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Embryonic stem cells as an ectodermal cellular model of human p63-related dysplasia syndromes

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ABSTRACT

Heterozygous mutations in the TP63 transcription factor underlie the molecular basis of several similar autosomal dominant ectodermal dysplasia (ED) syndromes. Here we provide a novel cellular model derived from embryonic stem (ES) cells that recapitulates *in vitro* the main steps of embryonic skin development. We show that ES cells carrying AEC or EEC mutations are unable to differentiate into the epidermal fate. Comparative transcriptome analysis strongly reveals an embryonic epidermal signature and suggests that mutations in the SAM domain (AEC) provide activating properties while mutations in the DBD domain (EEC) induce strong inhibitory capabilities. Our model uncovers the effect of relevant ED mutations that otherwise are difficult to evaluate on the ectodermal embryonic stage, an embryonic event critical for proper skin formation.

1. Introduction

The ectodermal dysplasia (ED) syndromes are a group of inherited human diseases. They are clinically characterized by the abnormal development or growth of tissues and structures that are developed from the embryonic ectoderm, affecting mainly the skin, hair, nails and teeth, and sweat glands and other glands [1,2]. The epidermis of patients can be very dry, itchy and sometimes large areas can be eroded. Different combinations of these features are observed in five ED syndromes linked to mutations in the transcription factor gene p63 [3]. The most common syndrome is defined by a combination of Ectrodactyly, Ectodermal dysplasia and Cleft lip/palate disorders (EEC). The second related syndrome is the Ankyloblepharon-Ectodermal dysplasia-Clefting (AEC) syndrome (or Hay-Wells syndrome) characterized by ectodermal dysplasia, generally not associated with ectrodactyly or other limb malformation. Most of the patients affected by AEC present patches of life-threatening congenital skin erosion. The Limb-Mammary Syndrome (LMS), Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT) and Rapp-Hodgkin Syndrome (RHS) are also p63-related ED syndromes. The disorders are congenital with a morbidity related to the absence or presence of eccrine and mucous glands while mortality is typically due to complications related to extensive skin erosions.

The phenotype of p63-knock out mice confirms a key role for p63 in skin development. Mice in which both alleles of the p63 gene have been deleted are born lacking limbs and fail to develop stratified epithelia and epithelial appendages, such as teeth, hair follicles and mammary glands [1,2]. Transgenic mice and embryonic stem cell model experiments strongly suggested that ΔNp63 is required for both embryonic ectodermal commitment [4,5] and epidermal stem cell/progenitors self renewal [6].

Interestingly, there is a striking genotype–phenotype correlation between the ED patient disabilities and the particular domain mutated on the p63 gene [7]. EEC syndrome results from missense mutations in the DNA-binding domain (DBD) of p63 [8,9]. AEC patients present a clustering mutations giving rise to amino acid substitutions in the Sterile Alpha Motif (SAM) domain [10]. These mutations are predicted to disrupt protein–protein interactions of the domain with as yet unknown partners. The mutations in ADULT, SHFM and LMS syndromes are distinct to the former syndromes. Thus, the pattern of mutations has suggested that the C-terminal SAM domain of p63 might be particularly important

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for skin development, whereas the DBD could be crucial for a wide range of ectodermal development.

The effects of ED mutations on cell growth, cell death and epidermal differentiation have been evaluated by different groups on adult epithelial cell lines [11–14]. However, even if a skin is formed in ED patients, it is well known that biological compensation mechanisms occur when developmental events are defective. The congenital ectodermal defect might influence the epidermal commitment, and thus the state of the resulting epidermal homeostasis. For that purpose, we have designed a cellular model from embryonic stem (ES) cells that recapitulate *in vitro* the main steps of skin development [15]. By serial dilutions of the committed ES cells, an ectodermal cell line that represents the epidermal progenitors has been isolated. It further allowed us to demonstrate that addition of ΔNp63 was sufficient to convert ectodermal progenitors into epidermal cells [16]. Here, we compared the ability of wild type p63 and ED-related mutants to induce epidermal commitment of ES-derived ectodermal cells and compared their respective transcriptome profiles.

