APR-246/PRIMA-1^{MET} rescues epidermal differentiation in skin keratinocytes derived from EEC syndrome patients with *p63* mutations

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p53 and p63 share extensive sequence and structure homology. *p53* is frequently mutated in cancer, whereas mutations in *p63* cause developmental disorders manifested in ectodermal dysplasia, limb defects, and orofacial clefting. We have established primary adult skin keratinocytes from ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome patients with *p63* mutations as an in vitro human model to study the disease mechanism in the skin of EEC patients. We show that these patient keratinocytes cultured either in submerged 2D cultures or in 3D skin equivalents have impaired epidermal differentiation and stratification. Treatment of these patient keratinocytes with the mutant p53-targeting compound APR-246/PRIMA-1^{MET} (p53 reactivation and induction of massive apoptosis) that has been successfully tested in a phase I/II clinical trial in cancer patients partially but consistently rescued morphological features and gene expression during epidermal stratification in both 2D and 3D models. This rescue coincides with restoration of p63 target-gene expression. Our data show that EEC patient keratinocytes with p63 mutations can be used for characterization of the abnormal molecular circuitry in patient skin and may open possibilities for the design of novel pharmacological treatment strategies for patients with mutant *p63*-associated developmental abnormalities.

The p53 family of transcription factors p53, p63, and p73 share high sequence and structure homology, especially in their DNA-binding domains. p53 plays a key role in tumor suppression and is mutated in around 50% of human tumors (1), whereas p63 is a master regulator during ectodermal and epidermal development (2, 3). p63 is expressed as several isoforms with different N-terminal ends, the TA and ΔN isoforms, and different C-terminal ends, designated α, β, γ, δ, and ε (4). ΔNp63α is the major isoform expressed in the epidermis (5).

The key role of p63 in ectodermal and epidermal development has been demonstrated in animal models as well as in human developmental disorders. Disruption of *p63* in mice and zebrafish causes severe epidermal and limb defects (2, 3, 6). In humans, heterozygous mutations in *p63* give rise to several clinical conditions with autosomal dominant inheritance (7). The three major characteristics of these developmental disorders are limb defects, orofacial clefting, and ectodermal dysplasia, which are comprised of defects in skin, hair, teeth, nails, and several exocrine glands. Among these disorders, the ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome displays all three major hallmarks. *p63* mutations associated with the EEC syndrome are point mutations in the DNA-binding domain affecting all isoforms. Some of the amino acid residues mutated in the EEC syndrome correspond to hotspot tumor-associated mutant residues in *p53*; p63 R204 corresponds to p53 R175H, p63 R304 to p53 R273, and p63 R279 to p53 R248 (8). Another p63-associated disorder is the ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome, which differs from the EEC syndrome mainly by the severity of the skin phenotype, the occurrence of an eyelid fusion at birth, and the absence of limb malformations. Mutations associated with the AEC syndrome are localized in the C-terminal SAM domain, which is absent in the p53 protein (7, 9).

The cellular mechanisms of epidermal differentiation and stratification controlled by p63 have mainly been studied in mouse models, including *p63*-deficient mice with or without rescue by p63 isoforms, and in mouse ES cells (10–12). However, these studies are not able to illustrate the disease mechanism underlying the phenotypes caused by heterozygous *p63* point mutations in humans (4). Two knock-in models of p63 mutations have been reported so far, L514F for the AEC syndrome (13) and R279H for the EEC syndrome (14). The L514F mice fully recapitulate the human AEC syndrome with abnormalities in skin and craniofacial structures. In the R279H knock-in model, limb phenotypes resembling that of p63 knock-out mice have been observed, whereas clear skin phenotypes have not been reported. Furthermore, there is a clear clinical variability among EEC syndrome patients with p63 mutations (15), which suggests that individual genetic background is one of the important determinants for the phenotypes. Therefore, mouse models established so far seem not be suitable to uncover the molecular circuitry in the skin that is affected in the EEC syndrome and to perform drug screening.

The current therapy for disorders caused by *p63* mutations is limited to cosmetic surgery. Opportunities for improved treatment may come from studies of the family member p53. Because of the important tumor-suppressor function of p53 (16, 17), novel strategies for targeting p53 for cancer therapy have been intensively pursued. Several compounds have been reported to restore wild-type function to mutant p53 and induce p53-dependent cell death (18). Previously, we found that the low molecular weight compounds PRIMA-1 and APR-246/PRIMA-1^{MET} (henceforth referred to as APR-246) restore conformation and tumor suppressor

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Conflict of interest statement: K.G.W. and V.J.N.B. are cofounders and shareholders of Aprea AB, a company that develops p53-based cancer therapy, including APR-246. K.G.W. is a member of its board.

