An RNAi Screen Reveals an Essential Role for HIPK4 in Human Skin Epithelial Differentiation from iPSCs

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SUMMARY

Molecular mechanisms responsible for the development of human skin epithelial cells are incompletely understood. As a consequence, the efficiency to establish a pure skin epithelial cell population from human induced pluripotent stem cells (hiPSCs) remains poor. Using an approach including RNAi and high-throughput imaging of early epithelial cells, we identified candidate kinases involved in their differentiation from hiPSCs. Among these, we found HIPK4 to be an important inhibitor of this process. Indeed, its silencing increased the amount of generated skin epithelial precursors at an early time point, increased the amount of generated keratinocytes at a later time point, and improved growth and differentiation of organotypic cultures, allowing for the formation of a denser basal layer and stratification with the expression of several keratins. Our data bring substantial input regarding regulation of human skin epithelial differentiation and for improving differentiation protocols from pluripotent stem cells.

INTRODUCTION

Lineage-specific differentiation protocols from either human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) toward the keratinocyte lineage represent a useful tool to generate human skin tissue (Green et al., 2003; Guenou et al., 2009; Itoh et al., 2011; Iuchi et al., 2006; Metallo et al., 2008; Tolar et al., 2011). Several applications can be found for these protocols, such as investigating the molecular mechanisms involved during the development of this cell lineage or in vitro modeling of skin diseases. Indeed, many iPSC-based models have already been reported for several tissues including brain, heart, blood, and skin (Carvajal-Vergara et al., 2010; Galach and Utikal, 2011; Kondo et al., 2013; Larribere et al., 2015; Nissan et al., 2011; Raya et al., 2009).

Current protocols on skin epithelial differentiation require the use of specific culture conditions as well as different coating or supportive feeder layers. They also need to be reproducible, robust, and efficient enough to deliver high amounts of pure differentiated cells, especially if the future goal is a clinical application. In addition, these protocols should be validated by functional assays, for example, grafting hiPSC-generated keratinocytes on organotypic cultures to prove their ability to build a whole stratified epithelium (Aberdam, 2004; Coraux et al., 2003; Guenou et al., 2009; Itoh et al., 2011). Nevertheless, the efficiency to establish not only a pure keratinocyte population from PSCs but also skin epithelial precursors remains poor to date, one of the reasons being the lack of knowledge of the molecular mechanisms regulating the early steps of the epithelial/ectodermal commitment.

Large-scale loss-of-function (RNAi) screening offers a systematic genetic approach to study lineage-specific differentiation mechanisms. To identify key actors of the epithelial/ectodermal commitment, we performed a kinome RNAi screen targeting a total of 719 kinases, followed by differentiation lasting 10 days (Erfle et al., 2007). We then used a live-cell assay and automatic microscopy based on keratin 18 (K18) expression to monitor the amount of generated epithelial cells. We identified 62 activators and 36 inhibitors of this differentiation, among which the homeodomain interacting protein kinase 4 (HIPK4) was validated as a novel negative regulator.

RESULTS

Characterization of Human Skin Epithelial Cells

hiPSCs were generated in our laboratory from fibroblasts of healthy donors (Larribere et al., 2015) and were differentiated for 5 days, 10 days, and 30 days toward the skin epithelial lineage by maintaining them in defined keratinocyte serum-free medium (DKSFM) containing retinoic acid...
(RA) (1 µM) and bone morphogenetic protein 4 (BMP4) (10 ng/mL), according to the protocol of Itoh et al. (2011) (Figure 1A). Gene expression profiling of the differentiated cells at day 5 and day 10 showed an increased expression of ectodermal, epithelial, and epidermal gene signatures compared with day 0 (hiPSCs) (Figure 1B). In particular, day-10 differentiated cells showed increased expression of several collagens, integrins, keratins, and laminins. Moreover, the pluripotency gene signature (including SOX2, OCT4, and NANOG) was downregulated and gene signatures of undesired lineages such as neuroectoderm, endoderm, or mesoderm were either not expressed or even downregulated compared with day 0 (Table S1). These data were then confirmed by qPCR for some of the ectodermal/epithelial markers: BMP4, BNC1, GATA2, FZD6, and AP2A were all upregulated at day 5 and day 10 compared with day 0 (Figure 1C, top panel). Conversely, mesodermal marker (KDR), endodermal markers (GATA4, AFP, UPK1B, and FOXA2), and neuroectodermal markers (PAX6, PARP1, and FGF2) were expressed at very low levels compared with day 0 (Figure 1C, bottom panel).

Furthermore, the differentiation at day 30 showed a cell phenotype and a gene expression profile closely resembling that of normal human keratinocytes (NHK). Indeed, a hierarchical clustering representation of the samples showed that cells differentiated at day 5, day 10, and day 30 presented closer similarities to NHK than at day 0 (Figure S1A). This suggests that cells were already committed to an epithelial identity as early as day 5 of differentiation. Additionally, we confirmed by qPCR at day 5 and day 10 an
increased expression of epithelial markers such as keratin 8 (K8) and K18, expression of which then decreased at day 30 and switched to an increased expression of keratinocyte markers such as keratin 5 (K5) and keratin 14 (K14). The expression of p63, a transcription factor involved in epidermal proliferation and stratification, was upregulated at day 10 and increased at day 30 (Figure 1D).

Finally, immunostaining of K18 at day 10 (subsequently used to monitor the RNAi screen, see below) presented 40% positive cells compared with 0% in the undifferentiated control condition (Figures 1E and S1B). As expected, day-10 differentiated cells were also positive for p63 staining (30%) but not for K14, since this marker’s expression increases later during the differentiation. Indeed, day-30 differentiated cells were positive for K14 (50%) and p63 (50%) staining but no longer for K18 due to its early transient expression.

Together, these data show that our differentiation protocol generates day-5 and day-10 differentiated cells with an ectodermal/skin epithelial identity and generates day 30 differentiated cells presenting a keratinocyte-like phenotype and identity. In the following experiments, we focused on day-10 differentiated cells to investigate the molecular mechanisms that regulate early skin epithelial differentiation.

High-Throughput RNAi Screen Analysis Identifies HIPK4 as an Inhibitor of Skin Epithelial Differentiation

We next performed an RNAi-based screen during early epithelial differentiation to identify key regulators, with the ultimate goal of improving its low efficiency. In brief, hiPSCs were single-cell seeded on Matrigel-coated 384-well plates and transfected directly in the differentiation medium with a kinase RNAi library containing small interfering RNAs (siRNAs) targeting kinases and kinase-regulatory proteins. After 10 days, the cells were stained against K18 and subsequently image processed with an automated microscope (Figure 2A).

