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Author(s)	Kitazawa, Koji; Hikichi, Takafusa; Nakamura, Takahiro; Mitsunaga, Kanae; Tanaka, Azusa; Nakamura, Masahiro; Yamakawa, Tatsuya; Furukawa, Shiori; Takasaka, Mieko; Goshima, Naoki; Watanabe, Akira; Okita, Keisuke; Kawasaki, Satoshi; Ueno, Morio; Kinoshita, Shigeru; Masui, Shinji
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Graphical Abstract
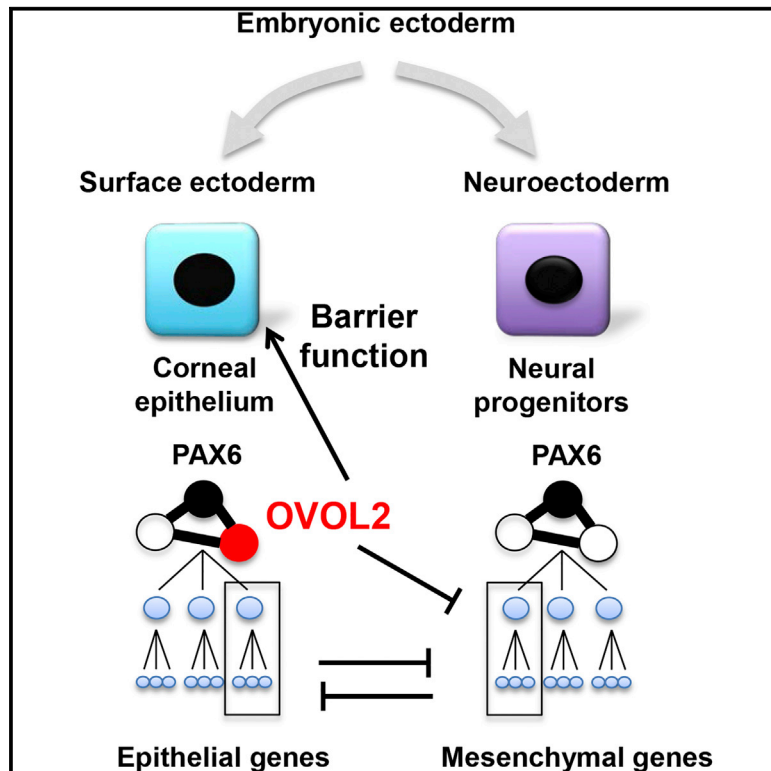
Authors

Koji Kitazawa, Takafusa Hikichi, Takahiro Nakamura, ..., Morio Ueno, Shigeru Kinoshita, Shinji Masui

Correspondence

shigeruk@koto.kpu-m.ac.jp (S.K.),
smasui@cira.kyoto-u.ac.jp (S.M.)

In Brief

Kitazawa et al. show that the transcription factor OVOL2 maintains the transcriptional program of corneal epithelial cells by repressing mesenchymal genes and EMT. This may allow OVOL2 to regulate the differential transcriptional programs of the surface ectoderm and the neuroectoderm.

Highlights

- Identification of a transcription factor that can functionally maintain human CECs

- OVOL2 can activate CEC-specific genes in heterologous cell types

- OVOL2 represses mesenchymal genes in CECs

- EMT may regulate transcriptional programs of surface and neuroectoderm

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OVOL2 Maintains the Transcriptional Program of Human Corneal Epithelium by Suppressing Epithelial-to-Mesenchymal Transition

Koji Kitazawa,[1,2,3,7] Takafusa Hikichi,[1] Takahiro Nakamura,[2,3] Kanae Mitsunaga,[1] Azusa Tanaka,[1] Masahiro Nakamura,[1] Tatsuya Yamakawa,[1] Shiori Furukawa,[1] Mieko Takasaka,[4] Naoki Goshima,[5] Akira Watanabe,[1] Keisuke Okita,[1] Satoshi Kawasaki,[2,6] Morio Ueno,[2] Shigeru Kinoshita,[2,3,*] and Shinji Masui[1,7,*]

[1]Center for iPS Cell Research and Application, Kyoto University, 53 Kawahara-cho Shogoin Sakyo-ku, Kyoto 606-8507, Japan
[2]Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho Hirokoji-agaru Kawaramachi-dori Kamigyo-ku, Kyoto 602-0841, Japan
[3]Department of Frontier Medical Science and Technology for Ophthalmology, Kyoto Prefectural University of Medicine, 465 Kajii-cho Hirokoji-agaru Kawaramachi-dori Kamigyo-ku, Kyoto 602-0841, Japan
[4]JBIC Research Institute, Japan Biological Informatics Consortium, TIME24 Building 10F 2-4-32 Aomi Koto-ku, Tokyo 135-8073, Japan
[5]Molecular Profiling Research Center for Drug Discovery, National Institute of Advanced Industrial Science and Technology, Waterfront Bio-IT Research Building, 2-4-7 Aomi Koto-ku, Tokyo 135-0064, Japan
[6]Department of Ophthalmology, Osaka University, 2-2 Yamadaoka Suita, Osaka 565-0871, Japan
[7]CREST (Core Research for Evolutional Science and Technology), JST (Japan Science and Technology Agency), Honcho 4-1-8 Kawaguchi, Saitama 332-0012, Japan
*Correspondence: shigeruk@koto.kpu-m.ac.jp (S.K.), smasui@cira.kyoto-u.ac.jp (S.M.)
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SUMMARY

In development, embryonic ectoderm differentiates into neuroectoderm and surface ectoderm using poorly understood mechanisms. Here, we show that the transcription factor OVOL2 maintains the transcriptional program of human corneal epithelium cells (CECs), a derivative of the surface ectoderm, and that OVOL2 may regulate the differential transcriptional programs of the two lineages. A functional screen identified OVOL2 as a repressor of mesenchymal genes to maintain CECs. Transduction of OVOL2 with several other transcription factors induced the transcriptional program of CECs in fibroblasts. Moreover, neuroectoderm derivatives were found to express mesenchymal genes, and OVOL2 alone could induce the transcriptional program of CECs in neural progenitors by repressing these genes while activating epithelial genes. Our data suggest that the difference between the transcriptional programs of some neuroectoderm- and surface ectoderm-derivative cells may be regulated in part by a reciprocally repressive mechanism between epithelial and mesenchymal genes, as seen in epithelial-to-mesenchymal transition.