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activity to mutant p53 (19, 20). APR-246 has recently been tested in a phase I/II clinical trial in patients with hematological malignancies or hormone-refractory prostate cancer (21). Interestingly, we found that APR-246 can restore the proapoptotic function to mutant TAp63γ in tumor cells (22). This finding suggests that APR-246–mediated rescue of mutants of the p53 family members involves a conserved molecular mechanism because of the extensive structural homology in the DNA-binding domains among these proteins. Therefore, APR-246 might be suitable as a lead compound to initiate development of new drugs targeting developmental and differentiation defects caused by p63 mutations, especially those located in the DNA-binding domain, such as mutations involved in the EEC syndrome.

Here we established an in vitro model to study the disease mechanism of the EEC skin phenotype, namely keratinocytes from EEC patients with p63 mutations. These keratinocytes showed differentiation defects that were consistently rescued upon APR-246 treatment.

Results

Deregulated Transcription Program in Keratinocytes from EEC Syndrome Patients with *p63* Mutations During Early Epidermal Differentiation. To study the epidermal defects in EEC syndrome patients with *p63* mutations, we established keratinocyte cultures from the epidermis of patients carrying *p63* mutations that are located in the DNA-binding domain of p63, R204W, R279H, and R304W (henceforth referred to as R204W, R279H, and R304W keratinocytes). Expression-array analysis of these EEC patient keratinocytes grown in submerged 2D cultures was performed at early differentiation stage (day 2 after initiation of differentiation) to detect deregulated gene expression caused by *p63* mutations, compared with keratinocytes from five normal controls with wild-type *p63*. As shown by exon array analysis, exons 3′ to 14 but not exons 1–3 and 15 were expressed at a comparable level above the detection threshold, demonstrating that the ΔNp63α is the only isoform expressed at a high level in primary keratinocytes (Fig. S1*C*). The low expression, if any, of other isoforms was also confirmed by RNA-seq data from the ENCODE consortium (Fig. S1 *A* and *B*) (23), as well as by our RNA polymerase II (RNAPII) ChIP followed by quantitative PCR (qPCR) analysis (ChIP-qPCR) at the *p63* TA- and ΔN promoters (Fig. S1*D*). Hierarchical clustering analysis showed that gene expression patterns in the five normal controls were relatively similar to each other, compared with those of keratinocytes with *p63* mutant R204W, R304W, or R279H (Fig. S1*E*). This finding indicates that the effect of *p63* mutations on the transcription program is more substantial than the influence of different genetic backgrounds. To identify aberrantly regulated genes common to the EEC syndrome and to minimize the effect of the genetic background of each individual patient, we analyzed expression profiles in keratinocytes from three EEC patients (Fig. 1*A*). A total number of 328 genes (66 and 89 genes of which were up- and down-regulated more than 1.5-fold, respectively) showed significant and consistent changes in expression in all three mutant cells, henceforth referred to as differentially expressed genes (Fig. 1 *A* and *B* and Dataset S1). The expression pattern in R279H cells was significantly different from those in R204W and R304W cells (Fig. 1*A* and Fig. S1*E*).

We searched for p63 binding sites in the vicinity of the 328 differentially expressed genes using our previously reported p63 binding profiles (24) and identified 208 potential p63 target genes (60%) that have p63 binding sites (BS) within the gene itself or in a region within 25 kb of the gene body (Fig. 1*B* and Dataset S1). Analysis using the DAVID bioinformatics tool (25) revealed that specific Gene Ontology (GO) terms, such as epithelium, tissue, organ development, and neurogenesis, were the most significant for the potential direct target genes (Dataset S2). With independent RT-qPCR analysis in all three patients, 16 of 20 genes were validated (Table S1). One of these genes, *Alcohol dehydrogenase 7* (*ADH7*), encodes subclass IV alcohol hydrogenase 7 subunits and possibly plays a role in retinol

