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Highly rapid and efficient conversion of human fibroblasts to keratinocyte like cells

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Abstract

Cell fate commitment during development is achieved through the expression of lineage specific transcription factors. Recent studies have suggested that expression of combinations of these lineage specific transcription factors can convert adult somatic cells from one type to another. Here we report that the combination of p63, a master regulator of epidermal development and differentiation, and KLF4, a regulator of epidermal differentiation is sufficient to convert dermal fibroblasts to a keratinocyte phenotype. Induced keratinocytes expressed keratinocyte specific proteins and had a transcriptome similar to keratinocytes. Reprogramming to a keratinocyte phenotype was rapid and efficient with a vast majority of cells morphologically resembling and expressing keratinocyte specific genes within a week of p63 and KLF4 transduction. Furthermore, p63 and KLF4 are capable of inducing a keratinocyte phenotype even in a cancerous cell line highlighting their importance for epidermal specification. The robustness of the conversion process also allows the use of this as a model system to study the mechanisms of reprogramming.

Keywords

Fibroblasts; p63; KLF4; ZNF750; GRHL3; OVOL1; PRDM1; keratinocytes; epidermis; direct conversion; reprogramming; cell fate; differentiation

Introduction

The distinct cell types that are present in adult mammals are a consequence of cell fate commitment and differentiation during embryonic development. During development, tightly regulated combinatorial expression of lineage specific transcription factors commits cells to certain fates, which is further reinforced and stabilized by changes in the epigenome. It was assumed that once a cell had committed and differentiated into a specific lineage, it would no longer be competent to be reprogrammed into another cell type. However, classic experiments such as the expression of a muscle specific transcription factor, MyoD, in

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Conflict of interest

The authors declare no conflict of interest

fibroblasts could induce muscle specific genes in those cells or the forced fusion of two unrelated cell types could cause the re-expression of previously silenced genes suggested that differentiated cells are more plastic than previously thought (Blau et al., 1983; Chiu and Blau, 1984; Davis et al., 1987). More recently, it was demonstrated that the expression of specific transcription factors could reprogram fibroblasts into induced pluripotent stem cells (iPS), hepatocytes, neurons, cardiomyocytes, angioblasts and neural and hematopoietic progenitor cells (Huang et al., 2011; Ieda et al., 2010; Kurian et al., 2013; Lujan et al., 2012; Sekiya and Suzuki, 2011; Szabo et al., 2010; Takahashi and Yamanaka, 2006; Vierbuchen et al., 2010). These studies suggest that cellular fate can be altered through the introduction of specific factors and that conversion to any cell type is potentially possible provided the correct factors are used. Despite these reports, the number of examples of reprogramming to certain cell types is relatively low in comparison to the number of distinct cell types in the body. Therefore, it is crucial to identify the factors necessary to generate all the important cell types in the body such as basal keratinocytes.

Basal keratinocytes are the stem and progenitor cells that maintain the self-renewing epidermis. They give rise to all the differentiated layers of the stratified epidermis including the suprabasal layer, granular layer, and the stratum corneum which provides the barrier that prevents dehydration as well as defense against pathogens (Blanpain and Fuchs, 2009; Sen, 2011). Many diseases afflict the epidermis including psoriasis, atopic dermatitis, epidermolysis bullosa, ichthyosis vulgaris, epidermal cancers, and chronic wounds in which a subset may benefit from cell replacement therapies (Segre, 2006; Uitto et al., 2012). Thus being able to directly reprogram cells into a keratinocyte fate is important scientifically as well as potentially clinically useful.

Results

KLF4 and p63 are sufficient to convert human dermal fibroblasts to an induced keratinocyte phenotype (iKC)

To screen for keratinocyte inducing factors, we selected 6 candidate transcription factors (PRDM1, p63, KLF4, OVOL1, ZNF750, and GRHL3) that we and others have previously published to be critical for epidermal fate determination and differentiation (Magnusdottir et al., 2007; Mills et al., 1999; Mistry et al., 2012; Nair et al., 2006; Segre et al., 1999; Sen et al., 2012; Ting et al., 2005; Truong et al., 2006; Yang et al., 1999). Of the p63 isoforms, Np63 α , was used as it is the predominant isoform expressed in neonatal keratinocytes (Sen et al., 2012; Truong et al., 2006). A mixture of all 6 retroviruses encoding each gene was transduced into neonatal dermal fibroblasts to determine if epidermal specific genes could be induced. At eleven days post-infection with the 6 retroviruses, strong induction of keratinocyte specific genes, *KRT14* and *GJB2*, could be detected by quantitative polymerase chain reaction (qPCR) (Figure 1a). This suggests that the combination of some or all of the factors, in the 6 gene pool, was sufficient for converting fibroblasts into an induced keratinocyte phenotype (iKC). To determine which of the 6 genes were critical to iKC formation, individual genes were removed from the pool of 6 candidate factors. Removal of KLF4 had the most dramatic effect, reducing *GJB2* and *KRT14* levels to near background levels of fibroblasts transduced with LacZ control (Figure 1a). Loss of p63 also reduced

induction of *GJB2* and *KRT14* by 3-4 fold as compared to cells transduced with all 6 factors (Figure 1a). Removal of any of the other 4 factors did not decrease the expression of the keratinocyte specific genes (Figure 1a). Because loss of KLF4 had the strongest impact on iKC formation, KLF4 was expressed in combination with each individual factor to determine the effects on *KRT14* and *GJB2* expression (Figure 1b). KLF4 expression with LacZ induced *KRT14* expression by ~80 fold and *GJB2* by ~60 fold over LacZ expressing fibroblasts (Figure 1b). Remarkably, the combination of KLF4 and p63 induced the expression of *KRT14* by over 2,000 fold and *GJB2* by over 1,400 fold over control levels (Figure 1b). The expression of KLF4 in combination with any of the 4 other factors did not significantly increase the levels of *GJB2* or *KRT14* over KLF4 and LacZ suggesting that of the 6 factors, KLF4 and p63 are the most critical to inducing iKC formation (Figure 1b). By eleven days post-transduction of p63+KLF4 (PK), the fibroblasts had converted from a spindle shaped cell to a cell morphologically similar to keratinocytes including tight cell-cell adhesion (Figure 1c). Staining with basal keratinocyte proteins, keratin 5 (K5) and keratin 14 (K14), showed that fibroblasts transduced with PK displayed the same level of expression and staining patterns as keratinocytes whereas no staining was detectable for fibroblasts transduced with LacZ (Figure 1d). DSG2 staining showed that iKC cells integrated with each other and formed desmosomes (Supplementary Figure S1).