INTRODUCTION

In development, embryonic ectoderm differentiates into either neuroectoderm or a non-neural ectoderm, such as surface ectoderm (Ozair et al., 2013), which gives rise to epidermal tissues including the corneal epithelium (Ordonez and Di Girolamo,

2012). However, the mechanism regulating specification between these lineages is largely unknown. Transcription factors (TFs) are crucial in lineage specification, as they regulate cell-type-specific transcriptional programs (Lee and Young, 2013). There are more than 2,000 TFs in the mammalian genome and hundreds are differentially expressed in each cell type. A handful of TFs, or master TFs, are able to regulate a whole transcriptional program (e.g., direct reprogramming) and play a central role in determining cellular identity (Lee and Young, 2013). The transcriptional program of neuroectoderm requires the paired box TF PAX6 (Zhang et al., 2010), which is also necessary for that of corneal epithelial cells (CECs) (Ouyang et al., 2014). The fact that these two closely related lineages require a common TF implies that a substantial part of their maintenance mechanisms is shared. TFs for neuroectodermal derivatives, such as neural progenitors, have been studied extensively (Xu et al., 2015) but those of human CECs are relatively unknown.

In the present study, we identified OVOL2 as a new TF that maintains CECs. OVOL2 functioned by mainly repressing mesenchymal genes. We also found that neuroectoderm lineage cells expressed mesenchymal genes, of which repression by OVOL2 and activation of epithelial genes could induce the transcriptional program of CECs. This process was likely regulated by a reciprocally repressing mechanism between epithelial and mesenchymal genes, as seen in epithelial-to-mesenchymal transition (EMT). These results should shed new light on the transcriptional programs determining neuroectoderm and surface ectoderm lineage.

RESULTS

TFs that Inhibit iPSC Induction in CECs Tend to Regulate Epithelial Development

Because TFs that strongly maintain cell-type-specific transcriptional programs are reported to inhibit the dedifferentiation
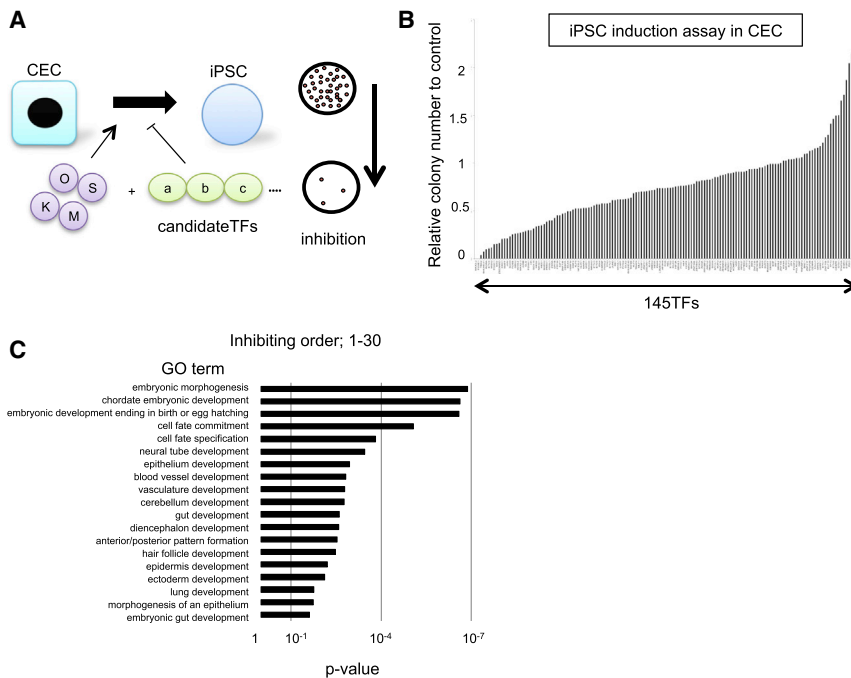
Figure 1. TFs that Inhibit iPSC Induction in CECs Tend to Regulate Epithelial Development

(A) Schematic figure of the iPSC induction assay.

(B) The ratio of AP-positive colonies induced from CECs (two independent experiments). OSKM with empty vector = 1.

(C) GO term analysis by dividing whole TFs into five groups according to the inhibition order. TFs involved in epithelial lineage development were enriched in TFs 1–30.

See also Figure S1 and Table S1.

triggered by induced pluripotent stem cell (iPSC) induction (Hanna et al., 2008; Hikichi et al., 2013), we sought TFs that inhibit iPSC induction in CECs to identify important TFs for CECs. Microarray data of human CECs and conjunctival epithelial cells were compared with those of cardiomyocytes, fibroblasts, and embryonic stem cells (ESCs) from a published database. We selected TFs that had at least 4-fold higher expression in CECs compared with the other cell types, which resulted in 145 candidate TFs. We coinfected each TF with OCT4, SOX2, KLF4, and MYC (OSKM) in a retrovirus and assayed inhibitory effects on iPSC colony numbers (Figure 1A). We arranged the TFs in order of the relative number of iPSC colonies (Figures 1B and S1A; Table S1). There was no correlation in the relative number of iPSC colonies for CECs and human fibroblasts using the same TFs, suggesting that the results reflected a cell-type-specific event (Figures S1B–S1D; Table S1). Using these data of CECs, we divided the 145 TFs into five groups according to their inhibitory effects on iPSC induction and analyzed the enrichment of Gene Ontology (GO) terms in each group. Only in the strongest inhibiting TF group, did we observe significant enrichment of many development-related terms including epithelial development (Figures 1C and S1E), suggesting that TFs that inhibited iPSC induction also tended to regulate differentiation, including possibly that of epithelial lineage.