2. Materials and methods

2.1. Cell lines and cell sorting

The ectodermal cell line isolated from the mouse CGR8 embryonic stem (ES) cell line used in this study and its culture condition have been described previously [16]. Transfection of the ES-derived ectodermal cell line was done with Lipofectamine 2000 (Invitrogen).

2.2. Real-time quantitative RT-PCR

RNAs extraction and quantitative RT-PCR analysis was performed as previously described [16]. Each gene was amplified using the appropriate specific primers:

36B4:	5′-TCCAGGCTTTGGGCATCA-3′	5′-CTTTATCAGCTGCACATCACTCAGA-3′
BMP-7:	5′-GGAAAAATGTCTGCCAGGAA-3′	5′-AGGCTTGCGATTACTCCTCA-3′
FGRF2b:	5′-CACCAACTGCACCAATGAAC-3′	5′-TGCTTGAATGTGGGTCTCTG-3′
GATA3:	5′-CCGAAACCGGAAGATGTCTA-3′	5′-GTTGAAGGAGCTGCTCTTGG-3′
Kl4:	5′-AAGGTCATGGATGTGCACGAT-3′	5′-CAGCATGTAGCAGCTTTAGTTCTTG-3′
K5:	5′-CCTGCAGAAGGCCAAGCA-3′	5′-TGGTGTTCATGAGCTCCTGGTA-3′
Kl8:	5′-AATCGAGGCACTCAAGGAAGAA-3′	5′-GGCATCCACTTCCACAGTCA-3′
Notchl:	5′-GCAATCTCAAGTCTGCCACA-3′	5′-GCTTCCTTGCTACCACAAGC-3′
Perp:	5′-GGATGGGAGGATGGACTAGG-3′	5′-GTGACAGTGGTGACCCTCCT-3′
Slug/snai2:	5′-GCTCCTTCCTGGTCAAGAAACAT-3′	5′-CCGAGGTGAGGATCTCTGGTT-3′
JARIDla:	5′-ATGTCGGAGGAAGAGGTGTTT-3′	5′-GTGATTGAAGTTGGCGGATAA-3′
LMO4:	5′-TCACTTGCAGGAATCGACTG-3′	5′-GGACCGCTTTCTGCTCTATG-3′