Conversion to a keratinocyte phenotype with p63 and KLF4 is efficient and rapid

To determine the kinetics and efficiency of reprogramming to iKC cells, a time course from day 1 to day 7 post-infection with PK was performed. At day 1, a small fraction (1.4%) of the cells were positively stained for K14 with none detectable in control LacZ cells (Figure 2a). By day 2, up to 7% of the cells began to express K14 although the cells still retained the spindle shaped morphology of a fibroblast (Figures 2a-2b). By day 4, cells with epithelial morphology that stained for K14 (30%) were readily detected which increased over time (Figures 2a-2b). By day 6, a majority of the cells had adopted an epithelial morphology and 53% stained for K14 (Figures 2a-2b) which increased to 67% by day 7. The mRNA levels of *KRT14* were induced by over 250 fold by day 1 after infection and steadily increased over the time-course with a peak of around 900 fold over LacZ controls at day 7 (Figure 2c). Similarly, *GJB2* was induced by day 2 and increased over time (Figure 2d). Fibroblast specific genes such as *MME* and *COL1A1* were downregulated by 50% by day 1 for *MME* and by over 60% for *COL1A1* by day 2 suggesting that silencing the fibroblast gene expression program and expression of the keratinocyte program occurs early during conversion to iKC cells (Figures 2e-2f). Interestingly, endogenous KLF4 and p63 expression was not induced during the conversion to iKC cells while the transgene expression remained similar throughout the 7 day timecourse (Supplementary Figure S2 a-c).

p63 and KLF4 reprogram fibroblasts to a keratinocyte gene expression program

To determine the extent of reprogramming to keratinocytes, global gene expression profiling was performed comparing fibroblasts (FB), iKC cells, and undifferentiated keratinocytes (KC). Keratinocyte specific genes were first identified by determining the differentially expressed genes between fibroblasts and keratinocytes. 2,772 genes (≥ 2 fold change; $\leq 5\%$ FDR) were differentially expressed between fibroblasts and keratinocytes which make up the keratinocyte (KC) gene expression signature (Figure 3a and Supplementary Excel 1).

1,429 genes were expressed 2 fold or greater in keratinocytes than in fibroblasts with enriched gene ontology terms such as cell-cell junction, cell adhesion, keratinocyte differentiation, and epidermis and ectoderm development (Figure 3a and Supplementary Excel 1). 1,343 genes were downregulated in keratinocytes as compared to fibroblasts with enriched gene ontology (GO) terms such as cell motion, extracellular matrix, and glycoprotein (Figure 3a and Supplementary Excel 1). Next, fibroblasts transduced with PK were compared to control fibroblast cells to determine the extent of reprogramming. 2,795 genes were differentially expressed which make up the iKC gene signature (Figure 3b and Supplementary Excel 2). Significantly, 1,190 genes were downregulated in iKC cells which were enriched in “fibroblast” specific gene ontology terms such as cell motion and glycoprotein whereas the 1,605 genes that were upregulated were enriched in “keratinocyte” specific GO terms such as keratinocyte differentiation, cell adhesion, and epidermis development (Figure 3b and Supplementary Excel 2). To determine the number of genes that were reprogrammed from a fibroblast to a keratinocyte signature, the iKC gene signature (2,795 genes) was overlapped with the 2,772 genes that made up the KC signature. Nearly half of the iKC gene signature (46%; 1,296/2,795) overlapped with the KC signature (Figure 3c and Supplementary Excel 3). Of those genes, 85% (1,100/1,296) were expressed in the same direction as keratinocytes and oppositely from the fibroblasts (Figure 3c and Supplementary Excel 4-5). 504 of the overlapped genes were upregulated in the same direction as keratinocytes, which were enriched in keratinocyte specific GO terms such as cell-cell junction and keratinocyte differentiation (Figure 3c and Supplementary Excel 4). These keratinocyte specific genes include desmosome genes (*DSC2*, *DSC3*, *DSG3*, *PKP1*, *DSP*, *PKP3*), gap junction genes (*GJB2*, *GJB3*, *GJB5*, *GJB6*), cell polarity and tight junction genes (*CLDN1*, *PARD6B*, *PARD6G*, *PRK CZ*), and ectoderm development genes (*EMP1*, *KRT14*, *KRT16*, *KRT17*, *KRT5*, *KRT6A*, *KRT6B*, *LAMA5*, *LAMB3*, *LAMC2*, *SFN*, *COL7A1*, *BNCL1*) (Supplementary Excel 4). 596 genes were downregulated similar to keratinocytes which were enriched in fibroblast specific GO terms such as cell motion (Supplementary Excel 5). To determine whether this reprogramming in gene expression was due to PK and not also due to chromosomal rearrangements/alterations, karyotyping was performed on iKC cells. Karyotyping analysis of the iKC cells showed a normal karyotype of 46 XY similar to fibroblasts transduced with LacZ (Supplementary Figure S3 a-b). These results suggest that there are no gross chromosomal abnormalities in iKC cells with a significant portion of the iKC gene expression signature similar to keratinocytes.

iKC cells are partially differentiated

Since 1,499 genes of the iKC gene signature did not overlap with the undifferentiated keratinocyte signature, we hypothesized that a subset of these genes may be keratinocyte differentiation genes. Of the 1,499 genes that did not overlap, 982 were upregulated relative to fibroblasts and may represent induced epidermal differentiation genes. The 982 genes were compared with our previously generated data set of 1,366 genes that are upregulated during calcium induced keratinocyte differentiation (Sen et al., 2010) (Figure 4a). Interestingly, 12% (118/982) of the upregulated genes are epidermal differentiation induced genes suggesting that iKC cells are partially differentiated (Figure 4a and Supplementary Excel 6). These genes include differentiation specific structural, enzymatic, and transcriptional regulators such as *CNFN*, *S100A7*, *GRHL1*, *SPINK6*, and *TGMI* (Figure 4a

and Supplementary Excel 6). Comparison of the expression level of differentiation induced genes *FLG* and *KRT10* for iKC cells (FB-PK), undifferentiated keratinocytes (KC), and keratinocytes differentiated with calcium (KC+Ca²⁺) demonstrated that iKC cells expressed *FLG* and *KRT10* mRNA to levels comparable to differentiated keratinocytes (Figure 4b). Immunostaining also shows that a portion of iKC cells express differentiation protein FLG but not K1 (*KRT1*) suggesting that a subset of the differentiation program is turned on (Figure 4c).