Combined Overexpression of Inhibitory TFs Induced the Transcriptional Program of CECs in Human Fibroblasts

Because important TFs are often capable of inducing a cell-type-specific transcriptional program (Xu et al., 2015), we next asked whether TFs that inhibited iPSC induction could induce the transcriptional program of CECs. We considered the top 21 inhibiting TFs from the above analysis along with PAX6 and KLF4, which

are known to maintain CECs (Swamynathan et al., 2007; Ouyang et al., 2014) and TP63 and MYC, resulting in a pool of 25 TFs. When these 25 TFs were overexpressed in human fibroblasts by lentivirus vector, fibroblasts started to gather together and show a morphology that resembled epithelial cells by day 10 (Figure 2A). We found that overexpression of the 25 TFs upregulated several CEC-specific genes, including CDH1, KRT3, and ALDH3A1 (Figure 2B). To find which TFs are necessary for the induction of these genes, we next removed individual factors from the pool and analyzed gene activation at 10 days after infection. In particular, removal of OVOL2 decreased CDH1, whereas removal of either PAX6 or KLF4 reduced KRT3. In addition, removal of SOX9 abolished ALDH3A1 (Figure 2B), suggesting that these four TFs are required for the activation of CEC-specific genes. Consistently, simultaneous overexpression of the four TFs in fibroblasts greatly upregulated CEC-specific genes (Figure S2A). To further enhance the activation of CEC-specific genes, we added TP63 and MYC, because they are thought to promote proliferation in CECs (Pellegrini et al., 2001; Perry et al., 2013), and because cell proliferation is likely to facilitate reprogramming, presumably by allowing TFs to access DNA during DNA replication (Xu et al., 2015). Overexpression of the six TFs in fibroblasts induced cells with epithelial morphology that expressed E-cadherin, K3, and K12 (Schermer et al., 1986) (Figures 2C and S2B). These cells expressed higher levels of CEC-specific genes (Figure S2A), suggesting the six TFs were able to induce the transcriptional program of CECs to some extent.

Approximately 5% of fibroblasts with the six TFs expressed CLDN1, a membrane protein specifically expressed in CECs (Yoshida et al., 2009) (Figure 2D). We next sorted CLDN1-positive cells (hereafter 6TFs-fib) by fluorescence-activated cell sorting (FACS) using anti-CLDN1 antibody. 6TFs-fib expressed CEC-specific genes including KRT12 at comparable levels as those in human primary CECs, while fibroblast-specific genes were suppressed (Figure 2E). Microarray analyses followed by unsupervised hierarchical analyses indicated that 6TFs-fib clustered with primary CECs but separated from fibroblasts (Figure 2F). Indeed, genes upregulated or downregulated in 6TFs-fib included a significant number of genes with highly different

expression levels between primary CECs and fibroblasts (Figures S2C and S2D). Moreover, principle component analysis (PCA) revealed that 6TFs-fib were mapped closer to primary CECs and distant from fibroblasts (Figure S2E), mostly because of the expression of genes involved in development or differentiation (Tables S1 and S2). This indicates that these six TFs were able to at least partially induce the transcriptional program of CECs.

In addition to global gene expression, the landscape of nucleosome-free regions highly reflects differences in cell lineages, likely because lineage-specific TFs bind and regulate gene expressions through these regions (Stergachis et al., 2013). Accordingly, we performed assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq), which provides genome-wide information on nucleosome-free regions (Buenrostro et al., 2013), using CECs, 6TFs-fib, and fibroblasts. Notably, in some CEC-specific genes, peaks in the promoter regions seen in primary CECs but not in fibroblasts appeared in 6TFs-fib, whereas peaks in the promoter regions of some fibroblast-specific genes seen in fibroblasts disappeared in 6TFs-fib (Figures 2G and S2F). Global comparison of peaks indicated that 17.3% of peaks that appeared in 6TFs-fib (6TFs-fib presence peaks) were primary CEC-specific peaks, while 61.3% of peaks that disappeared (6TFs-fib absence peaks) were fibroblast-specific peaks (Figures 2H and S2G), indicating that the six TFs were able to at least partially induce a CEC landscape of nucleosome-free regions in fibroblasts.

OVOL2 Repressed Mesenchymal Genes in CECs

Because the functions of PAX6 and KLF4 in CEC maintenance have already been reported (Graw, 2003; Swamynathan et al., 2007), we next examined how OVOL2 contributes to maintaining the transcriptional program of CECs using small interfering RNA (siRNA) in primary CECs. OVOL2 knockdown reduced the expression of CEC-specific genes (Figure 3A) and keratin 12 protein in primary CECs (Figure 3B). Morphologically, CECs with OVOL2 knockdown became elongated fibroblastic cells (Figure 3C). Immunocytochemistry of CECs with OVOL2 knockdown showed a reduction of K12 and E-cadherin proteins (Figure 3D). Moreover, OVOL2 knockdown significantly reduced the barrier function of primary CECs as measured by transepithelial electrical resistance (TER) (Figures 3E and 3F), suggesting that OVOL2 functionally maintains CECs. Global expression analyses between CECs and CECs treated with siOVOL2 by microarray revealed that among the 42,545 genes analyzed, 805 genes were differentially expressed by >1.5-fold, and 320 genes were upregulated and 485 genes were downregulated in CECs with siOVOL2 (Figure 3G). Downregulated genes enriched GO terms related to epithelial functions (Figure 3H), whereas upregulated genes were not significantly associated with any terms.