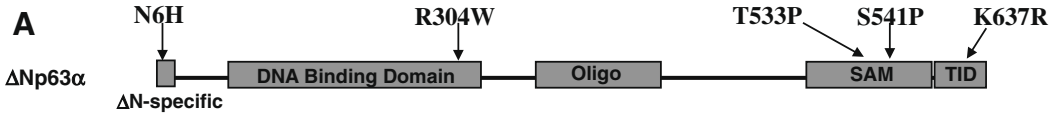
2.3. Agilent microarray

ES-derived ectodermal cells were transfected with wild type or mutant p63 constructs along with a construct expressing the fluorescent EGFP gene under the epidermal specific K5 promoter. The resulting fluorescent K5/K14+ keratinocytes were sorted using FACS Aria (Becton Dickinson). Total RNA was extracted by the Trizol (Invitrogen) method and purified on RNeasy columns (Qiagen, Hilden, Germany). RNA concentration was measured by spectrophotometry and its integrity checked using an Agilent Bioanalyzer instrument. One microgram total RNA was amplified and labeled with Cy3 and Cy5 fluorochromes using the Amino Allyl MessageAmp aRNA kit according to the manufacturer's (Ambion, Austin, TX) protocol. Cy3- and Cy5-labeled cRNAs were fragmented using fragmentation buffer (Agilent Technologies, Palo Alto, CA), dissolved in hybridization buffer (Agilent), and hybridized to pangenomic mouse microarrays of the RNG/MRC resource, as already described [17]. These microarrays harbor 24,109 50mer oligonucleotides. Cy3- and Cy5-labeled cRNAs dissolved in hybridization buffer were hybridized on previously processed slides (treated with 50 mM ethanolamine in 50 mM borate buffer, pH 9.0 at 20 °C for 1 h) in a total volume of 500 µl using the Microarray Hybridization Chamber (Agilent) at 62 °C for 17 h using a four rpm agitation. Slides were washed with washing buffer #1 (6× SSC, 0.005% Triton X-102) at 20 °C for 10 min and with washing buffer #2 (0.1× SSC, 0.005% Triton X-102) at 4 °C for 5 min, dried, and stocked under vacuum. Fluorescence data were acquired from slides using a GenePix 4000B instrument (Molecular Devices Corp., Union City, CA). TIF images containing the data from each fluorescence channel were quantified with the GenePix Pro 6.0 program (Axon Instruments, Union City, CA). Analysis was performed using the Significance Analysis of Microarrays (SAM) method on MeV 4.0 after percentage cutoff filtering (= 100%). Gene expression profiles were analyzed by two-color microarrays in CGR8 cells transfected with each p63 mutated construct and compared with cells transfected with the p63 wild type control in two replicate experiments at 48 h. For each experimental point, two technical replicates (dye-swaps) were examined.

3. Results and discussion

We generated expression vectors coding for the wild type p63 isoform in which mutations found in EEC (R304W), AEC (T533P and S541P), SHFM (K637R) and ADULT (N6H) patients have been introduced. ES-derived ectodermal cells were co-transfected with wild type ΔNp63 and disease-related p63 constructs and epidermal commitment was illustrated by K5/K14-positive cell appearance (Fig. 1A). While exogenous expression of ΔNp63, mutants N6H (ADULT) and K637R (SHFM) were able to induce keratinocyte differentiation, the mutants S541P (AEC) and R304W (EEC) failed to produce K14-positive keratinocytes (Fig. 1A).

Exogenous expression of a gene does not reflect fully the adult ED patient skin in which one allele is mutated and the second one is normal. Therefore, we tested ES cells in which an AEC mutation (L514F) has been knocked-in into the endogenous Trp63 gene. It allows the equimolar production of one wild type and one mutated allele, as happens in ED patients. When tested for epidermal commitment, the mutant cells were unable to differentiate into the epidermal fate, as compared to control (Fig. 1B). This inability to induce epidermal commitment prompted us to generate a comparative transcriptome analysis of ES-derived ectodermal cells



P63 constructs	Epidermal commitment (%)	Location of the mutation
ΔNp63α	21	
N6H	4.5	ΔN specific
K637R	5.6	TID
R304W	0	DBD
T533P	0	SAM
S541P	0	SAM

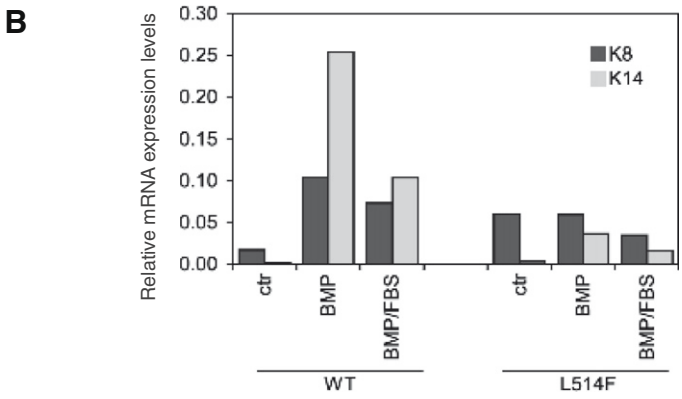
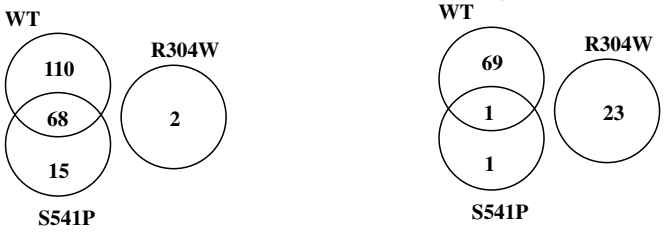