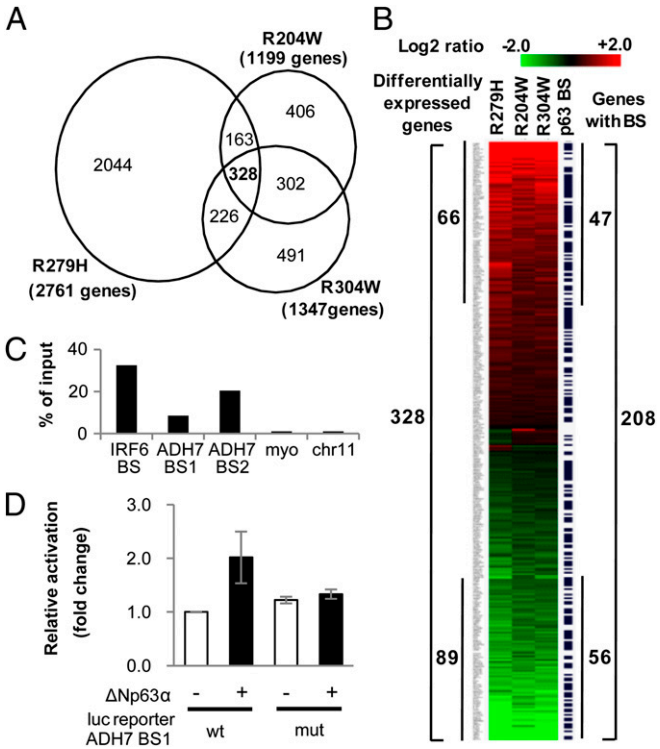

Fig. 1. Expression analysis of EEC patient keratinocytes carrying *p63* mutations identified *ADH7* as one of the potential p63 target genes. (*A*) Overlap of differentially expressed genes in three EEC patient keratinocyte cultures compared with the controls (ANOVA $P < 0.05$). (*B*) The expression pattern of 328 differentially regulated genes shown by heatmap. p63 binding sites represented in black bars are shown to the right of the heatmap. (*C*) Two binding sites near *ADH7* (BS1, BS2) (24) were validated using ChIP-qPCR. A p63 binding site near IRF6 was used as the positive control and myoglobin exon 2 and a no gene region on chromosome 11 as negative controls. (*D*) Transactivation by ΔNp63α through the wild-type ADH7 BS1 and BS1 with mutated p63 binding motif.

oxidation in the retinoic acid biosynthesis pathway. The expression of *ADH7* was almost completely lost in keratinocytes with mutations in the *p63* DNA-binding domain associated with the EEC syndrome (Table S1). Two p63 binding sites (BS1 and BS2) were found near the transcription start site of *ADH7* (see, for example, Fig. S4*A*) (24), and p63 binding at these regions was confirmed by ChIP-qPCR analyses (Fig. 1*C*). Transient transfection assays showed that the ADH7 BS1 was able to drive gene expression in a p63-dependent manner, and this activation was completely abolished by mutations in the p63 binding motif present in ADH7 BS1 (Fig. 1*D*; see also Fig.S4*A*). Taken together, these data suggest that *ADH7* is a p63 target gene.

Morphological Defects in Keratinocytes Carrying Mutations in the p63 DNA-Binding Domain Are Rescued by APR-246. Having detected deregulated gene expression in p63 mutant keratinocytes during early epidermal differentiation, we investigated whether these cells exhibit differentiation defects during epidermal stratification. Because of the different gene expression pattern in R279H keratinocytes compared with those in R204W and R304W keratinocytes (Fig. 1*A* and Fig. S1*E*), we decided to only use R304W and R204W keratinocytes. Under proliferative conditions, the morphology of R204W and R304W keratinocytes in submerged 2D cultures was less homogenous and cells were larger and flatter (Fig. S2 *B*, *a* and *d*) compared with the wild-type p63 cells (Fig. S2 *B*, *g*). On days 4 and 7 after differentiation, wild-type p63 cells started to form multilayer structures (Fig. S2 *B*, *h* and *i*), whereas many R204W and R304W keratinocytes were detached

from the surface of the culture dish, and the remaining cells retained as a single-layer with largely unchanged morphology (Fig. S2 *B*, *b* and *e*). This difference in morphology suggested differentiation defects in the R204W and R304W cells.

Next, we investigated the possibility of pharmacological rescue of the observed differentiation defects. We hypothesized that the compound APR-246, which has been shown to target mutant p53 with amino acid substitutions in the DNA-binding domain and restore p53 tumor suppressor activity (19, 20), would be a candidate compound for restoration of differentiation defects caused by corresponding mutations in the p63 DNA-binding domain. Thus, we treated keratinocytes carrying R204W and R304W mutations with 10–30 μM of APR-246 before initiating differentiation (Fig. S2*A*). On day 7 after initiation of differentiation, most of the APR-246–treated R204W and R304W keratinocytes remained attached to the surface of culture dish. Some cells changed to a flattened elongated morphology, started to establish cell-cell contacts, and formed multilayer structures (Fig. S2 *B*, *c* and *f*), resembling those seen in normally differentiating wild-type p63 keratinocytes (Fig. S2 *B*, *i*). Thus, these morphological changes in APR-246-treated keratinocytes suggest that APR-246 can at least partially restore differentiation in p63 mutant keratinocytes.

Restoration of Expression of Stratification Markers in p63 Mutant Keratinocytes by APR-246. To investigate whether APR-246 can restore differentiation in p63 mutant keratinocytes at the molecular level, we performed immunofluorescence staining of differentiation markers in 2D cultures. Terminal differentiation markers, such as involucrin and transglutaminase (TGM), were lowly expressed in mutant p63 R204W keratinocytes, and their expression was substantially enhanced upon APR-246 treatment, as assessed by immunofluorescence staining (Fig. 2*A*) and Western blotting (Fig. 2*C*). Suprabasal epidermal markers, such as Keratin 1 (K1) and Keratin 10 (K10), were expressed at low levels in untreated R304W keratinocytes and also induced on differentiation day 7 after APR-246 treatment (Fig. 2*B*). Of note, p63 expression in wild-type keratinocytes was reduced during differentiation, which is consistent with previous findings (2, 26). Treatment with APR-246 did not seem to influence p63 expression levels or to induce the TAp63 isoform in the mutant p63 keratinocytes (Fig. 2*C* and Fig. S1*D*). Quantitative analysis of protein expression in R304W cells by flow cytometry showed that expression of K1 was induced by APR-246 to an almost complete recovery compared with wild-type p63 cells, and that expression of K10 and TGM was also substantially rescued upon treatment with APR-246 (Fig. S3*G*, *Left*).