Because iKC cells are partially differentiated, we asked whether they could be further induced to differentiate upon calcium addition. Global gene expression analysis of iKC cells treated with high levels of calcium showed that 1,040 genes (485 genes induced; 555 genes repressed) were differentially expressed between iKC cells +/- calcium (Supplementary Figure S4a and Supplementary Excel 7). Induced genes fell into GO categories such as keratinocyte differentiation, cell adhesion and EGF-like calcium binding while genes repressed had functions in cell cycle and DNA replication (Supplementary Figure S4a and Supplementary Excel 7). This suggests that similar to keratinocytes treated with calcium, iKC cells also underwent the differentiation program by downregulation of proliferation and induction of structural and enzymatic differentiation genes. The 485 genes induced upon iKC treatment with calcium was compared with the 1,366 genes that are upregulated during calcium mediated keratinocyte differentiation (Sen et al., 2010). 22% (107/485) of the upregulated genes are keratinocyte differentiation induced genes (Supplementary Figure S4b and data available upon request). Comparison of the genes downregulated in calcium treated iKC cells and keratinocytes showed that 60% (335/555) of the downregulated genes in iKC cells are the same as those downregulated during keratinocyte differentiation (Supplementary Figure S4c and data available upon request). These results suggest that iKC cells are responsive to calcium induced differentiation with 42.5% (442/1,040) of its differentially expressed genes regulated in the same way as keratinocyte differentiation genes (Supplementary Figure S4d).

The zinc finger domain in the C-terminus of KLF4 is necessary for iKC formation

Because of the importance of KLF4 to the reprogramming process, we decided to determine the regions of KLF4 necessary for iKC formation (Supplementary Figure S5a). Deletion of 160 amino acids from the N terminus, C terminus as well as N and C terminus were made and transduced along with p63 to determine the region necessary for function (Supplementary Figures S5a-b). Removal of 160 amino acids from the N terminus did not significantly alter the reprogramming capability of KLF4 whereas loss of the C terminus abrogated the induction of epidermal specific proteins, K5 and K14 (Supplementary Figure S5b). Analysis of the C terminus showed an evolutionarily conserved C2H2 zinc finger domain. Conversion of the two histidines to alanines (H2 mutant) of the last C2H2 of the zinc finger domain blocked the reprogramming capacity of KLF4 suggesting that the DNA binding zinc finger domain is necessary for the conversion of fibroblasts to iKC cells (Supplementary Figures S5a-b).

p63 and KLF4 reprogram fibroblasts to a keratinocyte chromatin signature

To determine if the epigenetic signature of iKC cells have been reprogrammed to a state similar to keratinocytes, we analyzed the histone modifications in the promoter regions of keratinocyte specific genes, *KRT14*, *GJB2*, and *GJB3*. The enrichment for trimethylated histone H3 lysine 27 [H3K27me3(Li et al., 2007; Sen et al., 2008); repressive mark] and trimethylated histone H3 lysine 4 [H3K4me3(Li et al., 2007); active mark] were compared between fibroblasts-LacZ, fibroblasts-PK, and keratinocytes. Control fibroblasts had a significant enrichment for the repressive H3K27me3 mark on the promoter of all the tested keratinocyte specific genes and no enrichment for the activation mark H3K4me3 confirming that these genes are epigenetically silenced in fibroblasts (Figures 5a-b). In contrast, keratinocytes had a significant enrichment for H3K4me3 and minimal H3K27me3 in the promoter regions indicative of actively transcribed genes (Figures 5a-b). Importantly, fibroblasts transduced with PK lose the H3K27me3 repressive mark and gain the activation mark H3K4me3 in all tested keratinocyte specific genes. Thus, the epigenetic state of iKC cells has been reprogrammed to resemble keratinocytes.

p63 and KLF4 are sufficient to reprogram human colorectal carcinoma cell line, HCT116, to express keratinocyte specific genes

To date, most studies have tested the reprogramming potential of fibroblasts. It is unclear whether other cell types such as cancerous cells lines are also amenable to reprogramming. To determine if PK transduction can reprogram other cell types to iKC cells, we chose to use the human colon carcinoma cell line HCT-116. Transduction of HCT-116 with PK induced the expression of keratinocyte specific genes such as *KRT14* and *SPRR1A* and induced basal keratins K5 and K14 (Supplementary Figure S6a-b). This suggests that PK can reprogram different cell types to a keratinocyte phenotype.

Discussion

Here, we report that the combination of two transcription factors, p63 and KLF4 can convert fibroblasts to a keratinocyte phenotype. iKC cells are similar to keratinocytes in global gene expression, morphology, chromatin state, as well as protein expression. Conversion to iKC cells was rapid and efficient as cells with a keratinocyte morphology appeared by 4 days post-transduction with PK and a majority (53%) of the cells staining for the basal keratin marker K14 by day 6. Downregulation of fibroblast genes also occurred early during the reprogramming process. Global gene expression profiling demonstrated that nearly half (46%) of the iKC gene signature overlapped with the keratinocyte gene signature with a vast majority of the genes (85%) being regulated in the same direction. Genes that are upregulated in iKC cells are keratinocyte specific genes such as desmosome, gap junction, cell polarity and tight junction genes while genes involved in fibroblast function such as cell motion are downregulated. Staining with DSG2 also demonstrated that iKC cells functionally integrate with each other through desmosomes. Approximately half of the iKC gene signature did not overlap with the KC signature. We hypothesized that some of these non-overlapping genes may be upregulated keratinocyte differentiation genes since KLF4 has been shown to promote epidermal differentiation(Segre et al., 1999). Comparison with our previously published set (Sen et al., 2010) of upregulated epidermal differentiation

genes suggests that 118 of the 1,499 genes that did not overlap with the KC signature were actually induced during keratinocyte differentiation. This suggests that iKC cells are partially differentiated. Despite having a subset of differentiation genes already being expressed in iKC cells, they were still responsive to calcium. Addition of calcium could further differentiate the cells with over 40% (442/1,040) of the iKC +Ca²⁺ signature overlapping and regulated in the same way as the keratinocyte (KC+Ca²⁺) differentiation signature. This suggests that iKC cells are similar to keratinocytes in that they are responsive to calcium induced differentiation.