To gain insight into the function of OVOL2, we analyzed TF motif enrichment using the ATAC-seq data. In sequences among the fibroblast-specific peaks and the 6TFs-fib absence peaks, the OVOL2 motif (CCGTTA) (Watanabe et al., 2014) was significantly enriched (Figure S3A; Table S3A). Moreover, genes within the 6TFs-fib absence peaks and containing the OVOL2 motif enriched GO terms related to mesenchymal cells (Figure S3B). To address whether OVOL2 represses mesenchymal genes in

CECs, we examined OVOL2 effects in response to TGF-β, whose activation leads to a loss of CEC identity through EMT (Yamben et al., 2013). We used the doxycycline (Dox)-inducible system to conditionally overexpress OVOL2 in primary CECs. While CECs treated with TGF-β changed to fibroblastic morphology (Figure S3C) and showed an upregulation of mesenchymal genes (Figure S3D), OVOL2 overexpression at least partially blocked the morphological change and significantly repressed the upregulation (Figures S3C and S3D), indicating that OVOL2 represses mesenchymal genes in CECs. Indeed, in OVOL2 knockdown CECs, mesenchymal genes such as *ZEB1* and *ZEB2*, two well-known mesenchymal genes (De Craene and Berx, 2013), were upregulated (Figure S3E). Chromatin immunoprecipitation sequencing (ChIP-seq) analysis using CECs showed OVOL2 peaks on *ZEB1* and *ZEB2* (Figure 3I; Table S3B), suggesting that OVOL2 bound and repressed fibroblast-specific genes, particularly those with mesenchymal function. In accordance with this result, recent reports showed that OVOL2 is a strong epithelial regulator that maintains transcriptional programs in epidermal keratinocytes and mammary epithelial cells by repressing *ZEB1* and *ZEB2* (Lee et al., 2014; Watanabe et al., 2014). At the same time, overexpression of ZEB1 in CECs partially repressed the expression level of *CDH1* (Figure 3J), consistent with its effect in other epithelial cells (Shirakihara et al., 2007), and E-cadherin expression was weak in CECs overexpressing ZEB1 or ZEB2 compared to the control (Figure 3K). Additionally, barrier function assay demonstrated that CECs overexpressing ZEB1 or ZEB2 reduced the TER compared to the control (Figure 3L). These results suggested that OVOL2 maintains CECs in part by repressing mesenchymal genes such as ZEB genes.

OVOL2 Induced the Transcriptional Program of CECs in Neural Progenitor Cells

Interestingly, OVOL2 knockdown in CECs derepressed not only mesenchymal genes such as ZEB genes, but also neural progenitor cell (NPC)-specific genes including *SOX2* (Figure S3E). *ZEB* genes are required for neuroectoderm differentiation in vivo and in vitro (Chng et al., 2010; Miyoshi et al., 2006). We meta-analyzed published datasets of human cells and found that in neuroectoderm and NPCs, some mesenchymal genes including *ZEB* genes are highly expressed, whereas epithelial genes are repressed (Figure 4A). This expression pattern was similar to that of adipose-derived mesenchymal stem cells and fibroblasts (Figure 4A), which are known to express mesenchymal genes (Kfoury and Scadden, 2015). In contrast, in CECs and epidermal keratinocytes, which are derived from surface ectoderm (Koster and Roop, 2007), as well as in iPSCs and ESCs, which are thought to be epithelial cells (De Craene and Berx, 2013), the expression pattern was opposite; mesenchymal genes were repressed whereas epithelial genes were highly expressed (Figure 4A). We further meta-analyzed additional datasets of mouse cells and confirmed similar expression patterns (higher expression of mesenchymal genes; lower expression of epithelial genes) in various cell types derived from neuroectoderm (Figures S4A and S4B). These results suggest that mesenchymal genes might contribute to the maintenance of transcriptional programs of neuroectoderm lineage cells, and epithelial genes
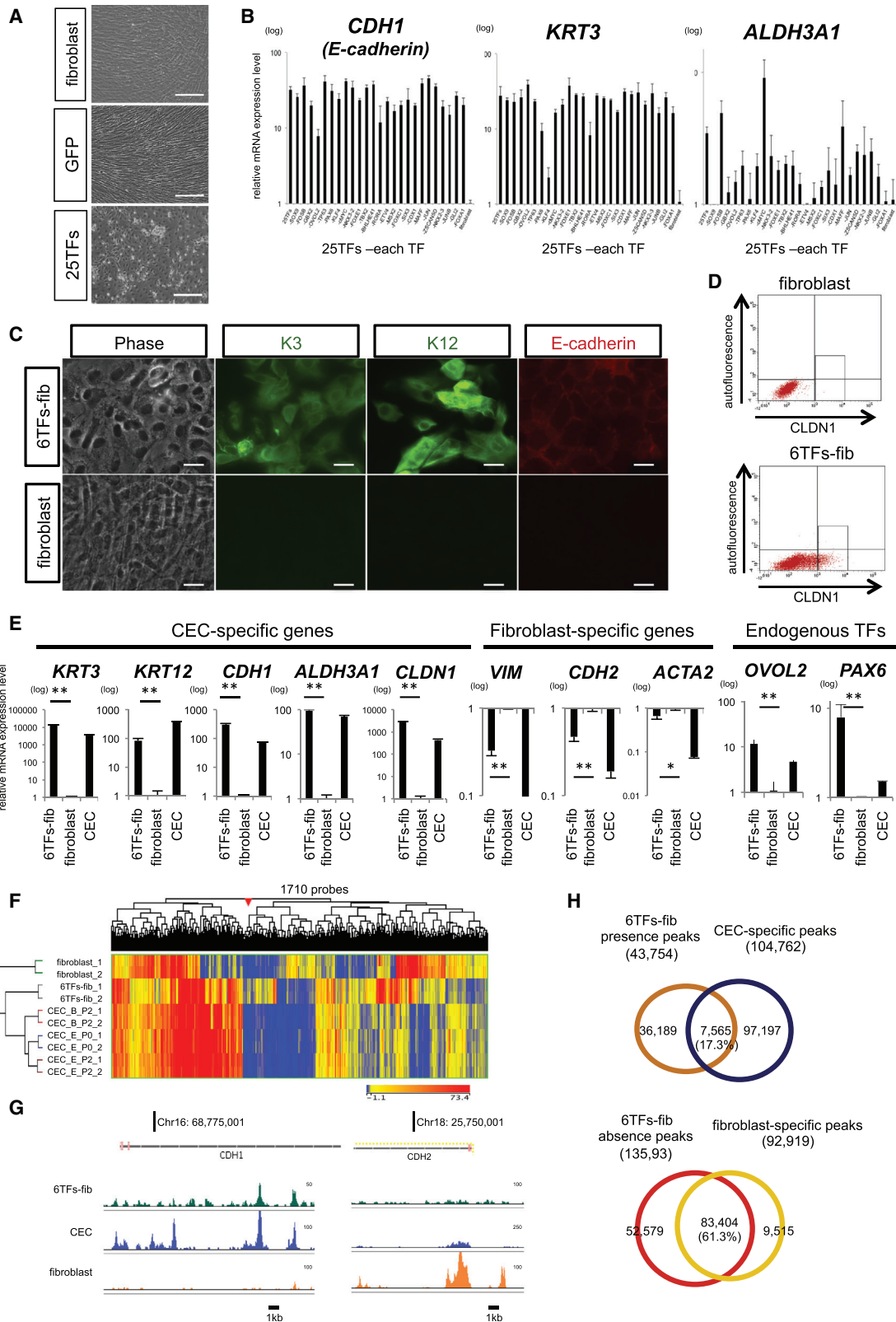
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expressed in surface ectodermal lineage cells possibly repress the transcriptional program of neuroectoderm cells by repressing mesenchymal genes using a mechanism similar to that in EMT (De Craene and Berx, 2013). We thus hypothesized that OVOL2 regulates cell-type-specific transcriptional programs between CECs and NPCs, which we used as representative cell types of surface ectoderm and neuroectoderm lineage, respectively, through the reciprocally repressive mechanism between epithelial and mesenchymal genes seen in EMT. To test this theory, we overexpressed OVOL2 alone in NPCs and analyzed the expression of CEC-specific genes. To our surprise, NPCs with OVOL2 overexpression started to form cell-cell junctions much like those in epithelial-like cells (Figure 4B). NPCs with OVOL2 overexpression exhibited a high expression of CEC-specific genes (*Krt12* and *Aldh3a1*) without any upregulation of *Krt1* or *Krt10*, while mesenchymal genes (*Snai2* and *Zeb2*) were downregulated (Figures 4C and S4C). Furthermore, by microarray analysis, we found that the expressions of most epithelial and mesenchymal genes were reciprocated in NPCs with OVOL2 and NPCs alone, with epithelial genes upregulated and mesenchymal genes downregulated in the former (Figure 4D). In light of the specificity of *Krt12* expression (as well as the phenotype of its knockout mice) seen only in corneal epithelium in the body (Kao et al., 1996), the upregulation of *Krt12* by OVOL2 overexpression suggests specific activation of the transcriptional program of CECs. We considered whether this activation was mediated, in part, by the repression of *Zeb1 and Zeb2*, because both genes were quickly downregulated in NPC overexpressing OVOL2 (Figure 4E), while at the same time Zeb1 overexpression partially blocked the upregulation of *Krt12* and *Cdh1* (Figure 4F). These results are consistent with our ChIP-seq data (Figure 3I) and demonstrate that ZEB1/2 were OVOL2 targets. At the global level, the upregulated and downregulated genes contained a significant number of genes highly expressed in CECs and NPCs, respectively (Figures S4D and S4E). These data indicated that forced expression of OVOL2 in NPCs could at least partially induce the transcriptional program of CECs by repressing mesenchymal genes. Moreover, overexpression of Grhl2, another epithelial TF (De Craene and Berx, 2013), induced epithelial-like cells in NPCs (Figure S4F) with an expression profile similar to that by OVOL2 overexpression (Figure S4G), suggesting the repression of mesenchymal genes and activation of epithelial genes (i.e., regulation of EMT-related genes) may be sufficient to activate the transcriptional program of CECs. Collectively, our data show that OVOL2 maintains the transcriptional program of CECs by repressing mesenchymal genes to maintain epithelial identity (Figure 4G). This result suggests that

