P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle

Yutaka Shimomura¹, Muhammad Wajid¹, Lawrence Shapiro² and Angela M. Christiano¹,³,⁎

P-cadherin is a member of the classical cadherin family that forms the transmembrane core of adherens junctions. Recently, mutations in the P-cadherin gene (CDH3) have been shown to cause two inherited diseases in humans: hypotrichosis with juvenile macular dystrophy (HUMD) and ectodermal dysplasia, ectrodactyly, macular dystrophy (EEM syndrome). The common features of both diseases are sparse hair and macular dystrophy of the retina, while only EEM syndrome shows the additional finding of split hand/foot malformation (SHFM). We identified five consanguineous Pakistani families with either HJMD or EEM syndrome, and detected pathogenic mutations in the CDH3 gene of all five families. In order to define the role of P-cadherin in hair follicle and limb development, we performed expression studies on P-cadherin in the mouse embryo, and demonstrated the predominant expression of P-cadherin not only in the hair follicle placode, but also at the apical ectodermal ridge (AER) of the limb bud. Based on the evidence that mutations in the p63 gene also result in hypotrichosis and SHFM, and that the expression patterns of p63 and P-cadherin overlap in the hair follicle placode and AER, we postulated that CDH3 could be a direct transcriptional target gene of p63. We performed promoter assays and ChIP, which revealed that p63 directly interacts with two distinct regions of the CDH3 promoter. We conclude that P-cadherin is a newly defined transcriptional target gene of p63, with a crucial role in hair follicle morphogenesis as well as the AER during limb bud outgrowth in humans, whereas it is not required for either in mice.

KEY WORDS: P-cadherin, p63, Apical ectodermal ridge, Hair follicle placode

INTRODUCTION

The classical cadherins are a major structural component of adherens junctions in many tissues. Previous studies have shown the involvement of the classical cadherins in many biological processes, such as cell recognition, cell signaling, morphogenesis and tumor development (Goodwin and Yap, 2004). Recently, two members of the classical cadherin family, E-cadherin and P-cadherin, encoded by the genes CDH1 and CDH3, have been shown to be involved in hair follicle (HF) morphogenesis (Jamora et al., 2003). HF development begins with the formation of a placode, which is composed of groups of rearranged epidermal cells, and subsequent formation of mesenchymal condensate just beneath the placode. The interaction between these specific structures further induces the downgrowth of the placode (Schmidt-Ullrich and Paus, 2005). In the epidermis, E-cadherin is abundantly expressed in the basal and suprabasal layers, while P-cadherin is expressed only weakly in the basal layer (Hirai et al., 1989). As the HF placode begins to form, the expression level of E-cadherin markedly decreases, whereas that of P-cadherin becomes much stronger in the placode than in the interfollicular epidermis (Jamora et al., 2003). The downregulation of E-cadherin, as well as the predominant expression of P-cadherin, continues throughout HF development (Muller-Rover et al., 1999). This ‘cadherin switch’ has been considered to be essential to change cell-cell contacts and potentially facilitate cell movement (Jamora et al., 2003).

The importance of P-cadherin in HF morphogenesis has been further highlighted by recent evidence that human P-cadherin mutations are associated with two congenital diseases affecting the HF. Recessively inherited mutations in the human CDH3 gene were identified in affected individuals with hypotrichosis with juvenile macular dystrophy (HUMD) characterized by congenital sparse hair and early blindness due to macular dystrophy of the retina (Sprecher et al., 2001; Indelman et al., 2002; Indelman et al., 2003; Indelman et al., 2005; Indelman et al., 2007). More recently, mutations in the CDH3 gene also result in hypotrichosis and SHFM, and that the expression patterns of p63 and P-cadherin overlap in the hair follicle placode and AER, we postulated that CDH3 could be a direct transcriptional target gene of p63. We performed promoter assays and ChIP, which revealed that p63 directly interacts with two distinct regions of the CDH3 promoter. We conclude that P-cadherin is a newly defined transcriptional target gene of p63, with a crucial role in hair follicle morphogenesis as well as the AER during limb bud outgrowth in humans, whereas it is not required for either in mice.