The reprogramming of fibroblasts to iKC cells also depends on the C2H2 zinc finger domain of KLF4 as mutating this sequence completely abolishes the conversion to iKC cells. This suggests that the recognition and binding of KLF4 to its DNA targets is essential for the reprogramming process. The chromatin marks of iKC cells are also similar to keratinocytes. The activation mark, H3K4me₃ is deposited in the promoter regions of keratinocyte specific genes while the repressive mark H3K27me₃ is lost in iKC cells. Our results also demonstrate that PK not only reprograms fibroblasts to iKC cells but also a cancerous cell line such as HCT-116 suggesting that p63 and KLF4 are capable of reprogramming cells even in different epigenetic backgrounds. This ability may be due to these proteins functioning as pioneer factors that can engage and turn on gene expression programs even in closed chromatin. KLF4 has recently been shown to act as a pioneer factor in reprogramming fibroblasts to IPS cells(Soufi et al., 2012). During the early stages of reprogramming, it can bind to closed chromatin to reprogram the epigenetic landscape and turn on previously silenced genes(Soufi et al., 2012). It is important to note that KLF4 in combination with specific transcription factors can lead to different cellular fates. Expression of KLF4 with OCT4, SOX2, and c-MYC can produce IPS cells while the combination of p63 and KLF4 can induce a keratinocyte phenotype. It will be interesting to determine how the combination of PK converts cells to a keratinocyte phenotype while the combination of KLF4, OCT4, SOX2, and c-MYC causes the conversion to IPS cells.

Despite the similarities between iKC and keratinocytes there are differences functionally. When keratinocytes are placed on dermis, the cells will stratify and differentiate into multilayered epidermis. However, when iKC cells are placed on dermis, the cells do not properly differentiate or stratify suggesting that the two cell types are not functionally equivalent (data not shown). This inability to regenerate epidermal tissue is likely due to the incomplete reprogramming of iKC cells to keratinocytes which could stem from a couple of issues. First, in most reported cases of complete reprogramming, the endogenous expression of the reprogramming factors is activated while the transgene is silenced(Hotta and Ellis, 2008). In iKC cells, the transgenes are not silenced and the expression of endogenous PK is not activated. Thus, our results suggest that the iKC phenotype is likely dependent upon continuous expression of the transgenes since the endogenous reprogramming genes are not activated. This is similar to the original IPS cells (generated under the *Fbx15* promoter and were not completely reprogrammed) reported by Takahashi and Yamanka where the reprogramming transgenes were not silenced while endogenous reprogramming genes were minimally activated(Hotta and Ellis, 2008; Takahashi and Yamanaka, 2006). However, not all complete reprogramming requires silencing of the transgenes as conversion of fibroblasts

to hepatocytes occurs without transgene silencing but does result in expression of endogenous reprogramming genes (Sekiya and Suzuki, 2011). Second, approximately half of the global gene expression program of the iKC cells did not overlap with keratinocytes. This suggests that other factors expressed in keratinocytes but not in iKC cells may help PK reprogram cells to functional keratinocytes. Potential candidates include INADL, which has been shown to be important for epithelial cell polarity and EHF, which is necessary for epithelial cell differentiation (Albino et al., 2012; Du et al., 2010; Wells et al., 2006). Both of these factors are highly expressed in keratinocytes but not in iKC cells (Supplementary Excel 1-2). Thus, our current studies may serve the basis for future studies to determine which factors in addition to PK will further convert iKC cells to functional keratinocytes which are capable of epidermal tissue formation and be used for potential regenerative therapies.

Materials and Methods

Tissue culture

Primary human fibroblasts and keratinocytes were derived from neonatal foreskin. Fibroblasts were grown in DMEM with 10% fetal bovine serum. Keratinocytes were grown in KSF-M (GIBCO-BRL) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE). Keratinocytes and iKC cells were induced to differentiate by the addition of 1.2 mM calcium for 3 days in full confluence. Amphotrophic phoenix cells and HCT-116 cells were maintained in DMEM and 10% fetal bovine serum. Fibroblasts transduced with LacZ or p63 and KLF4 were maintained in DMEM and 10% fetal bovine serum.

Gene transfer

Amphotrophic phoenix cells were transfected with 3 ug of each retroviral construct to overexpress genes. Transfections were done in 6 well plates using Fugene 6 (Roche). Viral supernatants were collected 48 hours post transfection and polybrene added (5ug/ml). These supernatants were placed on primary human fibroblasts or HCT-116 cells and centrifuged for 1 hour at 2,000rpm. Cells were transduced a total of 2 times. The day after the last transduction is referred to as day 1 post-transduction.

Gene Overexpression

Transcription factors, PRDM1, KLF4, OVOL1, and GRHL3, were cloned into the LZRS retroviral vector. The full-length open reading frames of the above listed genes were cloned in the ECORI/NOTI site of LZRS. The primers used were: PRDM1 FOR: ACGCAGAATTCGCCACCATGGAAAAGATCTATTCCAGAGGGGAGCTTC; PRDM1 REV: ACGCAGCGGCCGCTTAAGGATCCATTGGTTCAACTGTTTCTTG. KLF4 FOR: ACGCAGAATTCGCCACCATGAGGCAGCCACCTGGCGAGTCTGACATGG; KLF4 REV: ACGCAGCGGCCGCTTAAAAATGCCTCTTCATGTGTAAGGCGAGG. OVOL1 FOR: ACGCAGAATTCGCCACCATGCCCCGCGCTTCCTGGTGAAGAAGCCG; OVOL1 REV: ACGCAGCGGCCGCTCACAGGTGGGGGCTGCCCTGCAGCAGGGA. GRHL3 FOR: ACGCAGAATTCGCCACCATGATGAGAGTCAATGGAGATGATGACAGTG; GRHL3