differences between the transcriptional programs of some surface ectoderm- and neuroectoderm-derivative cells may be regulated in part by the reciprocally repressive mechanism between epithelial and mesenchymal genes seen in EMT.

DISCUSSION

The identification of master TFs has advanced our understanding of development and aided in therapeutic research (Lee and Young, 2013). Previously, PAX6 was the only identified TF that could induce CEC-lineage cells from a derivative of surface ectoderm (Ouyang et al., 2014). In the present work, we found PAX6 alone did not induce the transcriptional program of CECs in fibroblasts, whose lineage is presumably distant from surface ectoderm (Kfoury and Scadden, 2015) (Figure S2A). Instead, cotransduction with OVOL2, an epithelial regulator, successfully induced the transcriptional program. Overexpression of OVOL2 alone in fibroblasts, however, was not sufficient for the induction (Figure S2A) suggesting that PAX6 and OVOL2 may cooperatively activate the transcriptional program of CECs. Indeed, overexpression of OVOL2 with other TFs could induce CEC-like cells in a cell type (oral mucosal epithelial cell) other than fibroblasts (Figure S4H), suggesting that OVOL2 could provide important clues for future therapeutics.

Our data showed that OVOL2 maintained the transcriptional program of CECs by repressing mesenchymal genes, a function that may explain differences in the transcriptional programs of neuroectoderm and surface ectoderm. Several lines of evidence support this scheme. In *Ovol2* knockout embryo, surface ectoderm is reduced while neuroectoderm is expanded (Mackay et al., 2006). In addition, in an ESC differentiation system, *Ovol2* knockdown enhances neuroectoderm differentiation, and Ovol2 overexpression in chick embryo inhibits neuroectoderm differentiation (Mackay et al., 2006; Zhang et al., 2013). On the other hand, mesenchymal genes are required for the transcriptional program of neuroectoderm, as knockout of *ZEB1* and *ZEB2* in embryo causes defects in neural tube in which *Sox2* (a TF gene required for neuroectoderm) expression is repressed (Miyoshi et al., 2006), and *ZEB2* knockdown in ESCs suppresses neuroectoderm differentiation (Chng et al., 2010). Several other studies using ESCs have also indicated the involvement of mesenchymal genes in neuroectoderm differentiation (Di Micco et al., 2014; Du et al., 2013; Gill et al., 2011). Moreover, our analysis showed that various neuroectoderm derivatives expressed high levels of mesenchymal genes, while surface ectoderm derivatives instead expressed epithelial genes highly. These observations suggest that mesenchymal and epithelial genes, which might segregate