Restoration of differentiation marker-gene expression was also confirmed at the mRNA level. In wild-type cells, expression of all tested differentiation markers including K10 and Cystatin M/E (CysME, an inhibitor of asparaginyl endopeptidase required for envelope maturation) (27) were not significantly induced upon APR-246 treatment (Fig. S3*A*). In contrast, induction of K10 and CysME expression in R304W cells was consistent and statistically significant (Fig. S3 *A* and *C*). Expression of some differentiation markers including K10 and CysME was also increased in APR-246–treated R204W keratinocytes compared with untreated cells, but the effect was less pronounced than in R304W cells (Fig. S3*D*). When the total level of detected mRNA during differentiation was compared, the expression of K1 and K10 in APR-246–treated R304W cells almost reached the levels observed in the differentiating wild-type p63 keratinocytes (Fig. S3*G*, *Right*), whereas the rescue in R204 was less substantial (Fig. S3*G*, *Center*). Interestingly, the expression of differentiation markers was also slightly enhanced in an AEC keratinocyte cell line carrying the T533P mutation upon APR-246 treatment, to similar levels as in R204W cells (Fig. S3*E*).

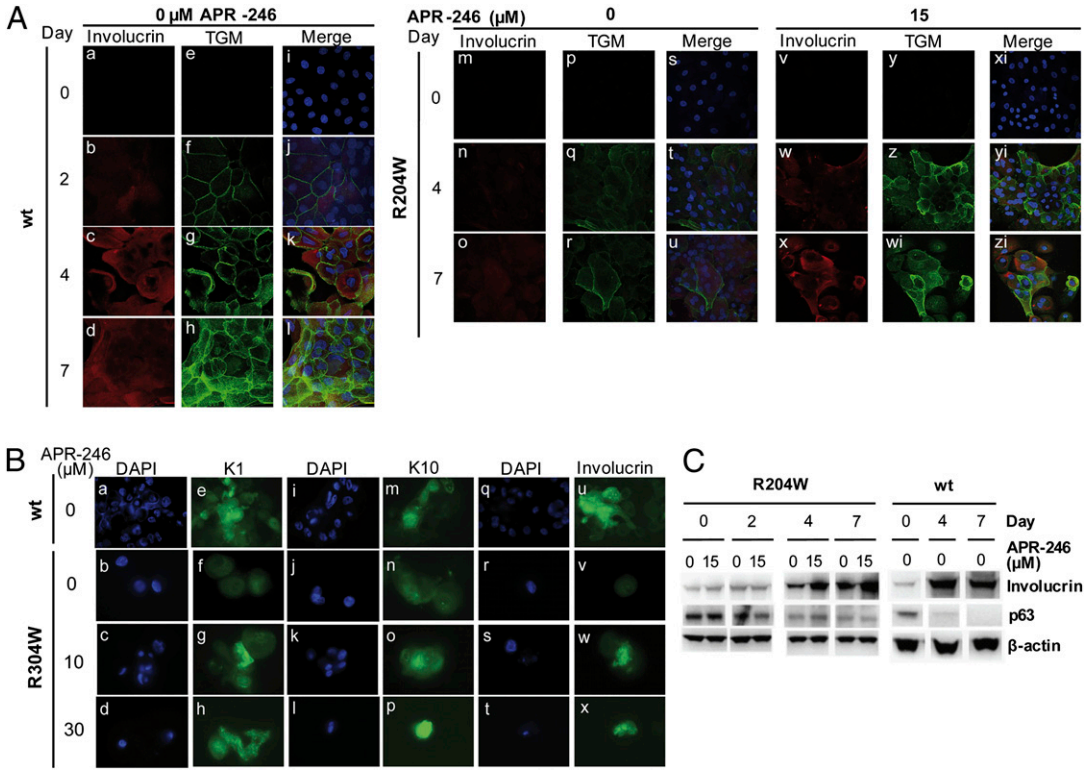

Fig. 2. Differentiation marker expression in APR-246–treated EEC patient keratinocytes. Control, R204W (*A*) and R304W (*B*) keratinocytes treated with APR-246 were stained for differentiation markers. (Magnification: 40×.) R304W keratinocytes were collected on differentiation day 7. Nuclei were stained with DAPI in blue. (*C*) Protein lysates of control and R204W keratinocytes treated with APR-246 were analyzed by Western blotting for Involucrin, p63, and β-actin (loading control).