REV: ACGCAGCGGCCGCTTACAGCTCCTTAAGGATGATCTGAATTTTG. The mutant KLF4 constructs were generated using the following primers: N160 KLF4: KLF4 FOR: ACGCAGAATTCGCCACCATGGGCGTGGCGCCGGGCGGCACGGGCG and KLF4 REV: ACGCAGCGGCCGCTTAAAAATGCCTCTTCATGTGTAAGGCGAGG. C160 KLF4: KLF4 FOR: ACGCAGAATTCGCCACCATGAGGCAGCCACCTGGCGAGTCTGACATGG; KLF4 REV: ACGCAGCGGCCGCTTACAGGGTTCGGGGTAGTCCTGCTGGGGA. N160/C160 KLF4: KLF4 FOR: ACGCAGAATTCGCCACCATGGGCGTGGCGCCGGGCGGCACGGGCG; KLF4 REV: ACGCA GCGGCCGCTTACAGGGTTCGGGGTAGTCCTGCTGGGGA. H2 Mutant KLF4: KLF4 FOR: ACGCAGAATTCGCCACCATGAGGCAGCCACCTGGCGAGTCTGACATGG; KLF4 REV: ACGCAGCGGCCGCTTAAAAAGCCCTCTTCATCGCTAAGGCGAGGTGGTCCGACCT GGA The LZRS-ZNF750 and LZRS-LacZ retroviral construct were generated as previously described (Sen et al., 2012). The LZRS- Np63 α retroviral construct was a generous gift from the Khavari laboratory.

Immunofluorescence

For immunofluorescence experiments, cells were fixed in 4% paraformaldehyde for 15 minutes followed by blocking in PBS with 2.5% normal goat serum, 0.3% triton X100, and 2% bovine serum albumin for 30 minutes. Sections were incubated in primary antibodies for 1 hour. Primary antibodies include keratin 5 (Covance: PRB-160P) at 1:300, keratin 14 (Abcam:ab7800) at 1:300, keratin 1 (Covance:PRB-149P) at 1:500, filaggrin (Abcam: ab3137) at 1:200, and desmoglein 2 (Santa Cruz: SC-80663) at 1:100. The secondary antibodies used were Alexa 555 conjugated goat anti-mouse IgG (Molecular Probes) (1:1000) and Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probes)(1:1000). Nuclear dye, Hoechst 33342, was used at 1:1000(Molecular Probes).

Quantitative reverse transcriptase-PCR analysis

Total RNA from cells was extracted using the Rneasy mini kit (Qiagen) and quantified by Nanodrop. One μ g of total RNA was reverse transcribed using the Maxima cDNA synthesis kit from Fermentas. Quantitative PCR was performed using the Roche 480 Light Cycler. Samples were run in triplicate and normalized to GAPDH. Primer sequences for *GAPDH*, *SPRR1A*, and *KRT10* were the same as previously published (Mistry et al., 2012; Sen et al., 2008). Sequences for *KRT14*, *FLG*, *KLF4*, *GJB2*, *MME*, *COL11A1*, *KLF4* 3'UTR, *p63* 3'UTR, and *LZRS* 5'UTR are as follows: KRT14 FOR: GCGGCCTGTCTGTCTCAT, KRT14 REV: CCACCAGAAGCCCATCAC; FLG FOR: GGCAAATCCTGAAGAATCCA, FLG REV: TGCTTTCTGTGCTTGTGTCC; KLF4 FOR: GCTGATGGCAAGTTCGT, KLF4 REV: AAGAGACCGCCTCCTGCT; GJB2 FOR: ACGCATTGCCAGTTGTT, GJB2 REV: AGGGGAGCAGAGCTCCAT; MME FOR: AGTGCCAGCAGTCCAAC, MME REV: CACCATGCACTGGGATTG; COL11A1 FOR: AAGTGGCATCGGGTAGCA, COL11A1 REV: GCTGAATGTCCCCCTCAA; KLF4 3' UTR FOR: AGGAGCCAGCCAGAAAG, KLF4 3' UTR REV: ACCCCCTTGGCATTTTGT; p63 3' UTR FOR:

GGTCCCCACAGAGCAAGA, p63 3' UTR REV: TGCAATGACAGCCCTTGA; LZRS 5' UTR FOR: CCTCACTCCTTCTCTAGGCG, LZRS 5' UTR REV: CTGCCGCCAGAGGTCTCCA.

Gene expression profiling

RNA was harvested from the cells 7 days post-transduction with p63 and KLF4 or LacZ. Microarray analysis was performed on duplicate samples. Labeling of cDNA and hybridization to Affymetrix HG-U133 2.0 plus arrays were performed at the UCSD Genechip Microarray Core Facility. For gene expression analysis, arrays were RMA normalized and differential expression was defined using the following filters: Significance Analysis of Microarrays 3.0 (Tusher et al., 2001) with a false discovery rate less than 5%, and an average raw expression intensity > 100 at any time point. Hierarchical clustering and heat map generation were performed as previously described (Sen et al., 2010). For future analyses, Affymetrix probe IDs were converted to unique Unigene IDs. GO term enrichment was performed using DAVID analysis (Huang et al., 2009) with the total set of genes on the appropriate microarray as the background. Comparisons were made to our previously published data sets on undifferentiated and differentiated keratinocytes (Sen et al., 2010). Gene expression data was deposited in GEO with accession number GSE47746.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations (ChIP) were performed as previously described (Sen et al., 2012; Sen et al., 2010; Sen et al., 2008). 3 millions cells were used for each ChIP. Antibodies used for ChIP were H3K4me3 (Abcam: ab8580) and H3K27me3 (Millipore: 07-449). qPCR primers for the promoter regions of *KRT14*, *GJB2*, and *GJB3* are as follows: *KRT14* for: GCTTAGCCAGGGTGACAGAG, *KRT14* rev: CAGCAGCAGGCTTCCTCTAC; *GJB2* for: AGCACGAAAGGGGAAAC, *GJB2* rev: ATCCCCAACCAAATGCAA; *GJB3* for: GCACCAAGGGTAGAAGATG, *GJB3* rev: ACCTGGTGGGAGTTCACAAG.