Figure 2. Combined Overexpression of Inhibitory TFs Induced the Transcriptional Program of CECs in Human Fibroblasts
(A) The morphology of neonatal human fibroblasts, fibroblasts transduced with GFP, and fibroblasts transduced with 25 TFs. Scale bars, 200 μm.
(B) The effect of removing individual TFs from the pool of 25 TFs was examined by qRT-PCR (all n = 4). Data are shown as means ± SD.
(C) Phase contrast and immunocytochemistry of K3, K12, and E-cadherin in fibroblasts and fibroblasts with 6 TFs (6TFs-fib). Scale bars, 20 μm.
(D) Fluorescence-activated cell sorting (FACS) analysis using Claudin 1 (CLDN1) antibody.
(E) Quantitative analysis of CEC-specific genes, fibroblast-specific genes, and endogenous *OVOL2* and *PAX6* in the indicated cells (all n = 4). Data were analyzed using the unpaired t test and are shown as means ± SD. *p < 0.05, **p < 0.01.
(F) Unsupervised hierarchical analyses of global gene expression.
(G) ATAC-seq peaks in the promoter regions of CEC-specific genes and fibroblastic-specific genes (all n = 4).
(H) Comparison of ATAC-seq peaks at the global level. Numbers represent peak numbers detected by Hotspot software.
See also Figure S2 and Table S2.
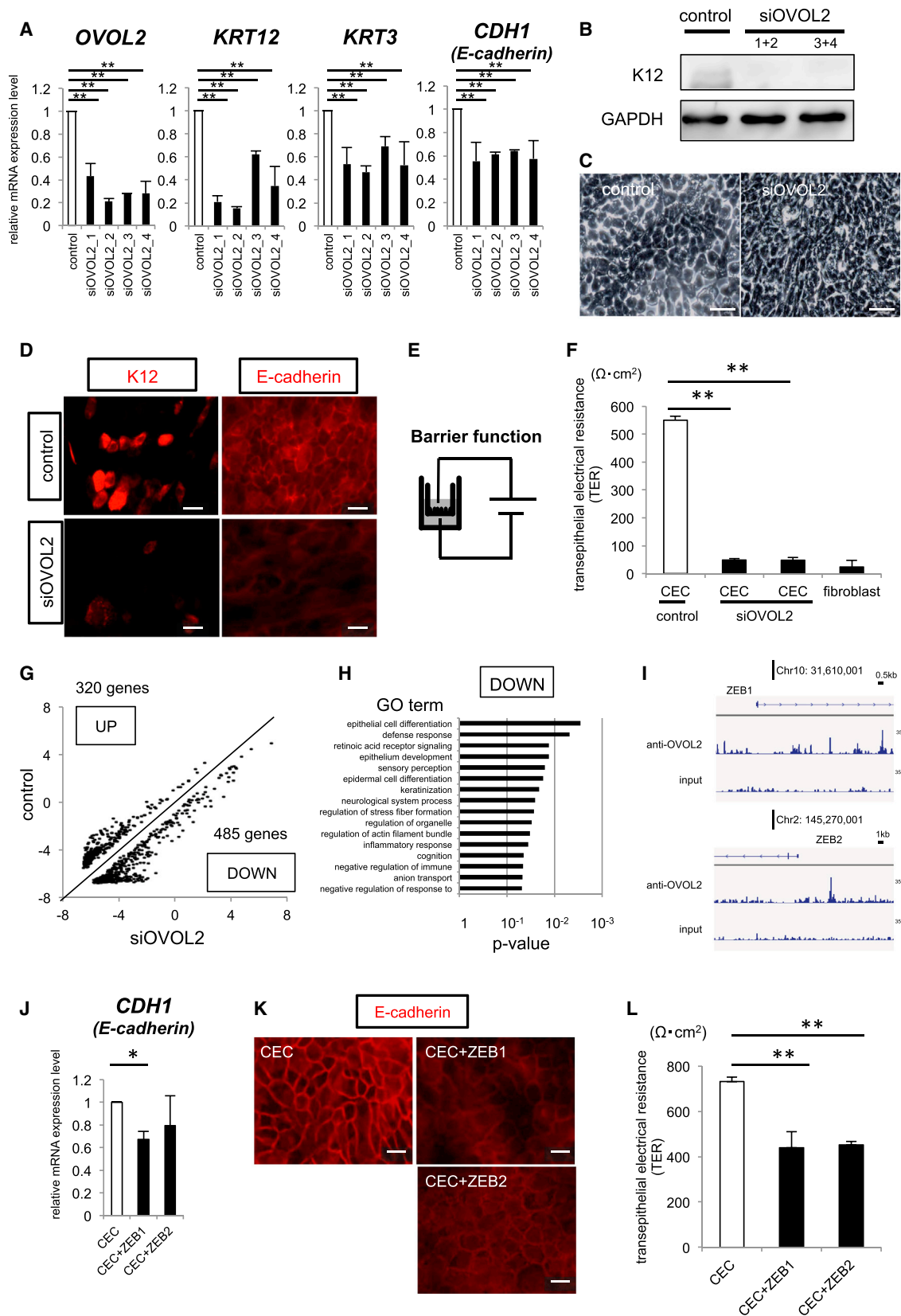
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neuroectoderm and surface ectoderm in early development, may still regulate the transcriptional programs in some derivative cells of these lineages. Recent reports have demonstrated that OVOL2 maintains epidermal keratinocytes and mammary epithelial cells by repressing mesenchymal genes, suggesting it an epithelial regulator (Lee et al., 2014; Watanabe et al., 2014). Our data showed that in CECs, OVOL2 activates epithelial genes by repressing mesenchymal genes whose expression might promote neuroectoderm lineage. Similarly to other epithelial cell types, OVOL2 bound to and could repress ZEB1 and ZEB2 in CECs, suggesting that both of these genes are direct targets of OVOL2. Because upregulation of ZEB1 and ZEB2 was modest in OVOL2 knockdown, these two genes might constitute only a subgroup of causative genes for the loss of epithelial identity, as seen in keratinocytes (Lee et al., 2014). Taken together, we propose that OVOL2 might regulate differences in the transcriptional programs between surface ectoderm and neuroectoderm through a reciprocally repressing mechanism between epithelial and mesenchymal genes, as seen in the switch-like reversion of the expression status in EMT.