Previous studies have clearly demonstrated the expression of P-cadherin in the HF placode (Jamora et al., 2003) and retinal pigmented epithelium (RPE) of the eye (Xu et al., 2002) during mouse embryogenesis, which corresponds to the sites of the human phenotype caused by dysfunction of P-cadherin. To date, however, the precise expression of P-cadherin during limb formation has not been determined. The development of the HF and limb bud share some characteristic features. Several common molecules such as Wnt, sonic hedgehog and bone morphogenic proteins contribute to generate both structures (Dupin et al., 2003). Morphologically, the apical ectodermal ridge (AER), which is the thickened rim of ectoderm at the tip of limb bud, may correspond in some ways to the HF placode. The AER mediates limb bud outgrowth via the interaction with underlying mesenchyme (Sanz-Ezquerro and Tickle, 2001).

Interestingly, the only other gene known to cause SHFM in humans is p63. Mice that lack p63 have striking developmental defects and die shortly after birth from dehydration. They lack stratified squamous epithelia and associated appendages (Mills et al., 1999; Yang et al., 1999). They also display severe defects in limb

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and craniofacial development, including cleft lip. Humans that possess dominant mutations in \( p63 \) similarly have dramatic developmental defects, depending on the location of the mutation within the \( p63 \) gene. One example is ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC) syndrome. Most patients who are affected with EEC have mutations that abolish the ability of \( p63 \) to bind to DNA (Celli et al., 1999). Other syndromes that result from mutations in \( p63 \) include ankylolobepharon-ectodermal dysplasia-clefting (AEC) syndrome, acro-dermato-ungual-lacrimal-tooth syndrome, non-syndromic SHFM and limb-mammary syndrome (Rinne et al., 2006). All of these syndromes are autosomal dominant and can exhibit SHFM, with the exception of AEC.

The transcription factor \( p63 \) is primarily expressed in epithelia. It was first identified as a member of the \( p53 \) family (Augustin et al., 1998; Yang et al., 1998; Osada et al., 1998). \( p53 \) is a tumor suppressor that is mutated in many human cancers. Although there is a high degree of similarity in sequence and structure between \( p53 \) and \( p63 \), they are functionally divergent. \( p63 \) can be expressed from two different promoters and transcriptional start sites, generating the TA and \( \Delta N \) isoforms of \( p63 \). Each isoform has three additional splice variants, designated \( \alpha \), \( \beta \) and \( \gamma \) (Yang et al., 1998). All \( p63 \) proteins possess a DNA-binding domain, which is 60% identical to that of \( p53 \). The TA\( p63 \) isoforms additionally have an N-terminal transactivation region, which is only weakly homologous (22%) to that of \( p53 \). The \( \Delta Np63 \) isoforms are truncated in this region. The diversity of isoforms, splice variants and expression suggests complexity in the function of \( p63 \).

The role of \( p63 \) during development is incompletely understood. The two \( p63 \) isoforms are differentially expressed in embryonic mouse epidermis. One model is that the \( TA-p63 \) isoform, which is expressed earlier in development, has a role in the commitment to epithelial stratification, whereas the \( \Delta N-p63 \) is involved in terminal differentiation (Koster et al., 2004; Koster et al., 2005). Although the expression patterns of \( p63 \) in normal epithelium are well-documented (Koster et al., 2004; Koster et al., 2005), those in epithelial tumors have been described in part (Nylander et al., 2000; Nylander et al., 2002; Di Como et al., 2002), the targets of \( p63 \) regulation have remained largely elusive. Recently, however, several studies using microarrays, chromatin immunoprecipitation and/or RNA interference approaches have identified several candidate \( p63 \)-target genes, such as EGFIR, ICAM3 and \( \beta \)4 integrin (Carroll et al., 2006; Barbieri et al., 2006; Vigano et al., 2006; Yang et al., 2006; Testoni et al., 2006). Interestingly, these studies have gradually disclosed that \( p63 \) regulates gene expression programs involved in cell adhesion (Carroll et al., 2006).

