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Fluctuating levels of reprogramming factor expression in cultured human undifferentiated keratinocytes

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Although human undifferentiated keratinocytes (HUKs) can be reprogrammed to become induced pluripotent stem cells (iPSCs) with high efficiency and rapid kinetics by transducing reprogramming factors (RFs), the endogenous expression of reprogramming factors in cultured HUKs is not clear at different stages. In this study, keratinocytes were isolated from foreskin of adult subject (18 years old) and cultured on collagen type IV-coated culture dishes in a low-calcium, serum-free medium (Epilife, Invitrogen). In order to clarify the expression patterns of RFs and other stem cell markers in cultured human keratinocytes, total RNA was extracted using Trizol reagent, and polymerase chain reaction (PCR) was performed using established GenBank sequences to design primers. The subsequent PCR analysis was carried out by agarose gel electrophoresis. The expression levels of RFs and other stem cell markers in human HUKs clearly fluctuated during culturing, which supports the hypothesis that HUKs might be reprogrammed into a pluripotent state when the maximum levels of RFs expression are maintained by appropriate culture conditions.

Key words: Human undifferentiated keratinocytes, reprogramming factors, expression fluctuation.

INTRODUCTION

Human somatic cells can be directly reprogrammed to become induced pluripotent stem cells (iPSCs) by ectopic expression of reprogramming factors (RFs). Results from many independent groups have suggested that human iPSCs, once established, generally exhibit a normal karyotype, are transcriptionally and epigenetically similar to embryonic stem cells (ESCs) and maintain the potential to differentiate into derivatives of all germ layers (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). This strategy, therefore, represents a significant breakthrough toward the practical use of iPSCs in regenerative medicine. For instance, iPSCs

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Abbreviations: HUKs, Human undifferentiated keratinocytes; **iPSCs,** induced pluripotent stem cells; **RFs,** reprogramming factors; **PCR,** polymerase chain reaction; **ESCs,** embryonic stem cells; **Oct4,** octamer-binding transcription factor 4; **Sox2,** SRY (sex determining region Y)-box 2; **Klf4,** Krüeppel-like factor 4; **c-Myc,** v-myc myelocytomatosis viral oncogene homolog; **CRIPTO,** teratocarcinoma-derived growth factor 1; **REX1,** RNA exonuclease 1; **KRT,** keratin; **RT-PCR,** reverse transcription polymerase chain reaction.

could serve as disease models and aid in the discovery of drugs and genes; furthermore, this approach to generating patient-specific iPSCs would undoubtedly transform regenerative medicine in numerous ways. Current reprogramming protocols, however, predominantly involve the expression of exogenous RFs using virus-mediated transduction. The potentially harmful genomic insertion of these viruses has been the major barrier to the clinical use of iPSCs generated using this approach. Therefore, recruiting endogenous Oct4 (octamer-binding transcription factor 4), Sox2 (SRY <sex determining region Y>-box 2), Klf4 (Krüeppel-like factor 4) and c-Myc (v-myc myelocytomatosis viral oncogene homolog) genes for reprogramming, eliminating the need for transgenes and other factors, would be a more practical approach with regard to the therapeutic use of iPSCs. Such an approach would make it possible for safe, viral-free human iPSCs to be derived routinely in the near future. The high efficiency and rapid kinetics of the generation of iPSCs from human undifferentiated keratinocytes (HUKs) involve retrovirus-mediated expression of three (Oct4, Sox2 and Klf4) or four (Oct4, Sox2, Klf4 and c-Myc) RFs (Aasen et al., 2008), suggesting that an accessible source of more easily reprogrammable cells from epidermal biopsies should facilitate progress toward the goal of safe, efficient iPSCs generation using non-transgenic approaches. Until recently, it was shown that mouse somatic cells-derived iPSCs can be generated using a combination of small-molecule compounds(Hou, Li et al. 2013).

In addition, the endogenous expression of RFs in HUKs should be characterized before non-transgenic reprogramming approaches are developed. In this study, therefore, we clarified the expression patterns of RFs and other stem cell markers in cultured human keratinocytes.