APR-246 Treatment Enhances Transcription of p63 Target Genes. To understand the mechanism behind the rescue of mutant p63 by APR-246, we examined the effect of APR-246 on *ADH7*, a p63 target gene identified in our expression array analysis. *ADH7* expression was undetectable at the proliferation stage and strongly induced when differentiation was initiated and this was almost completely lost in R204W and R304W keratinocytes (Fig. 3*A*). Restoration of *ADH7* expression was readily detected in APR-246–treated R304W keratinocytes, approximately fivefold, and reached statistical significance at day 4 ($P = 0.02781$), whereas the slight induction of *ADH7* expression in APR-246–treated R204W keratinocytes was not statistically significant (Fig. 3*A*). Rescue of expression was also observed for some other potential target genes identified in our expression array study, such as glutathione peroxidase 2 (GPX2) (Fig. S3*B*). To obtain an overview of APR-246 effects on gene expression, we assessed the total mRNA synthesis (or accumulation) of all of the tested genes over time. Expression of many tested genes, including the unique target gene *ADH7*, *GPX2*, and differentiation markers, was partially rescued by APR-246 in a dose-dependent manner but to variable degrees (Fig. S3*F*, *Top* and *Middle*). We also examined Claudin-1 protein expression in R204W keratinocytes with or without APR-246 treatment. Claudin-1 was previously reported as a direct target gene of p63 and is involved in epidermal differentiation (28). Expression of the Claudin-1 protein was enhanced in the APR-246–treated R204W cells after differentiation (Fig. 3*B* and Fig. S3*H*). The overall rescue of all tested genes at the mRNA (Fig. 3*C*, *Left*) and the protein level (quantitated by flow-cytometry analysis) (Fig. 3*C*, *Right*) were calculated and summarized in the box-whisker plot. The gene expression in APR-246–treated R304W keratinocytes was clearly induced, and more pronounced than in R204W cells (Fig. 3*C*). Consistent with the slightly enhanced expression of differentiation markers in AEC T533P keratinocytes (Fig. S3*E*), we also observed an induction of ADH7 expression in these cells (Fig. S3*F*, *Lower*).

To further investigate the effect of APR-246 on p63 target genes, we tested transcription driven by either wild-type or mutant p63 through ADH7 BS1 in transient transfection assays. Wild-type p63 but not p63 mutants R204W, R279H, R304W, and T533P activated transcription via ADH7 BS1 (Fig. S4*B*, *Left*). We observed a slight increase of transcription upon APR-246 treatment in mock or wild-type p63-transfected cells. Interestingly, however, APR-246 induced more pronounced enhancement of transcription at ADH7 BS1 in cells transfected with p63 mutants R204W, R279H, R304W, and T533P and the enhancement of treatment with 30 μM APR-246 was statistically significant ($P = 0.03$) (Fig. S4*B*, *Right*). This finding is consistent with the ability of APR-246 to induce expression of the EGFP reporter driven by a p53 consensus motif in the presence of mutant p63 (Fig. S4*E*). These findings indicate that treatment with APR-246 has a stronger effect on p63 mutants than on wild-type p63. To assess whether APR-246 enhances DNA binding of mutant p63 to its target sequences, we performed ChIP-qPCR analysis in SAOS2 cells stably transfected with R304W mutant p63. In two independent SAOS2 R304W cell lines, we observed a moderate increase of DNA binding at both ADH7 binding sites at high concentrations of APR-246 (Fig. S4*C*). This enhanced DNA binding induced by APR-246 was corroborated by the data from an in vitro DNA-binding assay (TransAM) based on a synthetic p53/p63 consensus sequence (Fig. S4*D*).

APR-246 Improves Stratification in 3D Human Skin Equivalents from p63 Mutant Keratinocytes. The partial but consistent APR-246–mediated rescue of keratinocyte differentiation in submerged 2D culture prompted us to test the effect of APR-246 on differentiation/stratification in a 3D human skin model. Compared with human skin equivalents established from wild-type p63 cells (Fig. 4 *A*, *a* and *d*), those established from R204W keratinocytes showed a thin and poorly stratified epidermis that lacked a granular layer and showed parakeratosis in the cornified layer