During the screen’s image analysis, the number of K18+ cells was determined, the values normalized per assay plate, and the Z scores calculated. Figure 2B shows the distribution of the control groups by their Z score and indicates a clear distinction between the positive control, in which conditions were applied to promote differentiation (cultured with DKSFM containing RA + BMP4) and the negative control, in which conditions were used to maintain pluripotency (cultured with mTeSR medium for 10 days). Two independent screens were performed, and only the results that increased or decreased the percentage of K18+ cells in the sample median by at least two median absolute deviations were considered as hits. A gene was considered as a potential regulator of epithelial cells when both replicates were scored as hits.

Out of 232 hits, 35 genes were involved in tissue development functions (such as the fibroblast growth factor receptor [FGFR] family) as expected for this assay based on stem cells’ early differentiation. We also found BMPR1B (bone morphogenetic protein receptor, type IB), a member of the BMP pathway, which supports our epithelial differentiation. BMP targets are indeed known to influence the development, differentiation, and proliferation of the epidermis (Metallo et al., 2008), and BMPR1B was described to be associated with epidermal differentiation (Botchkarev et al., 1999; Panchision and Pickel, 2001).

In addition, we found genes associated with I KKs (inhibitor of kappa light polypeptide gene enhancer in B cells), such as IKBKAP (inhibitor of kappa light polypeptide gene enhancer in B cells, kinase complex-associated protein), which is a regulator of I KKs, and IKKE (inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon) which is a non-canonical IKK. Of note, IKKα can promote epidermal differentiation independently of its nuclear factor κB (NF-κB) function (Hu et al., 2001). Finally, members of the MAPK pathway were also involved in K18 regulation: MAPK1 (mitogen-activated protein kinase 1), MAP2K1 to MAP2K5 (mitogen-activated protein kinase 1–5), MAP3K3 and MAP3K12 (mitogen-activated protein kinase kinase kinase 3 and 12). Together, these data support the functional relevance of our screen’s hit list in the regulation of epithelial differentiation (Tables S2 and S3).

Next, genes with a Z score lower than the averaged positive controls were identified as inhibitor candidate genes and genes with a higher Z score than the averaged negative controls were identified as promoter candidate genes (Table S4). One of the promoter candidate genes was ADCK2 (AarF domain containing kinase 2), mutation of which is associated with Klippel-Feil syndrome, a disease involving segmentation defects during early development. This gene was used as a representative example of differentiation promoter gene as the K18+ cell number remained close to 1% after a differentiation of 10 days (Figure 2C). On the other hand, one inhibitor candidate gene, HIPK4, encodes for a conserved serine/ threonine kinase belonging to the HIPK family (homeodomain interacting protein kinase 1–4), which plays a role in a large set of cell functions, including differentiation (He et al., 2010). HIPK4 is described to be expressed in human skin according to the GeneCards online database (https://gene_cards.weizmann.ac.il/v3/cgi-bin/carddisp.pl?gene=HIPK4#expression). Moreover, we observed Hipk4 expression in the skin of mouse embryos, suggesting a potential role for this protein in the early development of the skin (Figure S2A).

Indeed, quantification of K18+ cells in HIPK4 knockout condition after 10 days of differentiation indicated 85%
positive cells compared with 50% in the positive control condition (siControl). As expected, no K18 expression was found in the negative control condition (cells maintained in pluripotency condition) (Figure 2C). Overall, this RNAi screen allowed us to identify several putative regulators of human skin epithelial differentiation, and specifically suggests HIPK4 as a strong inhibitor of this process.

Validation of HIPK4 as an Inhibitor of Skin Epithelial Differentiation

In the next step, we quantified by immunostaining the number of cells positive for K18, K14, and p63 at day 10 and day 30 of differentiation (Figures 3A and S2B). At day 10, HIPK4 silencing induced an increase of K18+ cells (80%) compared with the siControl condition (40%). As expected at this time point, no K14+ cells were observed. Similarly at day 30, HIPK4 silencing induced a small but significant increase of K14+ cells (65%) compared with the siControl condition (50%). Interestingly, at day 10, the number of p63+ cells was not affected by HIPK4 knockdown (around 30%) but increased significantly at day 30 compared with the siControl condition (from 50% to 65%). These data suggest that the inhibitory role of HIPK4 on K18 expression before day 10 is independent of p63. At a later time point, however (day 30), the increase in K14+ and p63+ cells in the siHIPK4 condition may simply be due to an increased pool of day-10 precursors that matured in keratinocytes.

Figure 2. High-Throughput RNAi Screen Identifies HIPK4 as an Inhibitor of Skin Epithelial Differentiation

(A) Schematic of the RNAi screen workflow. Single hiPSCs were reverse transfected with a kinome RNAi library and differentiated into epithelial precursors. At day 10, immunofluorescence staining against K18 was performed and cells were imaged by automated microscopy.

(B) Scatterplot of data where each siRNA from the screen at day 10 is represented as the Z-score value. Plot includes negative (red, Z-score = +1.3) and positive (blue, Z-score = −1) controls. Positive control condition corresponds to cells transfected with siControl and differentiated for 10 days. Negative control condition corresponds to cells maintained in pluripotency condition for 10 days. Arrow marks siRNA against HIPK4 as a sample with increased numbers of K18+ cells compared with the positive control.

(C) Representative images of K18 immunostaining and quantification of percentage of positive cells after 10 days of differentiation with or without siHIPK4 or siADCK2. Scale bar, 100 μm.
We also analyzed the expression of epithelial markers at day 10 or keratinocyte markers at day 30 in these samples. In addition to K8 and K18, the expression of BMP4, ZEB2, FOXC1, and FRZB was upregulated by at least 2-fold at day 10 under HIPK4 knockdown compared with the siControl condition. The regulation of K18 expression by HIPK4 was also analyzed by western blot in a kinetic experiment from day 5 to day 10 (Figure 3C). HIPK4 expression, which starts at day 6 in the control condition, was delayed to day 9 under HIPK4 silencing. The restoration of HIPK4 expression after day 9 in the knockdown condition was probably due to the combined effects of transient siRNA silencing and increased expression during differentiation. Consequently, K18 expression, which started at day 7 in the control condition, was already expressed at day 6 in the HIPK4 knockdown condition, and accumulated at day 10 to higher levels than in the control condition.