MATERIALS AND METHODS

Cell resources and culture medium

Foreskin removed from adult subject (18 years old) who underwent a routine circumcision was provided by the Chinese People's Liberation Army General Hospital. The patient and his family members signed an informed consent form. The fresh foreskin was collected, and the subcutaneous tissue was removed. Keratinocytes were separated from the foreskin using dispase and trypsin and cultured on collagen type IV-coated culture dishes in low-calcium, serum-free defined medium (Epilife, Invitrogen) as described below.

Isolation and culture of keratinocytes

Human adult foreskin from routine circumcisions was processed within 2 h of collection. Epithelial sheets were obtained after overnight incubation with 3 mg/ml dispase at 4°C, and the separated epidermal sheet was placed in 0.25% trypsin for 30 min at 37°C. Medium was then added to inactivate the trypsin, and the tubes were shaken violently to dissociate individual basal keratinocytes. The keratinocytes were plated on collagen type IV-coated culture dishes in low-calcium, serum-free defined medium (Epilife, Invitrogen).

RNA preparation and RT-PCR

The expression of selected genes (seven stem cell markers: Oct4, Sox2, c-Myc, Klf4, Nanog, teratocarcinoma-derived growth factor 1 (CRIPTO) and RNA exonuclease 1 (REX1), and six keratinocyte-specific markers: keratin (KRT) 1, KRT 5, KRT 10, KRT 14, KRT 15, and KRT 19) was analyzed by reverse transcription polymerase chain reaction (RT-PCR). Three wells of human keratinocytes (in 6-well plates) were collected on days 0, 1, 5, 18 and 33 of culture. Total cellular RNA was isolated and reversely transcribed using conventional protocols. PCR amplification was performed using the primer sets shown in Table 1. All primer sequences were determined using established GenBank sequences. Duplicate PCR reactions were amplified using primers designed against β-actin as a control to assess PCR efficiency and for subsequent analysis by agarose gel electrophoresis.

RESULTS

Morphological inspection of human keratinocytes during differentiation

Figure 1 shows the morphological changes observed in human keratinocytes after cultivation in low-calcium, serum-free defined medium for 33 days. The undifferentiated monolayer cultures showed small, uniform, polygonal cells attached to the bottom of collagen type IV-coated plastic dishes soon after plating. From about the fourth or fifth day in culture, the keratinocytes grew and formed colonies at random over the small, uniform, polygonal cells (Figure 1A and B). In a few days, some keratinocytes developed into a stratified epithelium, and intact contaminating fibroblasts were seen among disaggregated keratinocytes via cell proliferation and differentiation (Figure 1C, D, and E).

Expression patterns of genes involved in stem cell defferentiation in human keratinocytes

In order to examine the expression patterns of stem cell markers, human keratinocytes were cultured in low-calcium, serum-free defined medium. The results of the RT-PCR analysis are summarized in Figure 2. The expression profiles of selected genes (seven stem cell markers: Oct4, Sox2, c-Myc, Klf4, Nanog, CRIPTO and REX1, and six keratinocyte-specific markers: KRT 1, KRT 5, KRT 10, KRT 14, KRT 15, and KRT 19) involved in the stem cell development pathway of keratinocytes were analyzed. The expression of each gene was analyzed during the development of keratinocyte stem cells from day 0 to day 33 in order to show the stability and reproducibility of the selected genes in culture.

The stem cell markers for undifferentiated cells, including Oct4, Sox2 and Nanog, and the keratinocyte-

Table 1. PCR primers used in this study.

Gene	Primer sequence	Annealing temperature (°C)	PCR product size (bp)
Oct4	FP:CGTGAAGCTGGAGAAGGAGAAGCTG RP:GAACATGTGTAAGCTGCGGCCCTTG	62	247
Sox2	FP:GCTGCACATGAAGGAGCACCC RP: CGGACTTGACCACCGAACCCA	62	443
c-Myc	FP:ACTCTGAGGAGGAACAAGAA RP:TGGAGACGTGGCACCTCTT	58	159
Klf4	FP:CCAGAGGAGCCCAAGCCAAAG RP:CGAGGTGGTCCGACCTGGAAA	58	289
Nanog	FP:CCCAAAGGCAAACAACCCACT RP:ATTGCTATTCTTCGGCCAGTT	58	276
CRIPTO	FP:TGCCCAAGAAGTGTTCCCTGT RP: GCAGCAGCCTTTACTGGTCAT	60	269
REX1	FP: CGCTGACACCATCCTCATCGG RP: GGCGTCATCGCTTGGTCTTGG	62	269
KRT1	FP: AGGATGTGGATGGTGCTTAT RP: GCTTTGCTCTTCTGGGCTAT	58	235
KRT5	FP: CTGGACACCAAGTGGACCCT RP: GCTCCGCATCAAAGAACATC	60	346
KRT10	FP: TGATAATGCCAACATCCTGC RP: CCTCCTCGTGGTTCTTCTTC	60	224
KRT14	FP: GGAGATGATTGGCAGCGTGGA RP: GGACCTGCTCGTGGGTGGACA	68	281
KRT15	FP: AGCCTACCTGAAGAAGAACCACG RP: TGGCATAGCGGCACTCTGTCT	62	365
KRT19	FP: GCGACTACAGCCACTACTACACGAC RP: CGACCTCCCGGTTCAATTCTT	58	474
β-actin	FP: AAAGACCTGTACGCCAACAC RP: GTCATACTCCTGCTTGCTGAT	62	219