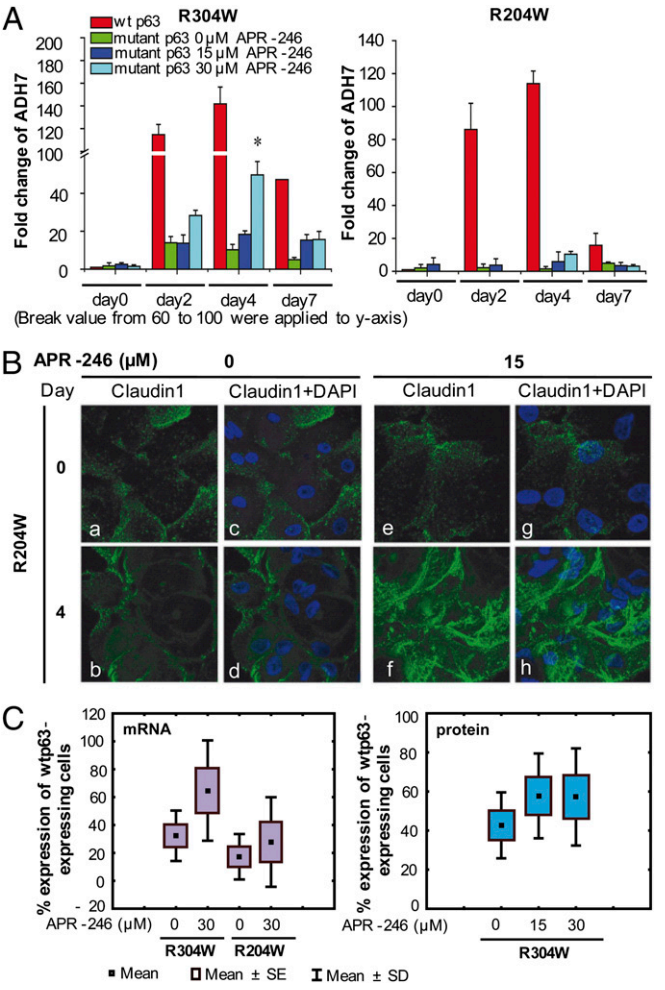

Fig. 3. Expression of p63 target genes upon APR-246 treatment. (*A*) RT-qPCR analysis of *ADH7* expression at different stages of differentiation in R304W and R204W keratinocytes treated with APR-246. (*B*) APR-246–treated R204W keratinocytes were stained for Claudin-1 (green). (Magnification: 63×.) Nuclei were stained with DAPI (blue). (*C*) A summary of induction of the mRNA (RT-qPCR, *Left*) and proteins (flow cytometry, *Right*) of all tested genes (GPX2, ADH7, K10, CysMe, involucrin, and K1 for mRNA and K10, TGM, K1, Claudin-1, and involucrin for protein) over 7-d differentiation is shown in the box-whisker plot. Expression levels of mRNA or protein in wild-type p63 cells were set as 100%. (*Left*) The paired *t* test between untreated cells and APR-246-treated cells shows that the rescue reached statistical significance in R304W keratinocytes ($P = 0.025$) but not in R204W keratinocytes ($P = 0.22$). (*Right*) The paired *t* test showed significant rescue in R304W cells treated with 15μM APR-246 compared with untreated cells ($P = 0.0796$, according to the Wilcoxon matched-pairs test).

(Fig. 4 *A*, *b* and *e*). Treatment of R204W human skin equivalents with 10 μM APR-246 during the air–liquid interface markedly improved epidermal morphology to a multilayered structure with a visible stratum spinosum. The stratum granulosum was still absent but the stratum corneum appeared more continuous (Fig. 4 *A*, *c* and *f*).

To investigate whether differentiation was rescued in these skin equivalents, a panel of differentiation markers were tested by immunostaining. Expression of the epithelial marker Keratin 14 (K14) and proliferation marker Ki67 was not substantially affected in the basal layer of the R204W and R304W skin equivalents (see Fig. S5 *C* and *E* for K14 and Fig. S5 *Yi* for Ki67) compared with that of the wild-type cells (see Fig. S5*A* for K14 and Fig. S5 *Wi* for Ki67) and also not affected by APR-246 (Fig. S5 *B*, *D*, and *F* for K14, and Fig. S5 *Xi* and *Zi* for Ki67).

Expression of both early (K10) and late [Involucrin, CysME, Late Cornified Envelope protein (LCE2) and Filaggrin] differentiation markers in R204W (Fig. 4 *B*, *b*, *g*, and *l*, and Fig. S5 *I*, *O*, *U*, and *Si*), and R304W (Fig. 4 *B*, *d*, *i*, and *n*, and Fig. S5 *K*, *Q*, *W*, and *Ui*) skin equivalents appeared irregular and discontinious compared with wild-type human skin equivalents (Fig. 4 *B*, *a*, *f*, and *k*, and Fig. S5 *G*, *M*, *S*, and *Y*). APR-246 treatment in R204W (Fig. 4 *B*, *c*, *h*, and *m*, and Fig. S5 *J*, *P*, *V*, and *Ti*) and R304W (Fig. 4 *B*, *e*, *j*, and *o*, and Fig. S5 *L*, *R*, *X* and *Vi*) cells enhanced expression of these early- and late-differentiation markers. For K10, Involucrin, and CysME, APR-246 treatment resulted in a continuous expression pattern resembling that of wild-type epidermis, and for LCE2 and Filaggrin, the rescue was less pronounced. APR-246 treatment did not seem to affect expression of tested markers in skin equivalents from the wild-type keratinocytes (Fig. S5). Furthermore, protein expression of the unique p63 target gene *ADH7* was visible in wild-type skin equivalents (Fig. 4 *A*, *g*) but almost undetectable in R204W skin equivalents (Fig. 4 *A*, *h*). Treatment with APR-246, however, resulted in enhanced ADH7 expression in R204W skin equivalents (Fig. 4 *A*, *i*).