Fig. 1. Epidermal commitment of wild type and mutant cells. (A) Wild type and p63 constructs in which an ED-related point mutation have been introduced were transfected into ES-derived ectodermal cells. Epidermal commitment was monitored by keratinocyte marker appearance and illustrated by percentage of K5/K14-positive cells by FACS analysis. (B) Real-time RT-PCR analysis of K14 and K8 gene expression during epidermal commitment of wild type (WT) and AEC (L514F) ES cells. Values are expressed as Arbp-normalized mRNA levels. Cells were treated with 0.5 nm of BMP-4 from day 3 to 5 and FBS 2.5% from day 9 to 14 (BMP/FBS), of left untreated (ctr). Samples were collected at day 14. Similar results were obtained with an independent L514F ES clone.

A

p63 Isoform	Up-regulated(*)	Down-regulated (*)
WT	178	69
S541P	83	2
R304W	2	23

(*) data obtained on biological triplicates



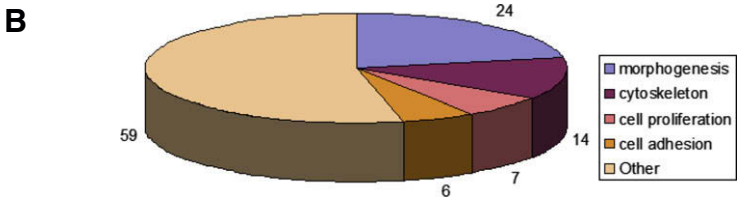


Fig. 2. Transcriptional profiling during epidermal commitment. (A) Results of transcriptome analysis 48 h post transfection. The numbers correspond to genes which expressions are modulated by at least 1.7-fold in biological triplicates with a robust p value ranging from 10^{-4} to 10^{-11}, with most of the modulated genes at 10^{-6}. Venn diagrams for genes whose expression was significantly altered in S541P and in R304W mutants as compared to wild type p63 construct. (Left) Overlapped up-regulated genes, and (right), overlapped down-regulated genes. (B) Five major biological processes that characterize the group of regulated and annotated genes by wild type p63.

expressing either wild type or mutant S541P and R304W ΔNp63 constructs. As illustrated in Fig. 2A, exogenous expression of wild type ΔNp63 modified the relative expression of at least 248 genes (178 up-regulated and 69 down-regulated). Ectopic expression of