specific marker KRT 19 were first detectable in vivo (day 0) and were expressed at maximum levels on day 5 of cultivation before becoming undetectable. Klf4 and the keratinocyte-specific marker KRT 1 were undetectable in vivo (day 0), and their maximum expression levels were observed on day 5 before becoming undetectable at day 10. The stem cell markers for undifferentiated cells, including c-Myc and CRIPTO, and keratinocyte-specific markers, including KRT 5, KRT 14 and KRT 15, were detectable *in vivo* (day 0) and were expressed at maximum levels on day 5 before showing reduced gene expression after day 10. In addition, the stem cell marker REX1 and keratinocyte-specific marker KRT 10 were undetectable *in vivo* (day 0) and were expressed at maximum levels on day 5 before showing reduced gene expression after day 10.

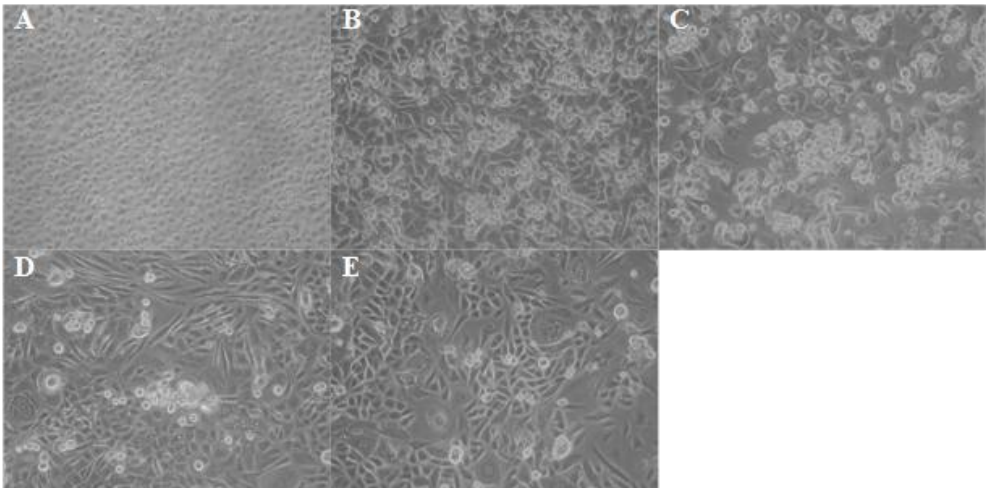

Figure 1. Morphological changes observed in human keratinocytes during cultivation in low-calcium, serum-free defined medium. (A) Morphology of human keratinocytes on day 1 in culture. (B) From the fifth day, the keratinocytes grew and formed colonies at random over the small, uniform, polygonal cells. (C-E) In a few days, some keratinocytes developed into a stratified epithelium (C: day 10), and intact contaminating fibroblasts were seen among disaggregated keratinocytes via cell proliferation and differentiation (D: day 18; E: day 33). Scale bar 100 μm.