Taken together, our results show that expression of both differentiation markers and p63 target genes was partially but consistently restored by APR-246 treatment.

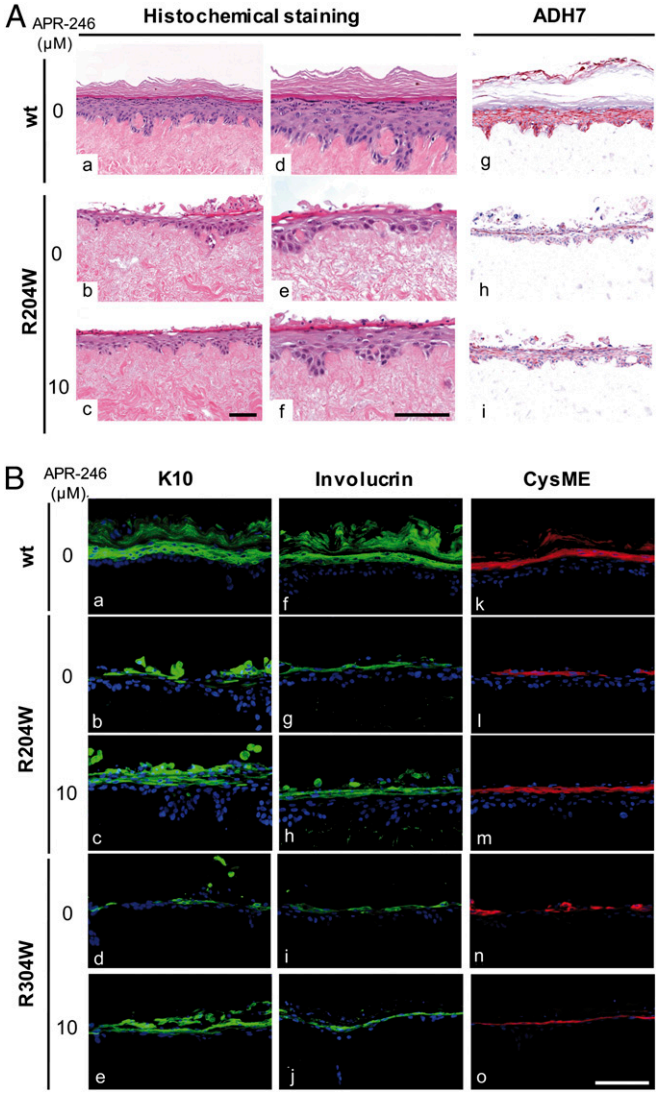

Fig. 4. Differentiation marker expression in APR-246–treated 3D human skin equivalents. (*A*) Human skin equivalents generated from p63 wild-type and R204W keratinocytes treated with APR-246 at the air–liquid interface culture were stained with H&E and an ADH7 antibody. (*B*) Proteins expression of K10, Involucrin, and CysME was examined by immunofluorescence staining of skin equivalents generated from wild-type keratinocytes and R204W and R304W keratinocytes, which were treated with APR-246. Nuclei were stained with DAPI (blue). (Scale bars, 50 μM.)

Discussion

Here we have established an in vitro model, primary skin keratinocytes from EEC patients with *p63* mutations in 2D submerged cultures and in 3D human skin equivalents. Using this unique model, we identified distinct clusters of potential direct target genes of p63, and tested our hypothesis that the differentiation defects caused by *p63* mutations may be rescued by the mutant p53-targeting compound APR-246.

The DNA-binding domains of p53 and p63 share extensive structural homology (2), and amino acid residues in p63 that correspond to tumor-associated hotspot mutations in p53 are often mutated in human developmental disorders, such as EEC (29). Various strategies have been developed to target mutant p53 for cancer treatment. Small molecules that bind to mutant p53 and restore wild-type function have been identified (30). In the present study, we asked whether one such compound, APR-246, could reactivate endogenous mutant p63 with amino acid substitutions in the DNA-binding domain in keratinocytes derived from patients with the EEC syndrome. Indeed, treatment with APR-246 resulted in significant rescue of morphology and gene expression in R304W and R204W keratinocytes during epidermal differentiation. We recently showed that APR-246 can restore DNA binding, target gene expression, and proapoptotic activity to mutant isoforms of TAp63γ in human tumor cells (22). As different isoforms of p63 may regulate distinct target genes, it can be envisaged that APR-246–mediated restoration of wild-type function to different isoforms of mutant p63 expressed in various cell types may result in distinct biological consequences. Our finding that a potential unique anticancer drug against mutant p53 can be used to target mutations in another p53 family member in a developmental disease raises the interesting possibility of developing targeted therapy for phenotypically distinct diseases that are caused by a similar underlying molecular mechanism.