Because the screen’s potential off-target effects are to be expected, the differentiation experiment was repeated with two independent HIPK4 siRNAs. The analysis at day 10 revealed that each single HIPK4-specific siRNA led to gene silencing (50% and 40%, respectively). Consistently, the increase in K18 expression under HIPK4 silencing was still observed (Figure S3A). Moreover, the negative regulation of HIPK4 on K18 expression was reproduced in a second hiPSC line (HD1). At day 5, transfection with siHIPK4 did not significantly increase K18 expression, likely due to the absence of HIPK4 at this time point; however, at day 10 siHIPK4 caused more than a 50% reduction in HIPK4 expression and a 2-fold increase of K18 expression compared with siControl (Figure S3B).
For better characterization of the differentiated cell populations, microarray analysis of transcriptome profiles from control and HIPK4 knockdown samples at day 5 and day 10 was conducted together with day-0 and NHK samples. To exclude a potential effect of HIPK4 silencing on other undesired lineage differentiation, we verified that endodermal, mesodermal, and neuroectodermal markers’ expression was minimal under HIPK4 silencing at day 10 compared with the control (Figure S3C and Table S5). A hierarchical clustering of the samples then gave two main points of information (Figure S4A): (1) HIPK4 knockdown at day 5 induced no major effect as HIPK4 was still not expressed at this time point (Figure 3C); and (2) HIPK4 knockdown at day 10 is clearly distinct from knockdown at day 5. Moreover, we were able to retrieve in a hierarchical gene clustering pluripotency markers, expression of which was high at day 0 and was then reduced during the differentiation process. Conversely, epithelial and epidermal markers were upregulated during the process and to a higher extent under HIPK4 knockdown than in the control at day 10 (Figure 3D). As explained above, these markers were not regulated at day 5. Furthermore, principal component analysis showed that differentiated samples were very different from day 0 and NHK samples (Figure S4B).

Finally, we performed a pathway analysis of the top regulated genes under HIPK4 knockdown at day 10 (Figure 3E and Table S6). Among the top regulated signaling pathways, we found Wnt/β-catenin, NF-κB, PTEN (phosphatase and tensin homolog), interleukin-6 (IL-6), and transforming growth factor β (TGF-β)/BMP. Interestingly, we were able to confirm by qPCR an upregulation of IL-6 under HIPK4 knockdown, as well as an upregulation of one TGF-β receptor (TGFBR2), although TGFBR1 and one downstream target CTGF were not regulated. These data suggest that IL-6 and TGF-β/BMP signaling may be involved in an HIPK4-dependent mechanism (Figure 3F).

Together, loss of HIPK4 during the early steps of epithelial differentiation leads to a significant enrichment in K18+ cell number and to an increase in epithelial and epidermal gene signature, confirming a negative role of HIPK4 in this process. In addition, loss of HIPK4 also leads to an increase in K14+ cell number after 30 days of differentiation and to an upregulation of keratinocyte markers. Furthermore, we hypothesize that IL-6 and/or TGF-β signaling could be involved in this mechanism, although this needs to be confirmed by additional investigation.

**Loss of HIPK4 Alone Is Not Sufficient to Induce Differentiation but Its Kinase Activity Is Necessary to Upregulate K8/K18**

We then tested the effect of the absence of RA and BMP4 in DKSFM medium on the differentiation. First, in the presence of the cytokines no effect of the knockdown was observed at day 5, but the expression of HIPK4 at day 10 was reduced by the knockdown and K18 expression was 2-fold upregulated (Figures 4A and 4B). Interestingly, the cells maintained in DKSFM medium without RA or BMP4 did not undergo differentiation and remained in stem cell colonies (Figure 4C). qPCR analysis showed that siHIPK4 had no effect on K18 expression not only at day 5 but also at day 10 compared with the control (Figure 4D). These results indicate that although HIPK4 silencing contributes to K18 regulation during early epithelial differentiation, it is not enough to induce differentiation from hiPSCs by itself.

To confirm the role of HIPK4 in K18 expression, we over-expressed a wild-type form and a kinase dead mutant of HIPK4 in hiPSCs and differentiated the cells for 10 days. In the control condition, K8 and K18 were upregulated as expected, compared with day 0. Interestingly, the overexpression of wild-type HIPK4 led to a decrease in K8 and K18 expression compared with the control condition (Figure 4E). Moreover, the overexpression of the mutant HIPK4 (kinase dead) restored the expression of K8 and K18 to levels almost similar to the control condition. These data confirm that HIPK4 functions as a brake to epithelial differentiation, and this involves its kinase activity.

**HIPK4 Silencing Promotes the Differentiation of Epithelial Precursors in Organotypic Cultures**

Lastly, we investigated the effect of epithelial precursors in organotypic cultures (OTCs) by evaluating the epithelial growth and morphogenesis. We expected an optimal induction of epithelial differentiation due to the inductive potential of the dermal equivalent and the air exposure in this 3-dimensional in vitro model (Stark et al., 2004). For establishment of OTCs, hiPSCs were transfected with HIPK4 siRNA, cultured for 4 or 10 days under differentiation conditions, and seeded on scaffold-based dermal equivalents. While a 10-day predifferentiation hampered successful growth in OTCs (data not shown), predifferentiation of 4 days allowed for a sufficient number of cells to attach and grow in OTCs for 3 more weeks (Figure 5A). OTCs were analyzed for epithelial markers by immunohistochemistry. In the siControl condition, cells demonstrate organoid structures of mixed populations of mesenchymal cells and K8/K18-positive epithelial cells. In contrast, cells transduced with HIPK4 siRNA showed an improved differentiation in early epithelium (Figures 5B and S5). The laminin staining indicates the basement membrane zone, on top of which epidermal cells should stratify. In the siControl condition, very few cells were stained for K8 or K18 because this differentiation time point was too early to generate epidermal progenitors. In the siHIPK4 condition, however, we observed a thicker basal layer with basal cell stratification, on top of which
lay a monolayer of K8- and K18-positive cells. Moreover, the analysis of later epidermal keratins such as K10 and K14 showed almost no staining in the siControl condition but strong staining in the basal layer under HIPK4 knock-down, suggesting that these keratins were already expressed at this time of differentiation and that a proper basal layer was formed (Figure 5C). Interestingly, the quantification of positive cells for early keratins (K8, K18, K19) and late keratins (K14, K10) showed an increase of all keratin expression whenever HIPK4 was silenced compared with the control condition (Figure 5C).