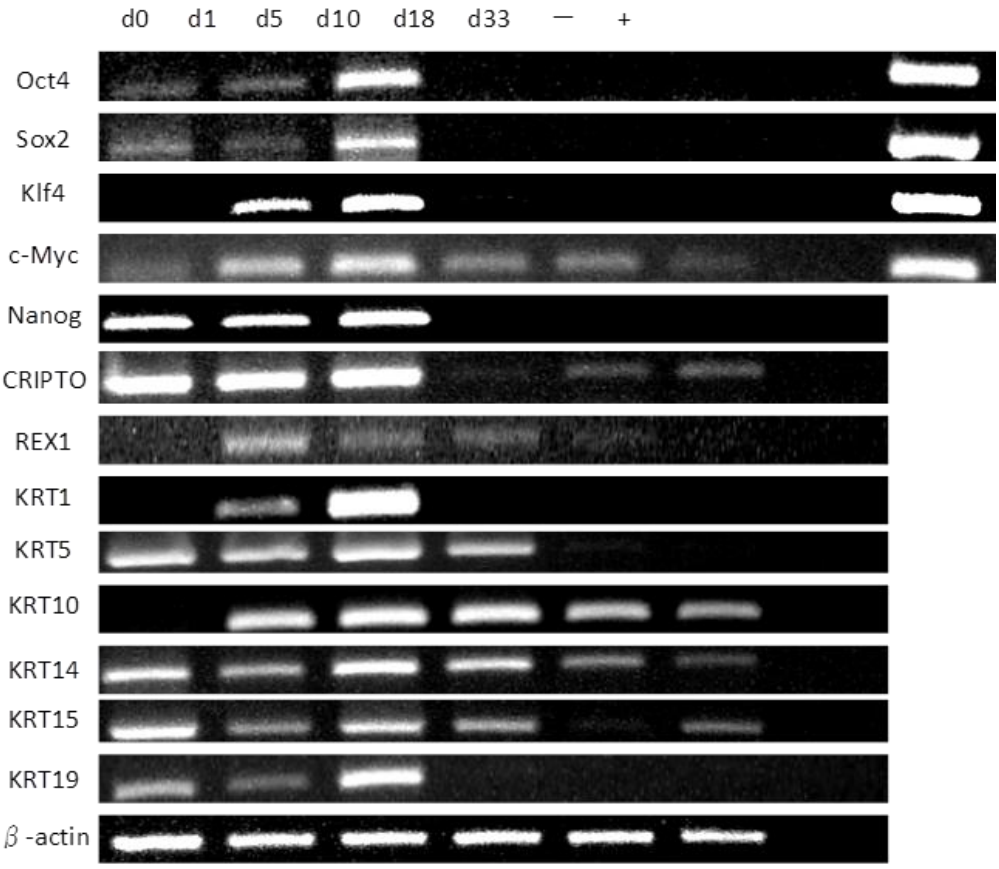

Figure 2. RT-PCR analysis of the expression of selected genes (seven stem cell markers: Oct4, Sox2, c-Myc, Klf4, Nanog, CRIPTO and REX1, and six keratinocyte-specific markers: KRT 1, KRT 5, KRT 10, KRT 14, KRT 15 and KRT 19) in human keratinocytes during cultivation in vitro. We used the pMIG-hOCT4, pMIG-hSOX2, pMIG-hKLF4 and pBABE-c-myc-zeo vectors as PCR templates for positive controls.

DISCUSSION

In this study, we evaluated the expression patterns of RFs and other stem cell markers in cultured human keratinocytes and found that the expression levels of almost all of the selected genes, including Oct4, Sox2, c-Myc and Klf4, were significantly increased in the early phase of culturing and decreased upon cellular differentiation. Our results strongly suggest that the fluctuation of stem cell marker expression is ubiquitous in cultured human keratinocytes. The culture characteristics of human keratinocytes support the hypothesis that the reprogramming process could be more easily achieved when pluripotent markers are expressed at maximum levels during cultivation *in vitro*. Oct4 expression is essential for the development of the inner cell mass (ICM) *in vivo*, the derivation of ESCs and the maintenance of a pluripotent state (Nichols et al., 1998), and the precise levels of Oct4 govern three distinct fates of ESCs (Niwa et al., 2000). A less than twofold increase in expression causes differentiation into primitive endoderm and mesoderm, whereas inhibition of Oct4 expression induces a loss of pluripotency and differentiation into trophectoderm (Niwa et al., 2000).