We examined expression of differentiation markers in different layers during stratification, including K1 and K10 as suprabasal epithelial markers, involucrin as a late-stage marker in the spinous layer, and TGM, CysME, LCE2, and Filaggrin as markers in the granular layer and in the terminally differentiated cells of the skin. The APR-246–mediated induction of expression of these markers in specific stratified epidermal layers in patient keratinocytes suggests that restoration of normal function to mutant p63 can rescue not only differentiation initiation but also differentiation at terminal stages.

We found that the p63 R304W mutant is more amenable to reactivation by APR-246 than the R204W mutant. This observation is in agreement with our previous study of APR-246–mediated rescue of proapoptotic activity in human tumor cell lines (22). The difference in the response to APR-246 might be a result of the molecular nature of the two mutations. R304 in p63 corresponds to R273 in p53, a residue that makes direct contact with DNA. R273H mutant p53 retains wild-type conformation to a large extent (31). R204 in p63 corresponds to p53 R175 that is important for the structural integrity of the DNA-binding domain. The p53 R175H mutant shows severe structural defects. The structural consequences of the R204W mutation in p63 are presumably similar to those of p53 R175H mutation, and extensive unfolding may make APR-246–mediated rescue less efficient. However, we cannot exclude that the observed differences are because of interindividual variation in the response to APR-246 that is unrelated to p63. Intriguingly, we also observed restoration of gene expression and DNA binding mediated by APR-246 for a *p63* mutation that is localized in the C-terminal SAM domain and associated with the AEC syndrome (Figs. S3 *E*

and *G* and S4*D*). This domain is not conserved in the p53 protein, and therefore the effect of APR-246 on this type of mutations has not been investigated so far. Previous studies suggest that the AEC mutations may affect the conformation of the p63 protein or protein–protein interactions of p63 with other cofactors to affect transactivation (32). Therefore, the rescue effect of APR-246 on these mutants may be because of stabilization of the overall protein conformation of p63 or by modulating critical protein–protein interactions.

Based on our expression analysis, we identified a cluster of potential target genes, the expression of which was deregulated in p63 mutant keratinocytes. One of these genes is *ADH7*, a class IV alcohol hydrogenase that may be involved in the biosynthesis of retinoic acid that is important for cellular differentiation. The expression of *ADH7* during early differentiation was almost completely lost in *p63* mutant keratinocytes (Table S1), indicating that p63 normally activates *ADH7* and that mutations in the *p63* DNA-binding domain disrupt this regulation. Consistent with this notion, we found that wild-type p63 normally binds to specific sites near the *ADH7* promoter (Fig. 1*C*) and activates transcription (Fig. 1*D*), whereas mutations in the DNA-binding domain of p63 abolished the transactivation (Figs. 1*D* and 3*C*). Interestingly, another enzyme in the retinoic acid biosynthesis pathway, retinal short-chain dehydrogenase (retSDR1, also known as dehydrogenase/reductase member 3, DHRS3) has recently been reported as a p63 target gene and its expression was shown to be affected by EEC syndrome *p63* mutations (33). These data suggest that p63 plays a role in the retinoic acid biosynthesis pathway and that EEC syndrome mutations disrupt cellular differentiation mediated by retinoic acid during embryonic development. *ADH7* and *Claudin-1* are probably two among a number of p63 target genes that are up-regulated in APR-246–treated mutant p63 keratinocytes. Their up-regulation suggests that reactivation of p63-dependent transcription is the underlying mechanism of APR-246–mediated rescue. Thus, we demonstrate that APR-246 partially restores wild-type function to endogenous mutant p63 in a physiological context where p63 is normally expressed at high levels and essential for epidermal differentiation. It will be of interest to identify other p63 target genes that are up-regulated upon APR-246 treatment in p63 mutant keratinocytes in a genome-wide scale.

Our findings may open new opportunities for drug screening and development of therapy for patients with developmental disorders associated with *p63* mutations. This possibility is relevant not only to EEC but also to ectodermal dysplasia, as APR-246 can target mutations associated with AEC, even though these mutations are not localized within the DNA-binding domain. AEC syndrome patients often suffer from severe skin phenotypes (6). Further in vivo studies of APR-246–mediated rescue of mutant p63 at various embryonic stages will be of great interest for exploring such therapy options. Because the retinoic acid biosynthesis pathway is affected by *p63* EEC syndrome mutations, retinoic acid and related compounds should also be further investigated for their effect on epithelial differentiation affected by *p63* mutations. In conclusion, we have shown that the mutant p53-targeting compound APR-246 partially restores epidermal differentiation of primary adult keratinocytes derived from patients with *p63* mutations, probably through restoration of p63 target gene expression. Our study provides a unique concept for development of treatment for the p63-related diseases. The notion of applying targeted therapy for phenotypically distinct diseases caused by similar molecular mechanisms could also be relevant to treatment of other diseases.

Materials and Methods

Materials and methods and all primers used in the experiments can be found in *SI Materials and Methods* and Dataset S3. Skin biopsies were taken from healthy volunteers or EEC syndrome patients with p63 mutations to establish primary keratinocyte cultures. APR-246 was applied to keratinocytes during the proliferation stage (Fig. S2*A*).

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