DISCUSSION

In this work, we have identified HIPK4 as a key inhibitor of human skin epithelial differentiation by performing a high-throughput RNAi screening analysis during this process and by monitoring the number of K18⁺ cells. K18 is a classical marker of simple epithelia during early embryonic development, which includes ectodermal epithelium (Baribault et al., 1993). Nevertheless, the results presented here showed an upregulation of ectodermal/skin epithelial markers and no expression of endodermal, mesodermal, or neuroectodermal markers at day 10 of differentiation. These data strongly suggest that K18⁺ cells monitored in the screen were mainly skin epithelial cells. Interestingly, several members of the MAPK signaling pathway were found among the screen’s candidates. This signaling pathway plays a central role in keratinocyte differentiation, since Map2k1/Map2k2 (Mek1/Mek2) double-knockout mice die 24 hr postnatally, due to dehydration and loss of barrier function of the skin (Scholl et al., 2007). Also, MAPKAPK2 and 3 (mitogen-activated protein kinase-activated protein kinase 2 and 3) are kinases which are regulated by the FGFR signaling pathway (Tan et al., 1996).
HIPK4 regulation has not been extensively studied to date. It was described to phosphorylate p53 at Ser9 and to be structurally related to HIPK1–3 for its catalytic domain (Arai et al., 2007). Although Hipk1/2 double-knockout mice die in utero, suggesting overlapping functions for these two family members (Isono et al., 2006), no Hipk4 knockout mouse has been deeply investigated. Based on the transcriptome analysis of the knockdown cells, we suggest that HIPK4 could influence several signaling pathways such as Wnt/β-catenin, NF-κB, PTEN, IL-6, and TGF-β/BMP. Wnt/β-catenin signaling is an important regulator during skin development and, in particular, catenins play a key role in epidermal development (Augustin, 2015; Perez-Moreno and Fuchs, 2006). Interestingly, NF-κB signaling, which was predicted to be inactivated in our analysis, can induce epithelial cell growth arrest (Seitz et al., 1998, 2000). PTEN signaling is involved in epithelial migration, proliferation, and morphogenesis (Castilho et al., 2013;
Although IL-6 signaling (and its downstream target \textit{STAT3}) was recently shown to be involved in lung epithelial development, its role in skin epithelial development is yet to be clarified (Kameyama et al., 2017). Of note, IL-6 can increase the phosphorylation of K18 in epithelial cells (Wang et al., 2007). Lastly, TGF-β/BMP signaling is known to be important in early development and particularly in epidermal differentiation (D’Souza et al., 2001; Li et al., 2003; McDonnell et al., 2001; Park and Morasso, 2002). More recently, inhibition of TGF-β signaling by a small compound was suggested to improve keratinocyte differentiation from hiPSCs (Shalom-Feuerstein et al., 2013).

In conclusion, after validation of a kinome RNAi screen, we demonstrated that loss of HIPK4 expression improves early skin epithelial differentiation accompanied by an increase in K8 and K18 expression, and improves late keratinocyte differentiation with an increase of epidermal identity (K5/K14/p63). Additionally we found that HIPK4 knockdown favors the epithelial differentiation in an organotypic culture, with an enhanced basal layer formation and an increased expression of several keratins, including late epidermal keratins such as K14 and K10. Therefore we propose that HIPK4 acts as a control mechanism of epithelial/epidermal differentiation by preventing excessive K18* precursor differentiation. This mechanism involving HIPK4 kinase activity and phosphorylation of its downstream targets should be examined more closely. This mechanism may also involve the activation of IL-6 and/or TGF-β signaling pathways. Taken together, these data present microscopy RNAi screens as a powerful tool to reveal novel regulators of human skin epithelial differentiation, and therefore will help to improve the current differentiation protocols from PSCs.

**EXPERIMENTAL PROCEDURES**

**Generation of hiPSCs**

Human iPSC D1 and HD1 lines were generated from fibroblasts of healthy donors (University Medical Center Mannheim, Germany; ethics committee approval no. 2009-350N-MA) as previously described (Larribere et al., 2015). In brief, human fibroblasts were infected with lentiviral particles carrying an inducible polycistronic cassette containing the reprogramming factors \textit{OCT4}, \textit{SOX2}, \textit{KL4}, and \textit{c-MYC}. Generated iPSCs were maintained in culture on Matrigel in mTeSR medium (Invitrogen Life Technologies, Darmstadt, Germany).

**Differentiation of hiPSCs into Skin Epithelial Cells**

For skin epithelial differentiation, the protocol was adapted from Itoh et al. (2011). Cells were trypsinized and seeded at single-cell level in DKSFM containing 1 μM all-trans RA, 10 ng/mL BMP4, and 50 μg/mL Normocin. The next day, medium was changed to fresh DKSFM containing RA and BMP4. On day 4, cells were washed twice with DMEM/F12 and fresh DKSFM supplemented with 50 μg/mL Normocin. The differentiation was stopped at different time points for RNA or protein isolation.

**High-Throughput RNAi Screen**

siRNAs (siGenome, Dharmacon) were pre aliquoted in Matrigel precoated 384-well plates to a final concentration of 250 nM using an automated workstation (Biomek FX, Beckman Coulter). hiPSCs were reverse transfected using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies). After trypsinization, cells were seeded either with a cytokine-containing DKSFM (for differentiation) or with mTeSR (for pluripotency maintenance). The plates were shortly spun down and kept at 37°C and 5% CO2. On culture day 5 an additional 50 μL of DKSFM without additional cytokines was added. The cells were cultured until day 10. Fixation (4% paraformaldehyde), blocking (3% BSA and 0.05% Triton X-100), and fluorescence staining were performed using an automated workstation. The K18 primary antibody dilution was 1:25. The plates were sealed and incubated over night at 4°C. The next day the cells were washed three times with PBS and incubated for 1 hr with the secondary antibody (anti-mouse Atto 594) together with Hoechst. After washing, the plates were sealed and stored at 4°C until imaging. The high-throughput screen was performed using a human RNAi library (siGenome, Dharmacon) consisting of a pool of four siRNAs per gene, targeting kinases, and kinase-regulatory proteins.

**Image Analysis of Screen Data**

Imaging was performed using an automated BD Pathway 855 Bioimaging System (Becton Dickinson) with a 20× objective (numerical aperture 0.75) and a Hamamatsu digital camera (Orca-ER). Per well, 25 fields were imaged for both filters, Hoechst (nuclei staining) and Atto 594 (K18 staining). Images were analyzed using the Cellprofiler image analysis software Version 2.0. Object selection was based on adaptive intensity and fixed-size thresholds for each individual object. The segmentation of objects was optimized in order to reach the best resolution even in dense cell clusters. For data analysis, parent objects were segmented in channel 1 (Hoechst), and in channel 2 (Atto 594) the mean intensity of child objects was measured. Based on the mean intensity of every nucleus, nuclei were categorized into a K18* and K18 cells, according to the manually set mean intensity threshold. The values of K18* cells were used for further analysis. To prevent strong viability effects, we excluded wells with less than 1,000 cells from the analysis. For data analysis, cellHTS2 software was used. The fraction of K18* cells per well was normalized per assay plate and the Z scores were calculated using the R package cellHTS2. The correlation between the data of replicates was estimated using the Spearman rank correlation coefficient. Inhibitor hit: Z score < positive control; promoter hit: Z score > 2.