EXPERIMENTAL PROCEDURES

Sample Collection and Cell Preparation

Human normal corneal tissues were obtained from donors from SightLife exclusively for research purposes and human normal conjunctival tissues from two patients who received conjunctival-chalasis surgery. Human oral mucosal tissues were obtained from healthy volunteers, and superfluous tissue was obtained from patients undergoing oral surgery. All patients provided informed consent following the guidelines of the Kyoto Prefectural University of Medicine Ethics Committee. CECs were isolated by treatment with 1,000 protease units (PU)/ml of dispase (Dispase type II; Godo Shusei) at 4°C overnight and TrypLE Express (Life Technologies) at 4°C for 30 min. Isolated primary CECs were seeded on a 60-mm dish or 6-well plate coated with 0.1% gelatin and were cultured in CEC medium, which consisted of DMEM and Ham's F-12 media (1:1 mixture) (Life Technologies) containing B27-supplement (Life Technologies), Rho-kinase (ROCK) inhibitor (10 mM) (Nacalai Tesque), epidermal growth factor (EGF) (10 ng/ml) (Life Technologies), Epigallocatechin gallate (10 ng/ml) (Sigma), and penicillin-streptomycin (50 IU/ml) (Nakalai Tesque), at 37°C in a 5% CO_2 incubator and then used for the following experiments after one passage using TrypLE Express at double dilution at room temperature upon reaching sub-confluence. For the functional screening of transcription factors, CECs from the same donors at the time of

first passage were stocked at −150°C until use. Neonatal human dermal fibroblasts were purchased from Lonza. NPCs have been previously established and cultured in N2B27 media with fibroblast growth factor (FGF) 2 and EGF (Conti et al., 2005; Hikichi et al., 2013).

Retroviral Preparation

Plat-GP cells were seeded at a density of 2.0×10^6 cells on a 10-cm dish in 10 ml of DMEM (Nacalai Tesque) with 10% FBS. Retroviral (6 µg) and 3 µg of VSV-G vector DNAs were transfected by using 27 µl of FuGENE 6 (Promega). A mixture of the retroviral supernatant and the cell culture medium in equal amounts was added to the cell in the presence of 5 µg/ml polybrene on the plate. The cell plate was set up in the centrifuge at 35°C and then centrifuged for 30 min at $800 \times g$.

iPSC Induction Assay

Yamanaka factors (OCT3/4, SOX2, KLF4, and cMYC) with each candidate TF (total, 5 TFs) were transduced by retroviruses to the CECs seeded at a density of 0.75 to 1.0×10^4 cells on a 6-well plate. At day 2, mitomycin-treated SNL feeder cells were added at a density of 5×10^5 cells, and the medium was replaced with CEC medium and 0.5% FBS. After day 6, the medium was switched to Primate ES cell medium (ReproCELL) supplemented with 20 ng/ml FGF2 and changed each day until day 21. iPSC colonies were stained by alkaline phosphatase (AP) Kit (Muto Pure Chemicals).

Induction of the Transcriptional Program of CECs in Fibroblasts

A combination of TFs was transduced by lentivirus or retrovirus to fibroblasts seeded at a density of 6.0×10^4 cells on a 6-well plate. After 3 days of infection, the medium was switched to CEC medium, and the cells were cultured until day 10. RNA was extracted using the RNeasy Mini Kit. Primers listed in Table S4 were used for qRT-PCR.

siRNA Treatment

ON-TARGETplus siRNA or siRNA-SMARTpool (25 nM) (GE Healthcare) was transfected into the primary CECs at a density of 2.5×10^5 cells on a 24-well plate using 2.5 µl of DharmaFECT 4 siRNA Transfection Reagent (GE Healthcare). The cells were harvested at 48 hr after the transfection.

Barrier Functional Assay

Cells were cultured on 12-well porous membrane filters (Transwell, 0.4 mm pore; Corning) at 5.0×10^5 cells. TER between the upper and lower chambers was assessed using a volt-ohm meter (EVOM; World Precision Instruments) as previously described (Kitazawa et al., 2013).

Induction of the Transcriptional Program of CECs in NPCs

Retroviruses were transduced to NPCs at 5.0×10^3 cells on a 6-well plate, and the next day 1 µg/ml puromycin or 500 µg/ml neomycin was added. For the

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Figure 3. OVOL2 Repressed Mesenchymal Genes in CECs

(A) Knockdown of OVOL2 using siRNA (siOVOL2) in CECs and downregulation of CEC-specific genes based on qRT-PCR (all n = 4). Data were analyzed using the unpaired t test and are shown as means ± SD. **p < 0.01.

(B) Western blotting of K12 in primary CECs treated with two siRNAs for OVOL2.

(C) The morphology of CECs and CECs treated with siOVOL2. Scale bars, 20 µm.

(D) Immunocytochemistry of K12 and E-cadherin in CECs and CECs treated with siOVOL2. Scale bars, 20 µm.

(E) Barrier function was assessed by measuring transepithelial electrical resistance (TER) with a volt-ohm meter.

(F) Barrier function assay demonstrated that siRNA for OVOL2 reduced the TER compared to control. Representative results of two independent experiments with technical triplicates are shown as means ± SD. Data were analyzed using the unpaired t test. **p < 0.01.

(G) Scatter plots analysis of gene expressions between CECs and CECs treated with siOVOL2 found upregulated genes (320 genes) and downregulated genes (485 genes).

(H) GO analysis showed that epithelial-related genes were enriched in DOWN genes.

(I) ChIP-seq analysis of OVOL2 in the CECs.

(J) Quantitative analysis of CDH1 in the CECs and CECs overexpressing ZEB1 or ZEB2 (all n = 3). Data were analyzed using the unpaired t test and are shown as means ± SD. *p < 0.05. GFP-transduced CECs were used as control.

(K) Immunocytochemistry of CDH1 in CECs and CECs overexpressing ZEB1 or ZEB2. GFP-transduced CECs were used as control.

