Supporting Information

Shalom-Feuerstein et al. 10.1073/pnas.1201753109

SI Materials and Methods

Pluripotency Characterization. Expression of pluripotency markers were determined by immunofluorescence using octamer-binding transcription factor 4 (OCT4) antibodies (Stemgent) and TRA-1-81 antibodies (Santa Cruz Biotechnology) and by real-time quantitative PCR (qRT-PCR), as described below. Alkaline phosphatase activity was revealed after 4% paraformaldehyde fixation by incubation with 0.01% Naphtol (Sigma) and 0.5 mg/mL Fast Blue (Sigma) in 100 mM Tris-HCL and 20 mM $MgCl_2$.

For whole-genome microarray and PluriTest analysis, we followed the same procedures as previously described (1, 2). RNA was isolated from two biological replicates per cell line (1×10^6 cells per sample) with the mirVana RNA isolation kit (Ambion). Illumina HT12v3 microarrays were hybridized following the manufacturers instructions and as previously described (1). The resulting raw data were processed with the PluriTest algorithm (1) for testing pluripotent features in induced pluripotent stem cell (iPSC) lines. Genetic integrity was evaluated by G-banded karyotype analysis.

In Vitro Differentiation Protocols. iPSC colonies were cultured in suspension in Petri dishes in differentiation medium (DMEM supplemented with 10% FBS, 1 mM Glutamine,100 μM nonessential amino acids, 100 μM β-mercaptoethanol) to allow the formation of . Embryoid bodies were plated on day 7 on gelatin-coated plates and further cultured until day 20. Directed trophoectoderm differentiation was performed by plating iPSC on matrigel in iPSC medium previously conditioned 24 h on mouse embryonic fibroblasts. The following day, medium was switched to serum-free medium [DMEM/F12 supplemented with 1 mM glutamine, 100 μM nonessential amino acids, 1× N2 and 1× B27 (Invitrogen)] with bone morphogenetic protein-4 (BMP-4) (25 ng/mL). Cells were collected 6 d later.

qRT-PCR. RNA was extracted using Aurum kit and cDNA was synthetized from 1 μg RNA using the iscript kit (Bio-Rad). qRT-PCR was performed in duplicate using SYBR Green (Bioline) and specific primers (sequences available upon request). Each reaction contained 12.5 μL SYBR-Green PCR Master Mix, 5 μL cDNA and 5 μL primer mix (0.5 μM), adjusted to 25 μL reaction volume. The value of each reaction was normalized to GAPDH and the relative expression of each transcript was calculated as a fold-change relative to control sample of undifferentiated cells.

Immunofluorescence. Differentiated cells that were grown on coverslips were fixed in cold methanol for 20 min. Blocking was for 20 min [2.5% BSA (Sigma), 2.5% naive Donkey serum (Jackson Laboratories)], primary and secondary antibodies were applied for 45 min followed by anti-fade DAPI mounting-gel fixation (Sigma). The following primary antibodies were used: rabbit anti-K14 (1:200) (Covance), rabbit anti-K5 (1:200) (Covance), mouse anti-K18 (1:200) [Chemicon (Millipore)], rabbit anti-pax6 (1:100) (Chemicon), mouse anti-K3(1:100) (Millipore), mouse anti-p63 (1:100) (Santa Cruz), and mouse anti–E-Cadherin (1:100) (R&D Systems).

Flow Cytometry. Cells were fixed in 2% paraformaldehyde for 20 min at room temperature. After washing with PBS, cells were incubated with 0.5% BSA, 0.5% saponin, and donkey serum for 30 min. Primary antibodies for K14 (Millipore) and K18 (Chemicon) were added for 45 min. Acquisition was performed on FACSCalibur using CellQuest software (BD Biosciences).

1. Rinne T, Hamel B, van Bokhoven H, Brunner HG (2006) Pattern of p63 mutations and their phenotypes—Update. *Am J Med Genet A* 140:1396–1406.

2. Petit I, et al. (2012) Induced pluripotent stem cells from hair follicles as a cellular model for neurodevelopmental disorders. *Stem Cell Res (Amst)* 8:134–140.