\( p63 \) and \( CDH3 \) are the only genes that have been known to be responsible for SHFM in humans. At present, the \( CDH3 \) mutations reported so far are limited, and the genes that control the expression of P-cadherin remain unknown. The phenotypic overlap led us to hypothesize that \( p63 \) might be a transcriptional regulator of \( CDH3 \). In this study, we identified pathogenic mutations in the \( CDH3 \) of five consanguineous Pakistani families affected with either HJMD or EEM syndrome. In addition, we determined the expression pattern of P-cadherin during hair and limb development in the mouse embryo. Finally, we show that P-cadherin is a direct transcriptional target gene of \( p63 \).

**MATERIALS AND METHODS**

**Mutation analysis of the \( CDH3 \) gene**

After obtaining informed consent, we collected peripheral blood samples from members of five Pakistani families (Families 1-5) (under institutional approval and in adherence to the Declaration of Helsinki Principles). Genomic DNA was isolated from these samples according to standard techniques. Using genomic DNA and primers described previously (Kjaer et al., 2005), all exons of \( CDH3 \) with adjacent sequences of exon-intron borders were amplified by PCR. The PCR products were directly sequenced on an ABI Prism 310 Automated Sequencer, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

**Indirect immunofluorescence and whole-mount immunohistochemistry**

Cryosections were prepared from \( C57BL/6 \) embryos at E9.5 (9.5 days post coitus, day of plug considered 0.5 days), E10.5, E15.5 and E17.5. Sections were fixed in cold methanol:acetone (1:1) for 7 minutes, followed by blocking with 10% normal goat serum in PBS for 1 hour at room temperature. Primary antibodies used were anti-P-cadherin rat monoclonal PCD-1 (1:100; ZYMED Laboratories), anti-p63 rabbit polyclonal H-129 (1:200; Santa Cruz Biotechnology), anti-N-cadherin rabbit polyclonal H-63 (1:200; Santa Cruz Biotechnology) and mouse monoclonal anti-E-cadherin (1:200; BD Biosciences). After the incubation with primary antibodies, FITC and/or Alexa-Fluor-labeled secondary antibodies (Jackson Immunoresearch) were applied to the sections. Nuclei were counterstained with DAPI. The images were processed with Adobe Photoshop (version 8.0) software. Whole-mount immunohistochemistry was performed as previously described (Yoshida et al., 1996) with some modifications. \( C57BL/6 \) embryos were incubated with anti-P-cadherin antibody PCD-1 (1:100), and subsequently with 1 \( \mu \)g/ml HRP-conjugated anti-rat-IgG antibody. Antibody binding was visualized with diaminobenzidine substrate.

**p63-expression vectors**

Expression constructs encoding c-myc-tagged mouse TA\( p63 \), \( \Delta Np63x \) and \( \Delta Np63y \) were kindly provided by Drs Dennis Roop and Maranke Koster (University of Colorado, Aurora, Colorado, USA). To generate the mutant (R280C) TA\( p63 \), the upstream coding region of TA\( p63 \) was PCR-amplified using the forward primer (5'-AAAAAGATCCCCTGCGAGACCCAGACAGA-3') and the reverse primer (5'-ATGTAAAAATGAGCAGCTCAGTA-3') and wild-type TA\( p63 \) expression construct as a template. Note that the mutation of interest was introduced into the reverse primer as underlined. The remaining coding region of TA\( p63 \) was also amplified by PCR using the forward primer (5'-AAATTTAAATCCTGTTACTCAGAACACAA-3') and the reverse primer (5'-AAATCACGACGTATGGGTACCGGAGTGT-3'). The two amplified fragments were then ligated at the MstI restriction enzyme site, which was subcloned in frame into the BsmI and XhoI sites of pCMV-Tag3 vector (Stratagene).