**Gene Expression and Ingenuity Analyses**

Illumina gene expression raw data from biological triplicates of NHK, D1 hiPSC (day 0), day-5, day-10, day-30 differentiated cells, and their counterpart with silenced HIPK4 were normalized in CHIPSTER software. Unsupervised hierarchical clustering was performed after filtering genes with a variance test (p < 0.05).
Differential gene expression between day-10 siHIPK4 sample and day-10 siControl sample was determined using an empirical Bayes statistical test. Gene signatures for ectodermal development or epithelial and epidermal differentiation were downloaded from the GO database (GO:0030856, GO:0072148). Gene signatures for pluripotency, or endodermal, mesodermal, and neuroectodermal development were downloaded from the GO database or from published transcriptome profiling (GO:0019827) (Tadeu et al., 2015).

Regulated genes (n = 391) in day-10 siHIPK4 sample were then uploaded to IPA (Ingenuity Pathway Analysis) software to evaluate the most regulated signaling pathways. All raw data have been uploaded on the public database under accession GEO: GSE102067.

siRNA Transfection

The cells were transfected 1 day after seeding and differentiated for 10 days. Transfection reagent (Lipofectamine RNAiMAX) was added to DMEM/F12 and incubated for 10 min. After incubation time, either the set of four siRNAs against HIPK4 or the non-targeting siControl were added and incubated for 20 min at room temperature. The transfection mix was then added to the cells with a final siRNA concentration of 25 nM. After 2 days, medium was aspirated, the cells were washed twice with PBS, and cytokine-containing DKSFM was added. At day 4 of differentiation the medium was aspirated, and the cells were washed again and cultured in DKSFM without cytokines. The medium was changed every 2 days. siRNA sequences were: HIPK4 (vector) as a control, and differentiated for 10 days. siRNA sequences were: HIPK4 dead mutant (mutant HPIK4), or with the empty vector (vector) as a control, and differentiated for 10 days.

Overexpression

Human iPSCs were transduced with a lentiviral expression vector (pTriEx-1) coding for wild-type HIPK4 (WT HIPK4 OE), kinase dead mutant HIPK4 (mutant HPIK4), or with the empty vector (vector) as a control, and differentiated for 10 days.

Immunofluorescence

Cells grown on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, and treated with blocking buffer (3% BSA, 0.1% Triton X-100 in 1x PBS) for 45 min. Antibody dilution (500 µL) was added per coverslip and incubated overnight at 4°C. Primary antibodies included keratin-14 (Covance), keratin-18 (Progen), and p63 (Abcam). Quantification of positive cells was calculated after counting a minimum of 300 cells per conditions.

Western Blot and qPCR

Whole-cell extracts representative of three independent experiments were prepared from hiPSCs, NHK, or from day-5, day-10, and day-30 differentiated cells that were extracted using an RNasey kit (QIAGEN). Real-time qPCR was performed using the SYBR Green Supermix (Applied Biosystems) on a 7500 Real-Time PCR system (Applied Biosystems). Primers used for qPCR were as follows: 18S, 5'-GAG GAT GAG GTG GAA CGT GT-3' (forward [fwd]) and 5'-TCT TCA GTC GCT CCA GGT CT-3' (reverse [rev]); GAPDH, 5'-GAG GAA GGA GAA CCG AGT C-3' (fwd) and 5'-GAA GAT GAG GTG GAT GGG ATT TC-3' (rev); K14, 5'-AGG AGA TCG CCA CCT ACC GCC-3' (fwd) and 5'-AGG AGG TCA CAT CTC TGG ATG ACT G-3' (rev); K18, 5'-GAG TAT GAG GCC CTG CTG AAC ATC A-3' (fwd) and 5'-GCC GGT GGT GGT CTT TTG GAT-3' (rev); K5, 5'-ATC TCT GAT GAG TAC CAG CAG ATG ATC-3' (fwd) and 5'-CAG ATT GGC GCA CTG TTT CTT-3' (rev); K8, 5'-GAT CGC CAC CTA CAG GAA GGT-3' (fwd) and 5'-ACT CAT GTG CTG CAT CCC AGA CT-3' (rev); p63, 5'-TTC TTA TGG AGG TGG TTG GCC TG-3' (fwd) and 5'-GAT CGC ATG TCG AAA TTG CTC-3' (rev). Involucrin, 5'-CCT CAT GTG TCA TGG GAT-3' (fwd) and 5'-TCA ACC TGA AAG ACA GAA GAG-3' (rev). Expression values were normalized to housekeeping genes 18S or GAPDH.

Organotypic Culture and Immunohistochemistry

The dermal equivalents were made in membrane insert-containing 6-well plates and comprised a circular piece of non-woven scaffold (22 mm), 750 µL of thrombin (10 U/mL) mixed with human dermal fibroblasts in fetal bovine serum (FBS), and 750 µL of fibrinogen (8 mg/mL). Thrombin and fibroblasts were soaked into the dry scaffold before the fibrinogen solution was added carefully and gently mixed. After combining the components, a soft fibrin clot formed in about 10 min at 37°C, which was then immersed in DMEM with 10% FBS containing 50 µg of L-ascorbic acid (siga) and 1 ng/mL TGFβ1 (R&D Systems). Precultivation was performed for 2–5 days with medium change every other day. The day before the epithelial cells were seeded, the dermal equivalents were shifted to rEAD medium (three-fourths DMEM and one-fourth Ham’s F12 supplemented with 10% FBS, 10–10 M cholaer toxin, 0.4 mg of hydrocortione, and 50 mg/mL L-ascorbic acid) mixed with DKSFM (1:1) to equilibrate them for the co-culture phase. In the organotypic co-cultures, the DKSFM was changed every other day during a period of 3 weeks. Specimens of OTCs were then embedded in Tissue Tek (Miles, Elkhart, IN, USA) and frozen in liquid nitrogen vapor. Cryostat sections (6 µm) were mounted on 3-aminopropyl-triethoxysilane-coated slides and air dried. The sections were fixed in 80% methanol at 4°C for 5 min followed by absolute acetone at −20°C for 2 min and preblocked with 3% BSA in PBS. The incubation with the primary antibody was carried out in a moist chamber either overnight at 4°C or at 37°C for 1 hr followed by 30 min at room temperature. Laminin antibody was gift from Prof. J.M. Foidart, University of Liege, Belgium. Reference numbers of other antibodies: Keratin 14 (BioLegend Inc., #905301), Keratin 10 (Progen, DE-K10, #11414), Keratin 8 (Progen, clone Ks 8.7, #61038), Keratin 18 (Progen, Ks 18.04, #61028), Keratin 19 (Progen, #GP-CK19). Secondary antibodies were applied together with 2 µg/mL DAPI for nuclear staining for 30 min at 37°C followed by 30 min at room temperature in a moist chamber. After washing thoroughly, the sections were mounted in Fluorescent Mounting Medium (Dako) and stored in the dark at 4°C.
The specimens were examined with a Leica DMRBE/RD microscope equipped with epifluorescence illumination, and micrographs were recorded with a CCD camera (F-View 12) applying Analysis Pro 6.0 software (Olympus Soft Imaging Solutions, Munster, Germany).