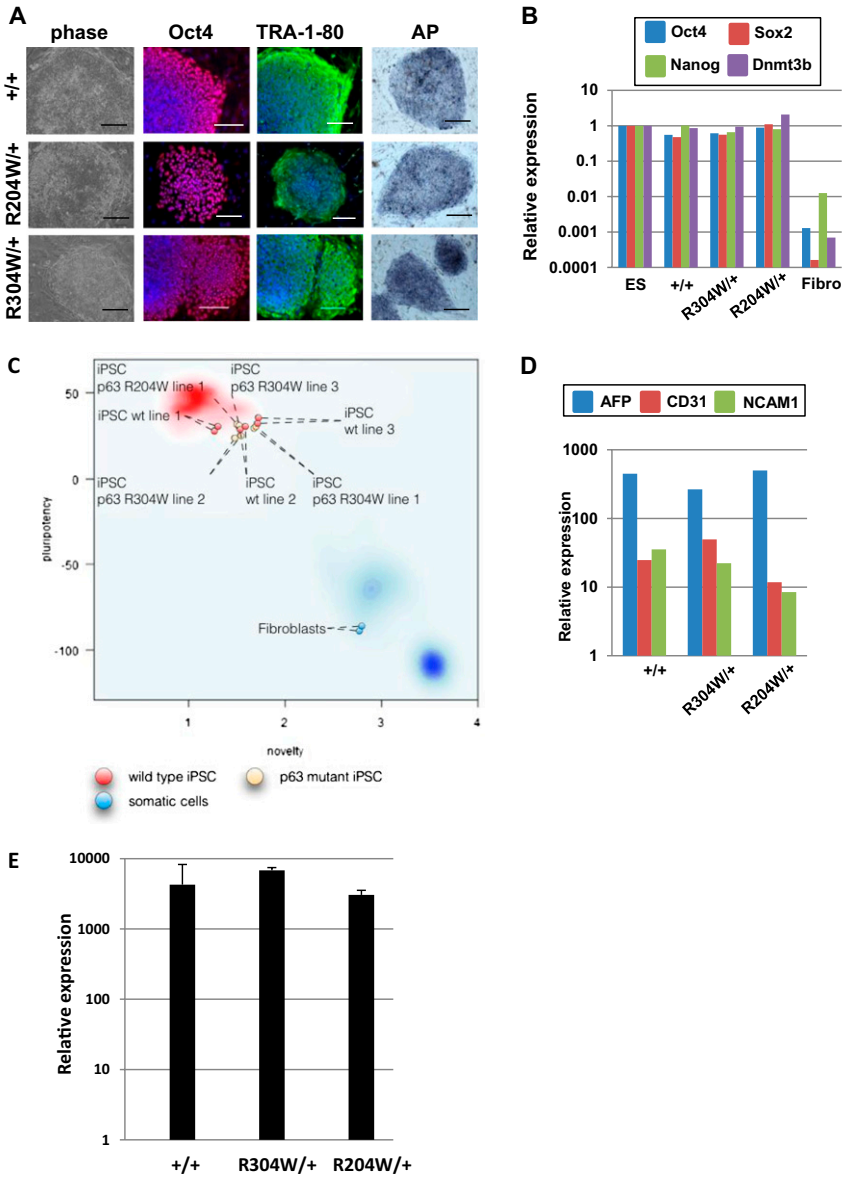

Fig. S1. Characterization of iPSC from EEC patients. (*A*) Morphology of iPSC$^{+/+}$, iPSC$^{R204W/+}$, and iPSC$^{R304W/+}$ cells and expression of the pluripotent markers Oct4, Tra-1-80, and alkaline phosphatase (AP). Immunofluorescence staining included DAPI staining (blue). (Scale bars, 100 mm.) (*B*) qRT-PCR for the pluripotent markers Oct4, sex-determining region Y box-2 (Sox2), Nanog, and DNA methyltransferase 3b (Dnmt3b). hES and fibroblasts were included as positive and negative controls, respectively. (*C*) Pluripotent transcriptional profile measured in PluriTest assay. PluriTest results are plotted in density distribution for previously referenced pluripotent cells (red cloud) and somatic cells (blue cloud). (*D*) In vitro differentiation of iPSC lines in embryoid bodies. iPSC were cultured in suspension for 7 d then plated on gelatin. At day 20 cells were analyzed by qRT-PCR for the germ layer markers AFP (endoderm), CD31 (mesoderm), and NCAM1 (neuroectoderm). Results show relative expression compared with undifferentiated iPSC. (*E*) Directed in vitro differentiation of iPSC toward trophoectoderm. iPSC$^{+/+}$, iPSC$^{R204W/+}$, and iPSC$^{R304W/+}$ cells were seeded on matrigel and cultured in presence of BMP-4 for 6 d. Expression of the trophoectoderm marker CDX2 was evaluated by qRT-PCR and results were expressed relative to CDX2 expression in undifferentiated iPSC. Results show average ± SE from two independent iPSC clones.

