,35). PKCδ/p38δ MAPK signaling, which is a key controller of keratinocyte proliferation and differentiation, increases p21 (Cip1) expression to suppress keratinocyte proliferation (36). A further study demonstrated that PKCδ/p38δ MAPK signaling suppresses methylosome protein 50 expression, leading to reduced H3/H4 arginine dimethylation at the p21 (Cip1) promoter; this was associated with enhanced p21 (Cip1) expression and reduced cell proliferation (37). Previous research has indicated that the MAPK signaling pathway may increases p21 (Cip1) expression to suppress keratinocyte proliferation, which indicated that hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-590-5p, hsa-let-7f-5p and hsa-miR-378c may be associated with p21 (Cip1) expression and keratinocyte proliferation. In addition, the PI3K-Akt and mTOR signaling pathways may regulate the growth and differentiation of...
Table VII. Pathways associated with the differentially expressed miRNAs.

| Pathway                          | Function                                                                                     | Related differentially expressed miRNAs                                                                 |
|----------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|
| PI3K-Akt signaling pathway       | Activated by many types of cellular stimuli or toxic insults; regulates fundamental           | hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-1297, hsa-miR-378e, hsa-miR-378c                               |
|                                  | cellular functions, including transcription, translation, proliferation, growth and survival   |                                                                                                         |
| MAPK signaling pathway           | Highly conserved module, involved in various cellular functions, including cell proliferation, | hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-590-5p, hsa-let-7f-5p, hsa-miR-378c, hsa-miR-299-3p |
|                                  | differentiation and migration                                                                  |                                                                                                         |
| Protein processing in            | Promotes cell apoptosis                                                                       | hsa-miR-181c-5p, hsa-miR-1297, hsa-miR-374a-5p, hsa-miR-222-3p, hsa-miR-135b-5p                   |
| endoplasmic reticulum            |                                                                                             |                                                                                                         |
| Focal adhesion                   | Serves essential roles in important biological processes, including cell motility, cell      | hsa-miR-181c-5p, hsa-miR-1297, hsa-miR-374a-5p, hsa-miR-378e, hsa-miR-4644, hsa-miR-378c            |
|                                  | proliferation, cell differentiation, regulation of gene expression and cell survival          |                                                                                                         |
| Hippo signaling pathway          | Promotes Mads localization in the cytoplasm, leading to cell apoptosis and restricting organ  | hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-4644                                                        |
|                                  | size overgrowth                                                                              |                                                                                                         |
| Wnt signaling pathway            | Required for basic developmental processes, including cell-fate specification, progenitor-    | hsa-miR-20b-5p, hsa-miR-1297, hsa-miR-374a-5p, hsa-miR-222-3p, hsa-miR-135b-5p                  |
|                                  | cell proliferation and control of asymmetric cell division                                      |                                                                                                         |
| Cell cycle                       | Regulation of cell mitosis                                                                     | hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-590-5p, hsa-miR-454-3p, hsa-miR-135b-5p |
| TGF-β signaling pathway          | TGF-β family members are involved in a wide spectrum of cellular functions, including        | hsa-miR-181c-5p, hsa-miR-20b-5p, hsa-miR-374a-5p, hsa-miR-590-5p, hsa-miR-454-3p, hsa-miR-135b-5p |
|                                  | proliferation, apoptosis, differentiation and migration                                         |                                                                                                         |
| Adherens junction                | Important for maintaining tissue architecture and cell polarity, and can limit cell           | hsa-miR-181c-5p, hsa-miR-135b-5p, hsa-miR-378e, hsa-miR-4644, hsa-miR-378c                      |
|                                  | movement and proliferation                                                                     |                                                                                                         |
| p53 signaling pathway            | p53 activation is induced by a number of stress signals, including DNA damage, oxidative      | hsa-miR-1297                                                                                           |
|                                  | stress and activated oncogenes, thus resulting in three major outputs: Cell cycle arrest,     |                                                                                                         |
|                                  | cellular senescence and apoptosis                                                              |                                                                                                         |
| Apoptosis                        | Apoptosis is a genetically controlled mechanism of cell death involved in the regulation of  | hsa-miR-20b-5p                                                                 | miRNA/miR, microRNA; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor.
keratinocytes (38,39), whereas the Hedgehog signaling pathway is a critical regulator of lineage-specific stem cells that maintains specialized sensory compartments in the epidermis (40) which may serve a key role in re-expression of biological characteristics in induced keratinocytes. 

The present study aimed to determine whether the differentially expressed miRNAs are associated with dedifferentiation of keratinocytes induced by GF109203X. It is well known that Oct-3/4, sex determine region Y-box 2 (Sox2), Nanog, c-Myc and Kruppel-like factor 4 (KLF4) are associated with dedifferentiation (41,42). In cancer cells and mouse embryonic stem cells, zinc finger E-box binding homeobox 1 links epithelial-mesenchymal transition activation and maintenance of stemness by suppressing stemness-inhibiting miRNAs, including miR-200c, miR-203 and miR-183, which cooperate to suppress expression of stem cell factors, such as Sox2 and KLF4 (43). In addition, miR-134, miR-296 and miR-470, which are upregulated during retnoicacid-induced differentiation of mouse embryonic stem cells, target the amino acid coding sequence of Nanog, Oct4 and Sox2 genes, leading to transcriptional and morphological alterations characteristic of differentiating mouse embryonic stem cells, and resulting in a novel phenotype (44). Lauschke et al (45) identified that miRNAs are important drivers of hepatic dedifferentiation. Taken together, dedifferentiation is a process associated with modulation of numerous genes, in which miRNAs may have an important role; this may explain why so many differentially expressed miRNAs were detected during GF109203X-induced keratinocyte dedifferentiation.

In conclusion, when treated with the PKC inhibitor GF109203X, keratinocytes exhibited a series of alterations, including altered morphology, expression of epidermal cell-specific markers and differentially expressed miRNAs. Bioinformatics analysis of the differentially expressed miRNAs indicated that inhibition of PKC signaling was associated with cell proliferation, differentiation and dedifferentiation. Considering that pre-clinical and clinical studies have demonstrated that modulation of miRNA expression by administration of specific miRNA mimics or inhibitors may be beneficial for treating diseases (46), the present study may offer novel miRNAs for regulation of the PKC pathway. However, the exact mechanisms underlying the differentially expressed miRNAs remain unclear and require further study.

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