Karyotype Analysis

Karyotype analysis on fibroblasts transduced with LacZ or p63 and KLF4 were performed at WiCell Cytogenetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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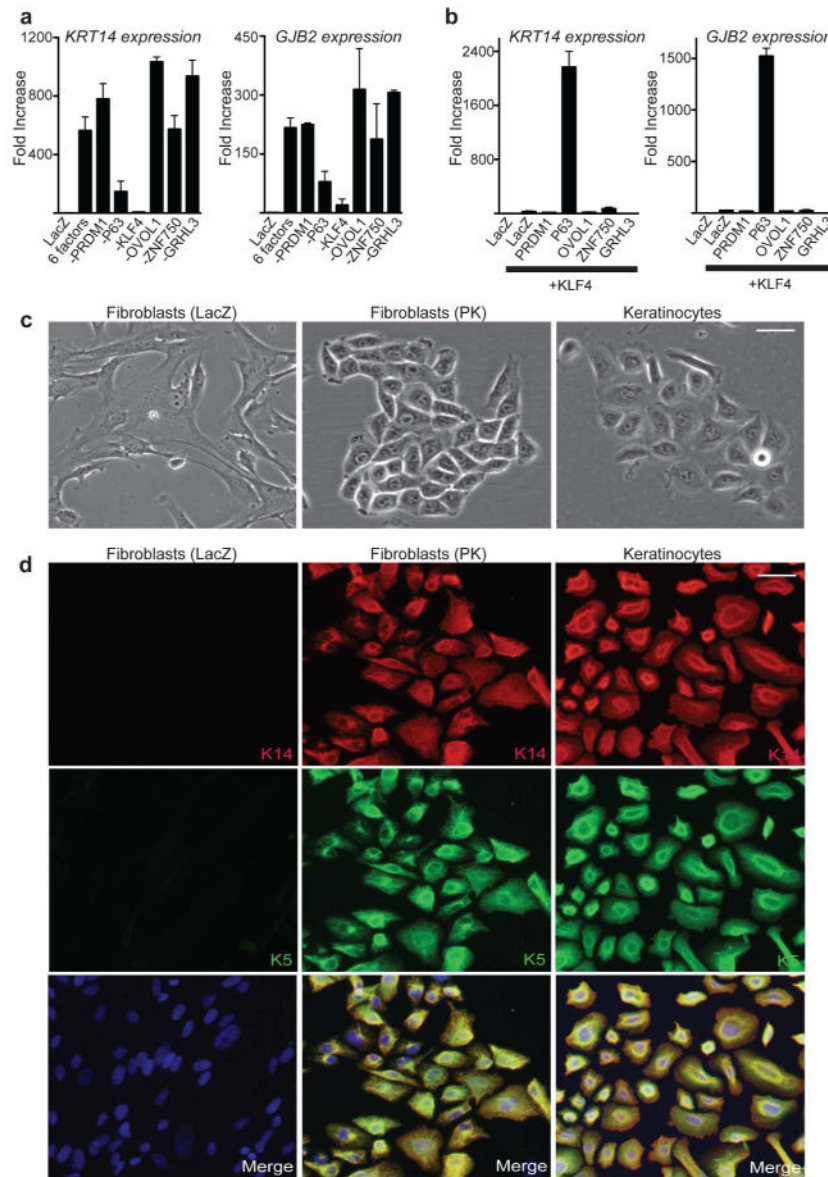


Figure 1. KLF4 and p63 are sufficient to convert human dermal fibroblasts to an induced keratinocyte phenotype (iKC)
a, RT-qPCR on expression of keratinocyte specific genes in fibroblasts transduced with control (LacZ) or combinations of keratinocyte specific transcription factors. Samples include all 6 transcription factors expressed at the same time or removal of each individual factor from the pool of 6. Expression data was calculated as increase over LacZ. Error bars=mean with SEM. **b**, RT-qPCR on expression of *GJB2* and *KRT14* in fibroblasts transduced with either LacZ or combinations of KLF4 with each of the remaining factors. **c**, Morphology of keratinocytes or fibroblasts (LacZ or p63+KLF4). Scale bar=15µm. **d**, Immunofluorescent staining of fibroblasts transduced with LacZ or PK(p63+KLF4) and keratinocytes for expression of K5 (green) and K14 (red). Hoechst=blue.