(L) Barrier function assay demonstrated that overexpression of ZEB1 or ZEB2 in CECs reduced the TER compared to the control (GFP-transduced CECs). Representative results of four independent experiments with technical triplicates are shown as means ± SD. Data were analyzed using the unpaired t test. **p < 0.01. See also Figure S3 and Table S3.
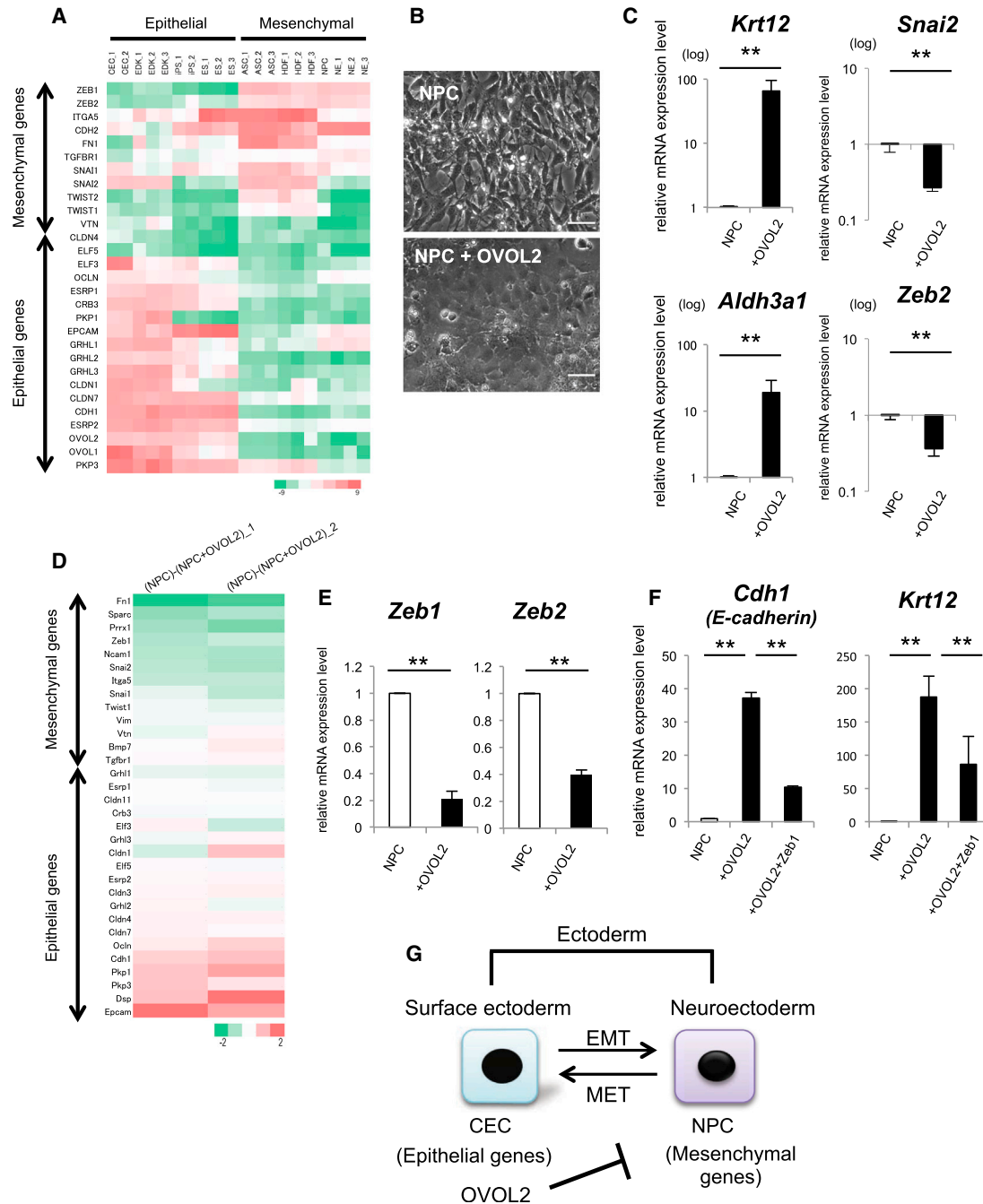
Figure 4. OVOL2 Overexpression Induced the Transcriptional Program of CECs in Neural Progenitor Cells by Regulating Genes Involved in EMT

(A) Microarray analyses of EMT-related genes among CECs, epidermal keratinocytes (EDK), adipose-derived stem cells (ASC), dermal fibroblasts (HDF), NPCs, neuroectoderm (NE), iPSCs, and ESCs.

(B–D) Ten days after viral infection. (B) The morphology of NPCs overexpressing OVOL2. Scale bars, 20 μm. (C) Quantitative analysis of CEC-specific genes and NPC-specific genes (all n = 4). Data were analyzed using the unpaired t test and are shown as means ± SD. **p < 0.01. (D) The expressions of EMT-related genes reciprocate with the overexpression of OVOL2. Two individual experiments were performed.

(E and F) Three days after viral infection. Data were analyzed using the unpaired t test and are shown as means ± SD. **p < 0.01. (E) Zeb1 and Zeb2 were downregulated in NPC overexpressing OVOL2. (F) OVOL2 activated, but Zeb1 repressed Krt12 and Cdh1 gene expressions in NPC.

(G) Schematic figure of the mechanism shows how the transcriptional profile between CEC and NPC is distinguished.

See also Figures S3 and S4.

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samples of 10 days after transduction, at 3 days after transduction the culture medium was switched to N2B27 medium with EGF but without FGF and cultured 7 days.

Statistics

Unpaired t tests were used for all qRT-PCR analyses. For the binding motif analysis of OVOL2 between CECs and fibroblasts, Fisher's exact test was used. Pearson's correlation coefficient was used for comparisons of iPSC induction activity between CECs and fibroblasts. All statistical results were verified independently using the Ekuseru-Toukei 2012 (Social Survey Research Information), and the hypergeometric distribution was analyzed using Keisan online calculator (http://keisan.casio.com).

ACCESSION NUMBERS

The accession number for the microarray, ATAC-seq and ChIP-seq data reported in this paper is GEO: GSE67823.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.020.

AUTHOR CONTRIBUTIONS

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