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2017.08.023.

AUTHOR CONTRIBUTIONS
Conception and Design, L.L., M.G., P.B., and J.U. Collection and/or Assembly of Data, L.L., M.G., D.N., K.A., H.C.V., H.-J.S., and J.U. Data Analysis and Interpretation, L.L., M.G., D.N., K.A., H.C.V., H.-J.S., P.B., M.B., and J.U. Provision of Study Material or Patients, J.U. Manuscript writing, L.L. and J.U. Final approval of manuscript, L.L., M.G., D.N., K.A., H.C.V., H.-J.S., P.B., M.B., and J.U. Financial support, P.B. and J.U. Administrative support, J.U.

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It further supports the importance of understanding how the epidermis develops from the pluristratified compartment and how it is maintained.

The specimens were examined with a Leica DMRBE/RD microscope equipped with epifluorescence illumination, and micrographs were recorded with a CCD camera (F-View 12) applying Analysis Pro 6.0 software (Olympus Soft Imaging Solutions, Munster, Germany).
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Supplemental Information

An RNAi Screen Reveals an Essential Role for HIPK4 in Human Skin Epithelial Differentiation from iPSCs

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Figure S1.

A. Related to Figure 1D: Dendrogram of samples using normalized data with Pearson correlation and average linkage method. The branches of the tree are colored according to the groups: hiPSCs (d0), normal human keratinocytes (NHK), day 5 (d5), day 10 (d10) and day 30 (d30) differentiated cells. Clustering is done using the function hcluster in which the parameter correlation enforces computation of Pearson type of distances.

B. Related to Figure 1E: Immunostaining of keratin 18 (K18), keratin 14 (K14) and p63 at day 10 and day 30 of differentiation. Nuclei were stained with DAPI. Scale bar: 100um.
Figure S2.
A. Related to Figure 2C: HIPK4 in vivo expression in skin sections of mouse embryos at day E17.5 in the brain region.

B. Related to Figure 3A: Upregulation of K18 and K14 under HIPK4 silencing. Immunofluorescence staining against K18 at day 10 and K14 at day 30 of differentiation in control condition (siControl) and under HIPK4 knockdown (siHIPK4). Scale bar: 100 μm.
A. Related to Figure 3C: HIPK4 silencing with single siRNAs. qPCR analysis of HIPK4 and Keratin 18 expression under HIPK4 knockdown with single siRNAs (siNo.9 and siNo.12) or in control condition (siControl) at day 10 of differentiation. Data represent a mean of three independent experiments ± SEM. Statistical analysis was performed using unpaired Student’s t-test (* P<0.05).

B. Related to Figure 3C: qPCR analysis of HIPK4 and K18 expression in a second hiPSC line (HD1) under HIPK4 silencing (siHIPK4) or in control condition (siControl) at day 5 and day 10 of differentiation. Data represent a mean of three independent experiments ± SEM. Statistical analysis was performed using unpaired Student’s t-test (* P<0.01).

C. Related to Figure 3E: qPCR analysis of mesodermal marker (KDR), endodermal markers (GATA4, AFP, UPK1B and FOXA2) and neuroectodermal markers (PAX6, PARP1 and FGF2) at day 5 and day 10 under HIPK4 knockdown. Data represent a mean of three independent experiments ± SEM.
**Figure S4. Related to Figure 3E:** Transcriptome analysis of *HIPK4* knockdown and control samples at day 5 and day 10 of differentiation.

**A.** Dendrogram of samples using normalized data with Pearson correlation and average linkage method. The branches of the tree are colored according to the groups: hiPSCs (d0), normal human keratinocytes (NHK), day 5 (d5) and day 10 (d10) differentiated cells under control or HIPK4 knockdown conditions. Clustering is done using the function hcluster in which the parameter correlation enforces computation of pearson type of distances.

**B.** Principal component analysis of gene expression profiles on the same samples as above. Proportion of Variance PC1: 0.44 and PC2: 0.21.
Figure S5. Related to Figure 5: *HIPK4* knockdown promotes epithelial differentiation of coculture with human dermal fibroblasts.

A. Immunostaining against Laminin b1, Keratin 8, 18, 10 and 14 in paraffin sections of 13-day old organotypic cultures (OTCs) from either *HIPK4* siRNA (si*HIPK4*) or a non-targeting siRNA (si*Control*) transfected cells. Scale bar: 100μm.

B. Immunostaining against Keratin 8 and 18 in paraffin sections of 21-day old organotypic cultures (OTCs) from either *HIPK4* siRNA (si*HIPK4*) or a non-targeting siRNA (si*Control*) transfected cells. OTCs of *HIPK4* siRNA treated cells show an improved directed differentiation. In contrast non-targeting siRNA treated cells demonstrate organoid structures of mixed populations of mesenchymal and epithelial cells. Scale bar: 100μm.
Table S1. Related to Figure 1B: Gene signature of endodermal, mesodermal, neuroectodermal tissue and pluripotency at day 10 of differentiation.