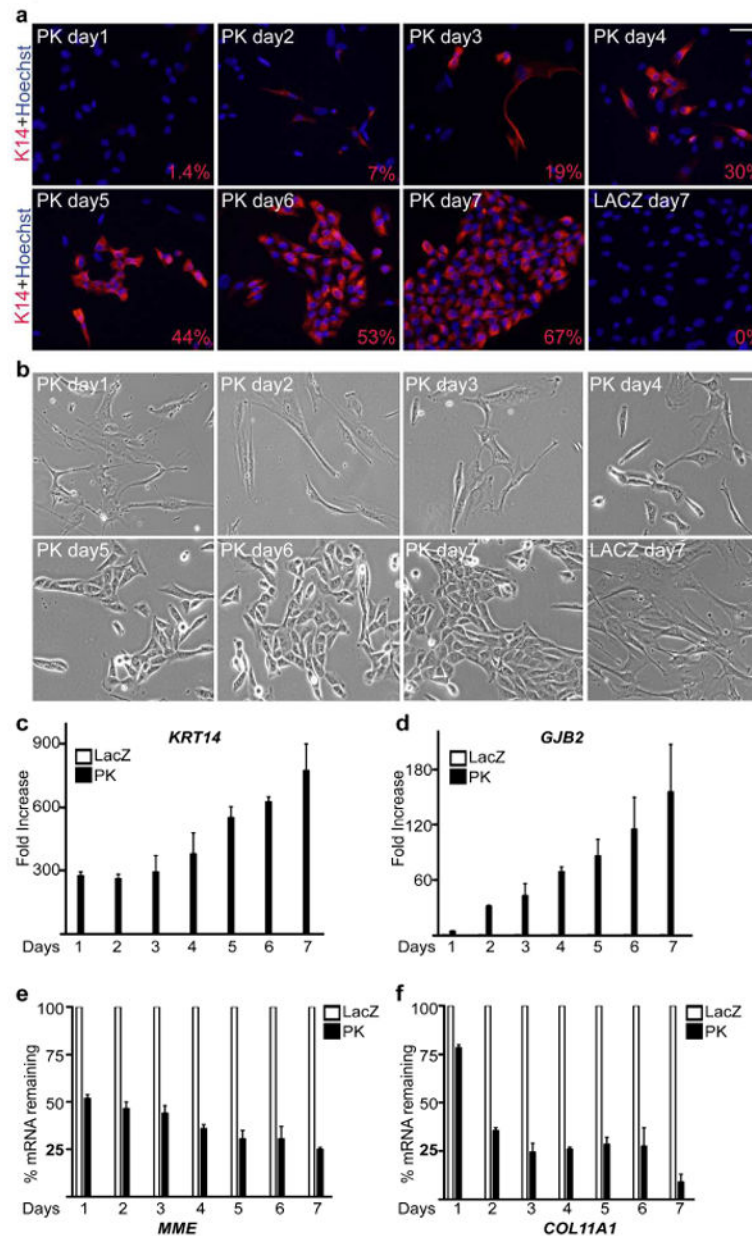


Figure 2. Conversion to a keratinocyte phenotype with p63 and KLF4 is efficient and rapid
a, Time course of immunofluorescent staining of PK transduced fibroblasts for expression of basal keratin, Keratin 14 (K14: red, Hoechst: blue). LacZ expressing fibroblasts stained with K14 is shown at day 7. Lower right hand corner= % of cells staining for K14 at each timepoint with a minimum of 300 cells counted per timepoint. Scale bar=30 μ m. **b**, Time course of the morphological changes of fibroblasts transduced with PK. **c-d**, RT-qPCR on increases in keratinocyte specific genes over time. Expression data was calculated as fold increase over LacZ. **e-f**, RT-qPCR on decreases in fibroblast specific genes over time. Expression data was calculated as a percent decrease over LacZ. Error bars=mean with SEM.

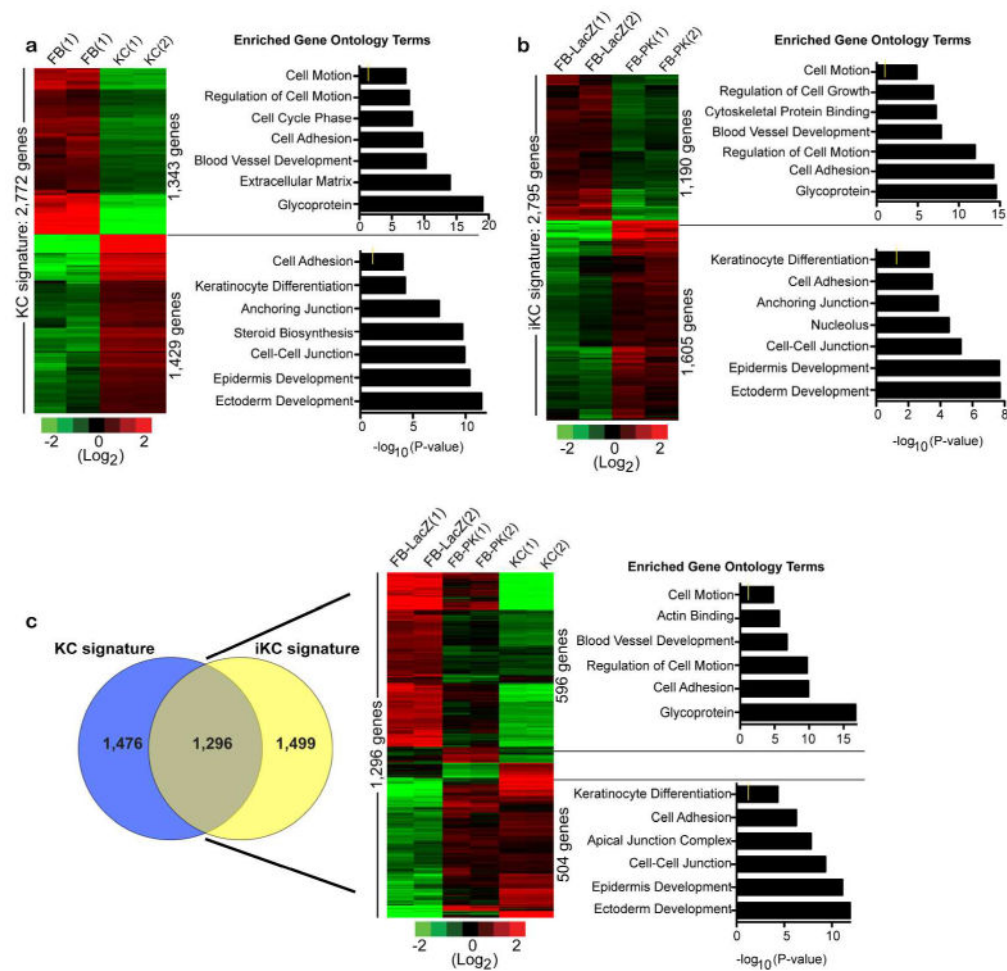


Figure 3. p63 and KLF4 reprogram fibroblasts to a keratinocyte gene expression program
a, Heat map (left panel) of the 2,772 genes (KC signature) that are significantly changed between fibroblasts (FB) and keratinocytes (KC). Heat map is shown in red (induced genes) and green (repressed genes) on a \log_2 -based scale. Gene ontology analysis (right panel). Yellow mark in bar graphs demark p value=0.5. P-values (Benjamini procedure) are represented in $-\log_{10}$. **b**, Heat map (left panel) of the 2,795 genes (iKC signature) that are changed between fibroblasts transduced with LacZ and PK. Gene ontology analysis (right panel). **c**, Overlap (left panel) between the KC and iKC gene signatures. Heat map (middle panel) of the 1,296 genes that overlap. Gene ontology analysis (right panel).