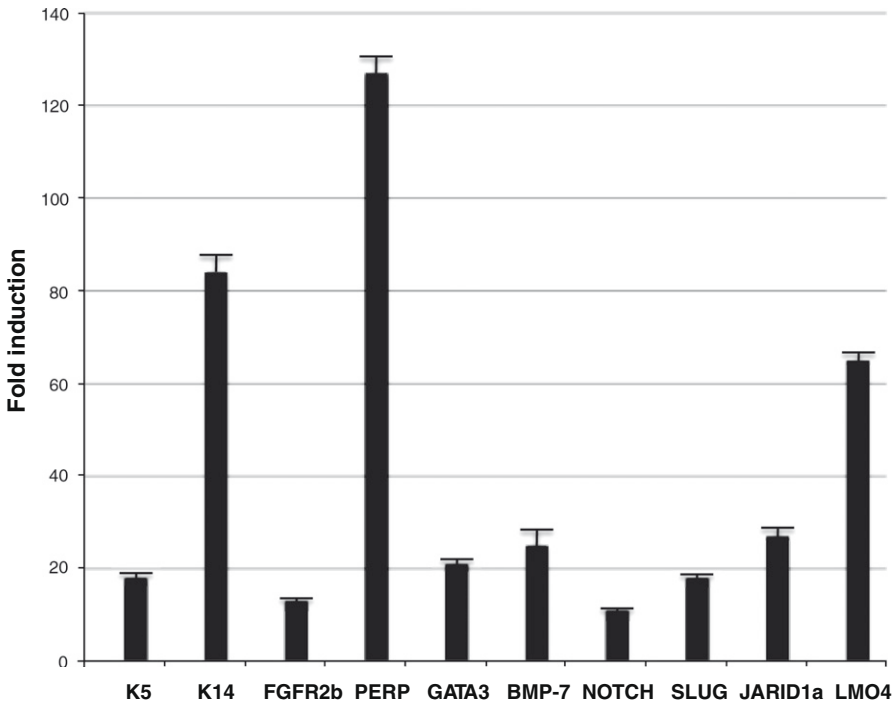


Fig. 3. p63-target gene expression during epidermal commitment. Real-time RT-PCR analysis 48 h after transfection of wild type and mutant ΔNp63 constructs. The results are represented as fold induction obtained in WT versus mutant cells. The data represent triplicate experiments.