| Symbol | ENDODERM |  | MESODERM |  | NEUROECTODERM |  |
|--------|----------|---|----------|---|---------------|---|
| Symbol | day 5    | day 10 | symbol | day 5 | day 10 | symbol | day 5 | day 10 |
| AFP    | -1.09    | -1.77 | ACVR1  | 0.15 | -0.11 | FGF1   | -0.05 | -0.05 |
| CD36   | -0.04    | -0.01 | ACVR1B | 0.06 | 0.30  | FGF2   | -1.59 | -2.48 |
| CDX2   | 0.81     | 0.02  | ACVR1C | 0.04 | 0.05  | FGF3   | -0.05 | -0.10 |
| CLDN6  | -0.39    | 0.41  | ACVR2A | -0.25| -0.14 | FGF4R1 | -0.63 | -0.78 |
| CTNNB1 | 0.26     | 0.22  | ACVR2B | -0.64| -0.78 | FGF4R2 | -0.18 | -0.11 |
| CXC4   | -0.01    | -0.12 | ACVR2B-AS | 0.18 | 0.18 | PARP1  | -0.56 | -1.47 |
| EOMES  | -0.70    | -0.76 | ACVRL1 | 0.13 | 0.03  | PAX6   | 0.11  | -0.14 |
| FABP1  | -0.06    | -0.06 | CDH2   | 0.55 | -0.06 |  |  |  |
| FOXA2  | -0.05    | -0.08 | CFC1   | -0.07| -0.06 |  |  |  |
| GATA4  | 0.04     | 0.03  | CFC1B  | 0.03 | -0.01 |  |  |  |
| GATA6  | 0.47     | 0.27  | EOMES  | -0.70| -0.76 |  |  |  |
| GDF3   | -0.98    | -1.01 | FABP4  | 0.12 | 0.28  |  |  |  |
| HNF1B  | -0.06    | -0.11 | FOXF1  | 0.18 | -0.04 |  |  |  |
| HNF4A  | 0.06     | 0.10  | GDF3   | -0.98| -1.01 |  |  |  |
| MCLX1  | -0.35    | -0.31 | GSC    | 0.02 | -0.08 |  |  |  |
| PECAM1 | -0.06    | -0.08 | GSC2   | 0.05 | 0.19  |  |  |  |
| SALL4  | -1.88    | -2.57 | KDR    | -1.24| -1.06 |  |  |  |
| SOX1   | -0.13    | -0.07 | MESDC1 | -0.03| -0.08 |  |  |  |
| SOX17  | -0.17    | -0.03 | MIXL1  | -0.35| -0.31 |  |  |  |
| UPK1B  | 0.06     | 0.03  | NCLN   | -0.67| -0.78 |  |  |  |
| VEGFA  | -0.31    | -0.11 | NOMO1  | -0.55| -0.37 |  |  |  |
| VEGFB  | 0.04     | 0.10  | NOMO2  | -0.17| -0.14 |  |  |  |
| VEGFC  | 0.45     | 0.29  | NOMO3  | -0.21| -0.33 |  |  |  |
| SNAI1  | 0.02     | 0.11  | SNAI3  | -0.07| -0.16 |  |  |  |
| T      | -0.10    | -0.12 | TBX6   | -0.04| -0.09 |  |  |  |
| TWIST2 | -0.09    | 0.29  | TRIM71 | -2.09| -2.46 |  |  |  |

**ENDODERM**
- AFP, CD36, CDX2, CLDN6, CTNNB1, CXC4, EOMES, FABP1, FOXA2, GATA4, GATA6, GDF3, HNF1B, HNF4A, MCLX1, PECAM1, SALL4, SOX1, SOX17, UPK1B, VEGFA, VEGFB, VEGFC

**MESODERM**
- ACVR1, ACVR1B, ACVR1C, ACVR2A, ACVR2B, ACVR2B-AS, ACVRL1, CDH2, CFC1, CFC1B, EOMES, FABP4, FOXF1, GDF3, GSC, GSC2, KDR, MESDC1, MIXL1, NCLN, NOMO1, NOMO2, NOMO3, SNAI1, SNAI3, TBX6, TRIM71

**NEUROECTODERM**
- FGF1, FGF2, FGF3, FGF4R1, PARP1, PAX6

**PLURIPOTENCY**
- ALPL, DNMT3B, LIN28A, LIN28B, nanog, POU5F1, SOX2, TRIM71
## Table S2. Related to Figure 2B: Screen’s hits associated with epithelial differentiation

| symbol    | gene                                                                 | Involvement (references)                                                                 |
|-----------|----------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| BMPR1B    | Bone Morphogenetic Protein Receptor, Type IB                         | associated with epidermis differentiation (Botchkarev et al., 1999; Panchision & Pickel, 2001) |
| IKBKAP    | Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells, Kinase Complex-Associated Protein | promote epidermal differentiation (Hu et al., 2001)                                      |
| IKBKE     | Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon |                                                                                       |
| MAPK1     | Mitogen-Activated Protein Kinase 1                                   |                                                                                       |
| MAP2K1    | Mitogen-Activated Protein Kinase Kinase 1                            | viability and proliferation of keratinocyte (Scholl et al., 2007)                        |
| MAP2K2    | Mitogen-Activated Protein Kinase Kinase 2                            |                                                                                       |
| MAP2K4    | Mitogen-Activated Protein Kinase Kinase 4                            |                                                                                       |
| MAP3K3    | Mitogen-Activated Protein Kinase Kinase Kinase 3                     |                                                                                       |
| MAP3K12   | Mitogen-Activated Protein Kinase Kinase Kinase 12                    |                                                                                       |
**Table S4. Related to Figure 2B: Screen’s top candidate gene list**

| GeneID  | z-score |
|---------|---------|
| STK33   | 4.04    |
| TEK     | 3.77    |
| MYLK    | 3.43    |
| GSK3B   | 3.38    |
| CDKN2B  | 3.3     |
| GCK     | 2.98    |
| MAP2K4  | 2.86    |
| MAPKAPK3| 2.78    |
| PAK1    | 2.75    |
| CDK1    | 2.72    |
| MYO3A   | 2.65    |
| SRPK2   | 2.64    |
| GTF2H1  | 2.62    |
| CDK11B,CDK11A | 2.52 |
| MAP2K2  | 2.43    |
| MAP2K3  | 2.41    |
| TYK2    | 2.38    |
| STYK1   | 2.3     |
| RBKS    | 2.29    |
| CDK7    | 2.25    |
| GRK7    | 2.25    |
| ADCK2   | 2.24    |
| ADRB2   | 2.2     |
| ZAP70   | 2.18    |
| PHKA2   | 2.17    |
| CDKN3   | 2.16    |
| MVK     | 2.1     |
| TRAT1   | 2.06    |
| WNK4    | 2.04    |
| GK2     | 2.02    |
| GMFB    | 2.02    |
| XYLB    | 2       |
| IPPK    | 1.99    |
| JAK1    | 1.99    |
| CAMK2B  | 1.98    |
| PIP5K1A | 1.97    |
| SOCS5   | 1.96    |
| DUSP10  | 1.92    |
| CDK5R1  | 1.9     |
| CSNK2B  | 1.89    |
| MAPK1   | 1.85    |
| SHPK    | 1.8     |
| IRAK3   | 1.77    |
| DGKQ    | 1.72    |
| DYRK2   | 1.72    |
| SCYL2   | 1.71    |
| NEK9    | 1.7     |