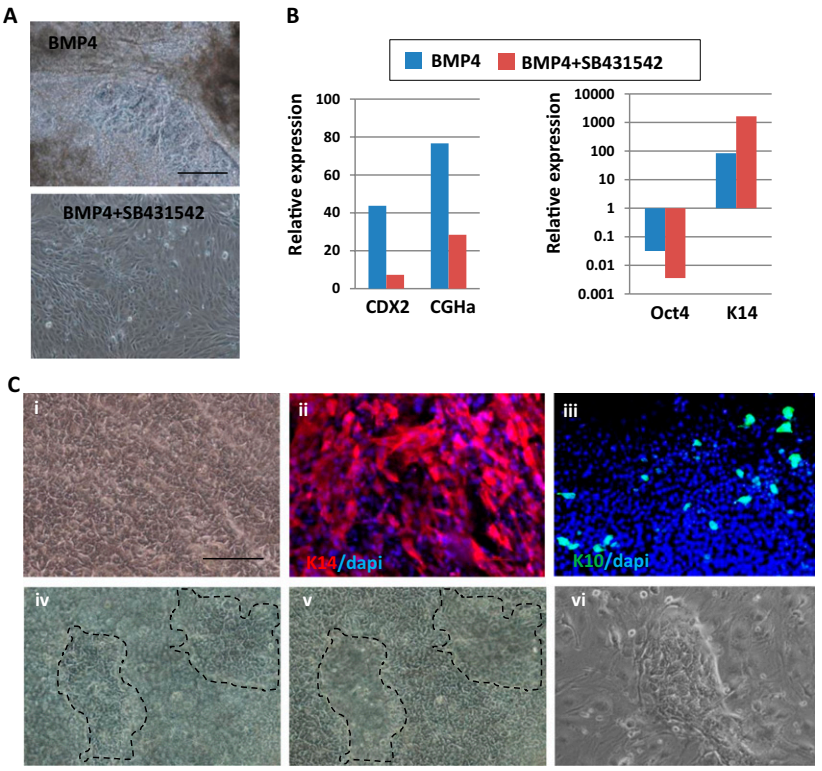

Fig. S2. Epidermal differentiation of iPSC by BMP-4 and TGF-β inhibition. iPSC$^{+/+}$ were subjected to epidermal differentiation in presence of BMP-4 alone or with BMP-4 and the TGF-β inhibitor SB431542. (*A*) Phase pictures at day 7. (*B*) qRT-PCR at day 10 for Oct4, K14, and the extraembryonic markers CDX2 and CGHa. (*C*) After 30 d of differentiation in presence of BMP-4 and SB431542, cells acquire a keratinocyte-like morphology (i) and highly express K14 (ii). Some mature K10^{+} cells are present (iii) and spontaneous stratification can be observed (iv and v) (Dashed lines outline stratified area in iv shows focus on the stratified keratinocytes, in v shows focus on the rest of the keratinocytes). Picked cells form typical keratinocyte colonies (vi). (Scare bars, 100 μm.)