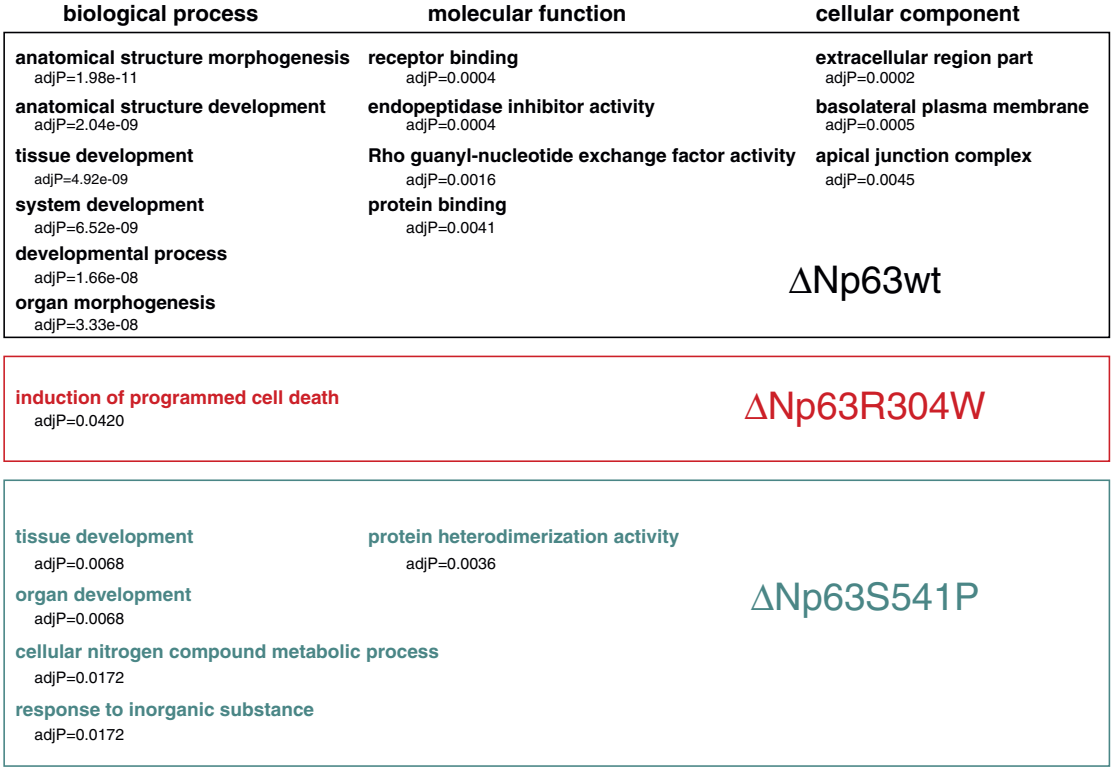


Fig. 4. GO analysis of the three different gene sets regulated by the ΔNp63 constructs. The table shows the most significantly enriched GO categories (and relative adjusted *p*-value) for each transcriptome generated by ΔNp63wt (black) and the two mutants R304W (red) and S541P (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the mutant S541P and R304W displayed different gene expression profiles, characterized by a lower number of regulated genes (85 and 25 for the S541P and R304W mutants, respectively). Interestingly, while the S541P mutant appeared to behave mainly as an inducer of gene expression (83 out of 85 genes up-regulated), the R304W worked as a major repressor (23 out of 25 genes down-regulated). These differences in the expression patterns induced by wild type and p63 mutants, strongly suggest that the amino acids

mutated in the AEC or EEC syndromes, contribute to dictate to p63 its trans-regulatory properties, indeed, mutation in the SAM domain provided activating properties while mutation in the DBD domain induced strong inhibitory capabilities. Accordingly, these modifications gave rise to novel mutant-specific gene profiles, with genes like PERP or FAS, known to be up-regulated by the wild type p63 protein, that were down-regulated by the R304W mutant. Further comparison of each expression profile revealed a subset of 110 genes solely regulated by wild type p63. The fact that S541P and R304W mutants were unable to regulate these p63-target genes suggests that these genes could be considered as an "embryonic epidermal signature", such as FGFR2, GATA3, PERP, NOTCH and BMP-7 (Fig. 3A). As a matter of fact, *FGFR2* gene was shown to be induced by the binding of p63 to a splicing protein through the SAM domain, unless the latter carried an AEC mutation [18]. BMP-7, FGFR2b and Notch were shown to be co-expressed with ΔNp63 *in vivo* and undetectable in the ectoderm of p63 KO embryos [19]. Interestingly, Slug, Jarid1a and LMO4 genes were also activated by wild type p63 but not by the ED mutant (Fig. 3A). Slug has been shown to be involved in the silencing of genes through histone deacetylation for proper adipogenesis [20]. Since Jarid1a and LMO4 are histone modifiers [21,22], our results suggest that the embryonic transition from ectoderm to epidermal fate requires the activation of Slug, Jarid1 and LMO4 for chromatin modification state. We also performed a gene ontology analysis through the GOTM tree machine (http://bioinfo.vanderbilt.edu/gotm) with the three differentially regulated gene sets and the most enriched categories for each construct are depicted in Fig. 4. The most striking observation is the lack of any developmental related category (at least with the default level of significance chosen) in the R304W mutant gene set and the appearance of the programmed cell death, a category which is not significantly enriched in either of the other two sets. Even taking into consideration the fewer number of regulated genes by the mutant R304W, it is tempting to speculate that this mutant fails to promote any developmental program, therefore partially explaining the limb defects observed in the EEC patients. It is also noteworthy that the molecular function categories enriched in the wt set are different in the S541P mutant set, confirming that mutations in the SAM domain might subvert specific protein–protein interactions therefore changing specific signaling pathways implicated, for example, in the correct differentiation of the epithelia and causing the skin phenotype of the AEC patients.

In conclusion, we described here an original cellular model that recapitulates *in vitro* an important step of embryonic skin development, relevant to study physiopathological congenital cases such as ectodermal dysplasia syndromes. It will complement the use of normal adult keratinocytes more suitable for the study of late differentiation/stratification events. Our model uncovers the effect of relevant ED mutations that otherwise are difficult to evaluate on the ectodermal embryonic stage, an embryonic event critical for proper skin formation. Further investigation of the differentially regulated genes identified in this study and their comparison to these obtained with adult keratinocytes will result in a better understanding of the molecular mechanism underlying each ED phenotype during embryonic epidermal commitment. Finally, this new cellular model will be powerful to screen for small compounds able to rescue normal p63 embryonic function for potential ED treatment, as already demonstrated for mutant p53 in solid tumors [23].

Conflict of interest

The authors state no conflict of interest.

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