**Promoter candidates**

| GeneID  | z-score |
|---------|---------|
| CSNK1E  | -1.55   |
| DBF4    | -1.56   |
| ACVR2A  | -1.57   |
| YWHAQ   | -1.57   |
| CINP    | -1.59   |
| MLKL    | -1.59   |
| AATK    | -1.6    |
| TRIB3   | -1.61   |
| AAK1    | -1.62   |
| PRKD1   | -1.64   |
| HIPK3   | -1.66   |
| EIF2AK3 | -1.69   |
| EPHA3   | -1.69   |
| FASTK   | -1.7    |
| GUCY2F  | -1.71   |
| POM121L10P,BCR | -1.71 |
| FGFR1   | -1.72   |
| SRPK1   | -1.72   |
| BMP2K   | -1.73   |
| CHEK2   | -1.73   |
| CHKA    | -1.73   |
| EPHA4   | -1.73   |
| EIF2AK1 | -1.74   |
| TEX14   | -1.74   |
| CHKB    | -1.75   |
| BLK     | -1.77   |
| HIPK4   | **-1.77** |
| TTBK2   | -1.79   |
| TAF1L,TAF1 | -1.83 |
| TPK1    | -2.01   |
| SLK     | -2.1    |
| TSSK1B,DGCR14,TSSK2 | -2.14 |
| UCK2    | -2.21   |
| TTK     | -2.38   |
| PBK     | -2.42   |
| TXK     | -3.01   |

**Inhibitor candidates**

| GeneID  | z-score |
|---------|---------|
| SCYL2   | 1.7     |
| Gene   | Value |
|--------|-------|
| CKS1B  | 1.69  |
| PAK2   | 1.69  |
| CARD10 | 1.68  |
| MST4   | 1.68  |
| TWF2   | 1.65  |
| AVPR1A | 1.62  |
| CD4    | 1.59  |
| ILKAP  | 1.56  |
| TLR1   | 1.56  |
| ARAF   | 1.54  |
| CDK10  | 1.54  |
| PRKDC  | 1.52  |
| PRKCQ  | 1.51  |
| CD7    | 1.5   |
| ZAK    | 1.5   |
### Table S5. Related to Figure 3D: Gene signature of endodermal, mesodermal, neuroectodermal tissue and of pluripotency.

| Symbol   | Endoderm day 5KD | Endoderm day 10KD | Mesoderm day 5KD | Mesoderm day 10KD | Neuroectodermal day 5KD | Neuroectodermal day 10KD |
|----------|------------------|------------------|------------------|------------------|------------------------|------------------------|
| KRT19    | 0.02             | -1.93            | HAND1            | -0.25            | FGF1                   | 0.01                   |
| CLDN6    | -0.11            | -1.80            | WNT3A            | 0.27             | FGF3                   | -0.03                  |
| SOX7     | 0.00             | -1.59            | NOMO1            | 0.15             | FGFR1                  | -0.08                  |
| CDH5     | 0.13             | -1.54            | ACVR1B           | 0.08             | FGFR2                  | 0.03                   |
| CTNNBL1  | -0.05            | -1.13            | TWIST1           | 0.02             | FGFR3                  | -0.13                  |
| KRT7     | 0.04             | -0.99            | NCLN             | 0.14             | FGFR4                  | 0.01                   |
| CD14     | -0.02            | -0.51            | GDF3             | -0.02            | PARP1                  | 0.04                   |
| SALL4    | 0.07             | -0.47            | TWIST2           | 0.08             | PAX6                   | -0.02                  |
| FOXA1    | -0.14            | -0.46            | GSC              | 0.02             |                       |                        |
| GDF3     | -0.02            | -0.23            | KDR              | 0.03             | -0.17                 |                        |
| SOX17    | 0.14             | -0.17            | ACVR2B-AS        | 0.01             | -0.17                 |                        |
| GATA6    | 0.04             | -0.14            | SNAI1            | 0.01             | -0.09                 | DNMT3B                 |
| CDX2     | 0.03             | -0.10            | SNAI2            | 0.02             | -0.08                 | KLF4                   |
| SOX1     | 0.01             | -0.05            | FOXF1            | -0.09            | -0.02                 | ABCG2                  |
| CXCR4    | 0.05             | -0.04            | MIXL1            | 0.17             | -0.02                 | ACVR1B                 |
| HNF4A    | -0.06            | -0.02            | SNAI3            | -0.05            | -0.01                 | TRIM71                 |
| MIXL1    | 0.17             | -0.02            | GSC              | -0.11            | -0.01                 | ALPL                   |
| FABP1    | -0.03            | 0.05             | NOMO2            | 0.02             | -0.01                 | POU5F1                 |
| GATA4    | 0.08             | 0.06             | CFC1             | 0.03             | 0.02                  | TPBG                   |
| VEGFA    | 0.06             | 0.07             | CFC1B            | 0.04             | 0.02                  | LEFTY1                 |
| FOXA2    | -0.01            | 0.07             | TBX6             | -0.10            | 0.07                  | ACVR2B                 |
| CD36     | -0.07            | 0.15             | T                | 0.06             | 0.07                  | FUT2                   |
| PECAM1   | -0.02            | 0.17             | ACVR1C           | 0.02             | 0.07                  | LEFTY2                 |
| AFP      | -0.27            | 0.17             | ACVR2B           | -0.07            | 0.10                  | CFC1                   |
| HNF1B    | 0.03             | 0.18             | WNT8A            | -0.02            | 0.10                  | TERT                   |
| UPK1B    | 0.06             | 0.28             | MESDC1           | -0.03            | 0.11                  | LIN28B                 |
| EOMES    | 0.03             | 0.35             | ACVR1L           | -0.21            | 0.15                  | SOX2                   |
| VEGFB    | -0.05            | 0.47             | ACVR2A           | 0.08             | 0.22                  | KLF5                   |

**ENDODERM**

**MESODERM**

**NEUROECTODERM**

**PLURIPOTENCY**

泽连

| Symbol    | Endoderm day 5KD | Endoderm day 10KD | Mesoderm day 5KD | Mesoderm day 10KD | Neuroectodermal day 5KD | Neuroectodermal day 10KD |
|-----------|------------------|------------------|------------------|------------------|------------------------|------------------------|
| EOMES     | 0.03             | 0.35             |                  |                  |                        |                        |
| NOMO3     | -0.05            | 0.43             |                  |                  |                        |                        |
| ACVR1     | 0.02             | 0.74             |                  |                  |                        |                        |