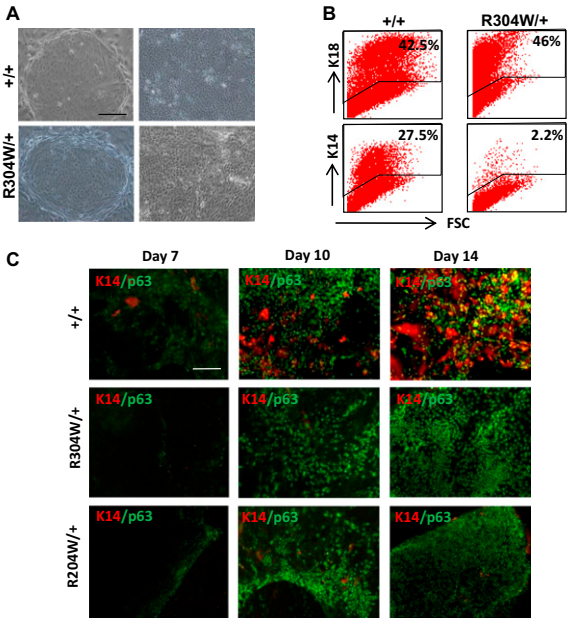

Fig. S3. Impaired epidermal differentiation of EEC-iPSC. iPSC$^{+/+}$, iPSC$^{R204W/+}$, and iPSC$^{R304W/+}$ cells were subjected to epidermal differentiation protocol in presence of BMP-4 and SB431542. (*A*) Phase pictures of iPSC$^{+/+}$ and iPSC$^{R204W/+}$ after 10 and 30 d of differentiation. (*B*) Flow cytometry analysis of iPSC$^{+/+}$ and iPSC^{EEC} after 25 d of differentiation. The data show one representative experiment. (*C*) Immunofluorescence staining for K14 and p63 during epidermal differentiation of iPSC$^{+/+}$ and iPSC^{EEC}. (Scale bars, 100 μm.)

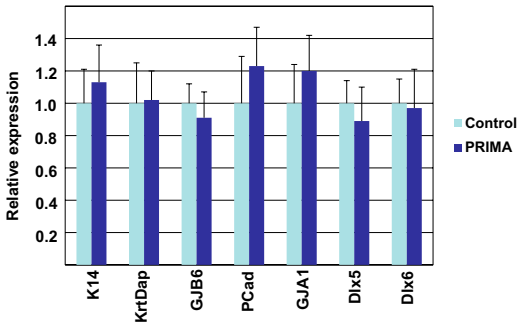

Fig. S4. APR-246 effect on corneal epithelial differentiation of iPSC$^{+/+}$cells. iPSC$^{+/+}$ were differentiated into corneal epithelial cells for 10 d in the presence (PRIMA) or absence (Control) of APR-246 treatment, as detailed in *Materials and Methods*. Real-time PCR analysis is showing the relative expression of the indicated epithelial transcripts. Results show average data obtained from three independent experiments ± SD.

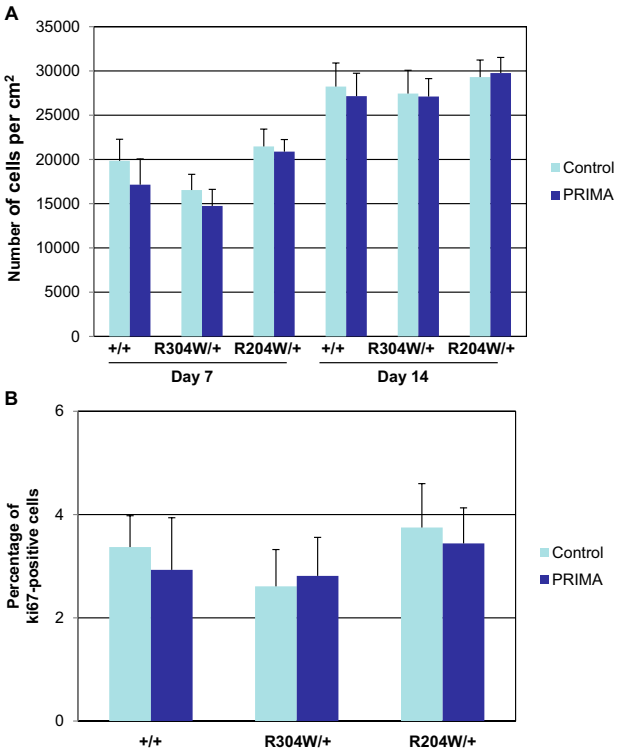

Fig. S5. APR-246 effect on proliferation during corneal epithelial differentiation of EEC-iPSC cells. The indicated iPSC lines were differentiated into corneal epithelial fate in the presence (PRIMA) or absence (Control) of APR-246 treatment, as detailed in *Materials and Methods*. Cells were at counted at day 7 and day 14 of differentiation. The averaged number of cells per square centimeter is shown in *A*. (*B*) Immunofluorescent staining of ki67 was performed at day 7 of differentiation followed by quantification of ki67^{+} cells. Data represents the percentage of ki67^{+} cells in the whole population (determined by DAPI staining). Five different fields were randomly pictured and counted for each sample. Results show average data obtained from two independent experiments ± SD.