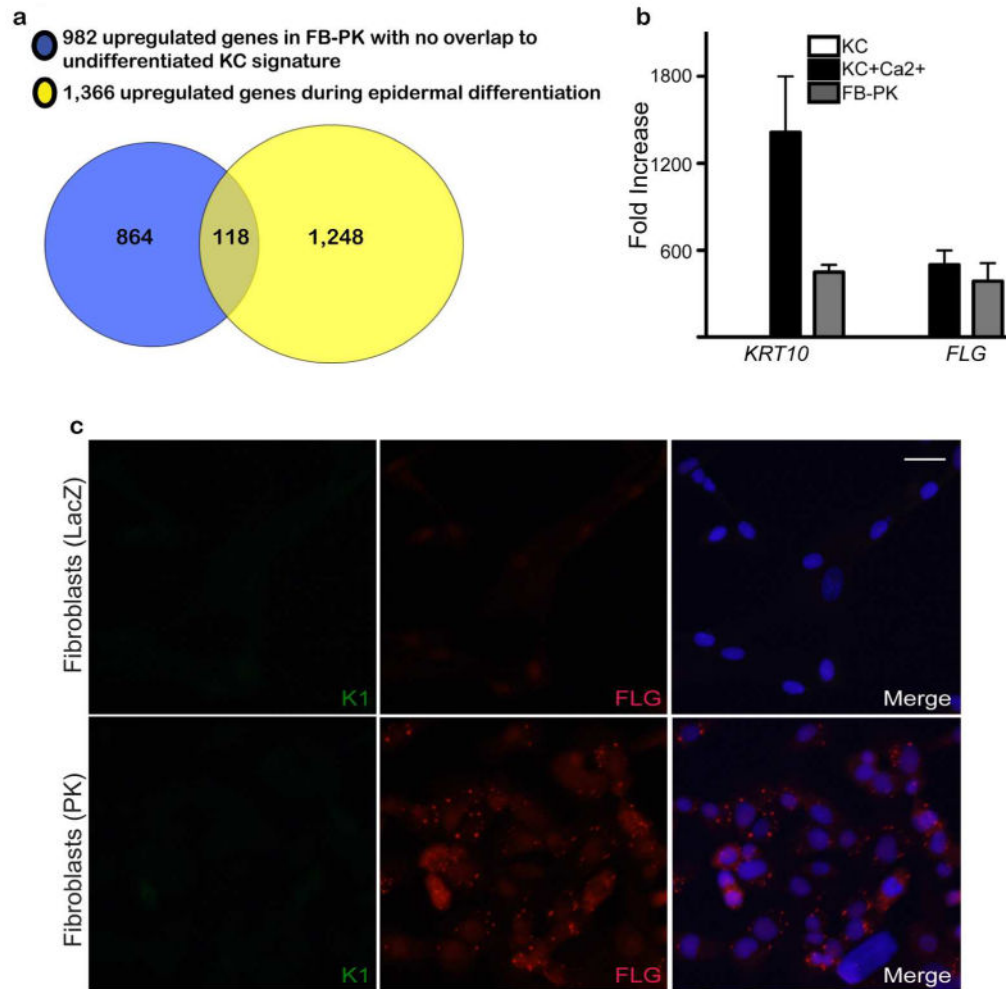


Figure 4. iKC cells are partially differentiated

a, Overlap between FB-PK upregulated genes and induced epidermal differentiation genes. 982 genes were upregulated in the FB-PK cells relative to FB-LacZ cells. These 982 genes were overlapped with the 1,366 genes that are upregulated during epidermal differentiation (gene set derived from *Sen et al.*, Nature 2010). **b**, RT-qPCR on expression of *KRT10* and *FLG* in undifferentiated keratinocytes (KC; white bar), differentiated keratinocytes (KC +Ca²⁺; black bar) and fibroblasts transduced with PK (FB-PK; grey bar). Expression data was calculated as a fold increase over undifferentiated keratinocyte samples (KC). Error bars=mean with SEM. **c**, Immunofluorescent staining of fibroblasts transduced with LacZ control or PK for expression of differentiation keratin 1 (K1:green) and filaggrin (FLG:red). Scale bar=15 μ m.

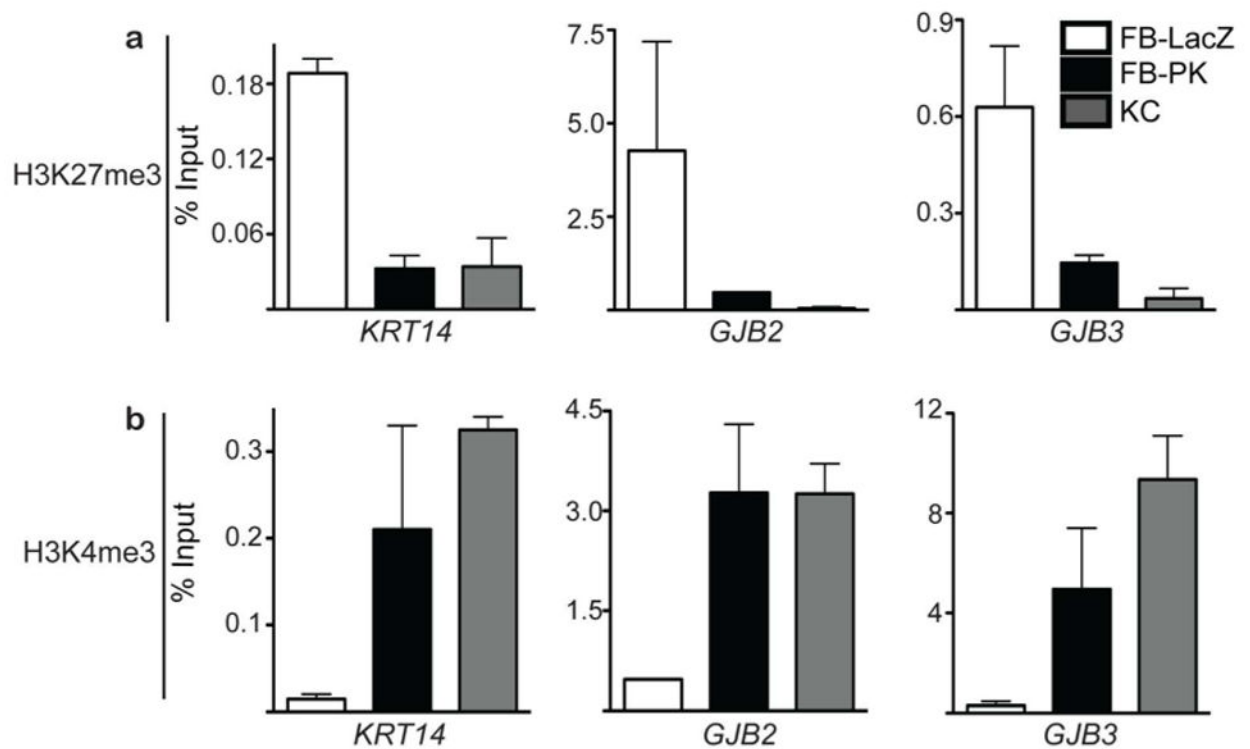


Figure 5. p63 and KLF4 reprogram fibroblasts to a keratinocyte chromatin signature
a, ChIP on fibroblasts transduced with LacZ (FB-LacZ), with p63 and KLF4 (FB-PK), and keratinocytes (KC) using an antibody against H3K27me3. QPCR was used to assess the levels of H3K27me3 on the promoters of *KRT14*, *GJB2*, and *GJB3*. Enrichment was calculated as a percent of the input. Error bars=mean with SEM. **b**, ChIP on FB-LacZ, FB-PK, and KC using an antibody against H3K4me3. The same parameters were used as described in (a).