Sox2 is a transcription factor involved in the self-renewal of ESCs. It plays an important role in maintaining ES-cell pluripotency and heterodimerizes in a complex with Oct4 (Yuan et al., 1995). Sox2 expression is restricted to cells with stem cell characteristics, supporting their self-renewal capability, and is no longer expressed in cells with more restricted developmental potential (Avilion et al., 2003). In addition, forced expression of Oct4 can compensate for the loss of Sox2 in ESCs (Masui et al., 2007).

c-Myc is a pleiotropic transcription factor and has been linked to several cellular functions, including cell-cycle regulation, proliferation, growth, differentiation and metabolism (Schmidt, 1999). It tends to be highly expressed in the majority of rapidly proliferating cells and is generally expressed at low levels or absent during quiescence (Murphy et al., 2005). The role of c-Myc in reprogramming is not yet clear. It is dispensable for the generation of iPSCs in mice and humans (Eminli et al., 2008; Huangfu et al., 2008; Kim et al., 2008; Nakagawa et al., 2008; Wernig et al., 2008), but the efficiency of reprogramming decreases dramatically without c-Myc.

Klf4 is a transcription factor expressed in a variety of tissues, including the epithelium of the intestine, kidney and skin (Segre et al., 1999). Depending on its interaction partner and the target gene, Klf4 can both activate and repress transcription (Rowland and Peeper, 2006), and a growing body of evidence suggests that Klf4 can function as both an oncoprotein and a tumor suppressor (Zhao et al., 2004). Constitutive expression of Klf4 suppresses cell proliferation by blocking G1–S progression through the cell cycle (Zhao et al., 2004). Recently, it has been demonstrated that forced over expression of Klf4 in ESCs inhibits differentiation in erythroid progenitors, suggesting a role for this factor in ES cell function (Li et al., 2005). Its exact role in the reprogramming process is still not fully understood.

Transcripts of human keratinocyte stem cell markers have been detected in vitro (Aasen et al., 2008), and keratinocytes treated with ESC-conditioned medium (CM) change their morphology and express the pluripotency regulator Oct4 and its target transcripts Sox2, Nanog and REX1 (Grinnell and Bickenbach, 2007). In our gene expression profile, no transcripts of Klf4 or REX1 were detected *in vivo* (day 0). The different expression levels observed for Klf4 and REX1, however, suggest that these two markers may be involved in the differentiation of epithelial cells.

It has been speculated that the expression of Oct4 fluctuates during the development of keratinocytes from cultured human ESCs (Green et al., 2003) as it is expressed at high levels in ESCs and shows reduced expression at day 6 of cellular differentiation (Pellizzer et al., 2004). Furthermore, key genes that control pluripotency, including Oct4, Sox2 and Nanog, undergo dynamic changes in transcript abundance during porcine embryo cleavage development (Magnani and Cabot 2008). In the course of keratinocyte differentiation, the expression levels of KRT 5 and KRT 14 decline, whereas the expression levels of KRT 1 and KRT 10 are augmented (Kartasova et al., 1992). It has also been reported that CaCl2 induces the differentiation of epidermal keratinocytes in culture (Hennings et al., 1980), with decreased expression of KRT 5 and 14, markers of the basal cell layers, and increased expression of KRT 1 and 10, markers of the prickle cell layers (Vellucci et al., 1995; Tennenbaum et al., 1996; Amoh et al., 2005; Yano et al., 2005). The expression of KRT 15, which has been reported to be a bulge stem cell marker, is significantly induced in both NHEKs and BDKs after CaCl2 treatment (Sasahara et al., 2009). Previous studies have evaluated the expression pattern of KRT 19 in the phase following the degeneration of the lowest part of the follicle. When epithelial cell populations were cultured, the percentage of KRT 19-positive keratinocytes significantly increased in primary culture, remained high for the first subcultures and then decreased prior to senescence (Michel et al., 1996). A similar expression pattern was observed in our study, except for the fact that no transcripts of KRT 1 or KRT 19 were detected after day 10.

Fluctuation in the expression levels of stem cell markers occurs during increased proliferation, rather than terminal differentiation, in their initial developmental stage, while the converse situation is observed in later stages. Our results show that the fluctuation in the expression levels of stem cell markers could provide an opportunity for reprogramming using non integrating viruses or transient episomal gene expression or, more favourably, for the generation of iPSCs by biochemical means alone.

In conclusion, this study highlights the expression pat-

patterns of stem cell markers that are present during early cellular development in cultured human keratinocytes, guiding future induction protocols for pluripotent stem cells and bioengineering research in this field.

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