**Transient transfections and quantitative PCR analysis**

HEK293 (human embryonic kidney epithelial) cells were plated in 12-well plates the day before transfection. Expression plasmids of TA\( p63a \), TA\( p63 \), \( \Delta Np63x \) and \( \Delta Np63y \) (1.6 \( \mu \)g each) were transfected with Lipotefectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Empty vector was also transfected as a control. Twenty-four hours after transfection, total RNA was isolated from the cells using the RNeasy Minikit (Qiagen). Total RNA (2 \( \mu \)g) was reverse-transcribed using random primers and SuperScript III (Invitrogen). Real-time PCR was performed on an ABI 7300 (Applied Biosystems). PCR reactions were performed using ABI SYBR Green PCR Master Mix, 300 nM primers, 200 ng cDNA at the following consecutive steps: (1) 50°C for 2 minutes; (2) 95°C for 10 minutes; (3) 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The samples were run in triplicate and normalized to an internal control (\( GAPDH \)) using the accompanying software. The following primers were used: \( CDH3 \) (forward 5'-CGAAGGACGACGGACTGA-3', reverse 5'-GTCTGTTTACGCGCTTCA-3'), \( GAPDH \) (forward 5'-TCACCAGGGCTGCTTTAACCTC-3', reverse 5'-GGGTGAACTCATTTGGAAATC-3'). The transfection and real-time PCR experiments were performed in triplicate. Statistical analysis was performed using the ANOVA procedure, followed by the Dunnett’s t-test. \( P<0.05 \) was considered significant.

**Promoter analyses and reporter gene assays**

The whole genome rVISTA tool (http://genome.lbl.gov/vista/index.shtml) evaluates conservation of genomes between pairs of species (specified as mouse and humans) and pinpoints transcription-factor-binding sites that are
over-represented in upstream regions of genes of interest. PCR-amplified CDH3 promoter regions were cloned into the Nilel and Xhol sites of the pGL3 basic luciferase reporter vector (Promega). To generate the mutated constructs, mutations of interest were introduced into the forward primers. HeLa (human cervical cell carcinoma) cells were seeded in a 12-well plate and the next day transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen). A constitutive β-galactosidase reporter was used for normalization of transfection efficiency. The cells were lysed 24 hours after transfection and the signals were assayed using the appropriate substrates for luciferase (Steady-Glo Luciferase Assay System) and β-galactosidase (Promega) on a 20/20 luminometer (Turner Biosystems) for luciferase and a Model 680 microplate reader (BioRad) for β-galactosidase.

Chromatin immunoprecipitation (ChIP)
HEK293 cells were seeded at 1 × 10^5 per 60 mm dish and transfected 24 hours later with 8 μg Tap63α or Tap63γ expression vector, or empty vector as described above. After 24 hours, the cells were fixed in 1% formaldehyde for 10 minutes and the Chromatin Immunoprecipitation Kit (Upstate) was used per the manufacturer’s recommendations. In addition, HaCaT (human epidermal keratinocytes) cells were cultured in a 100 mm dish and used for in vivo ChIP. An anti-p63 antibody mouse monoclonal 4A4 (Santa Cruz Biotechnology) was used for immunoprecipitation. Cell lysates were also precipitated with normal mouse IgG as a negative control. PCR was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen).

RESULTS
Clinical features
Families 1–3 are large consanguineous Pakistani pedigrees with four, twelve and six affected individuals, respectively, who were born with sparse hair that fails to grow (Fig. 1A). Retinal degeneration manifested in all affected individuals with decreased visual acuity during the first decade of life, whereas none of them had limb or finger anomalies. Family 4 has five affected individuals whose parents are first-degree cousins (Fig. 1A). All affected individuals have sparse hair, weak eyesight, and the additional phenotype of severe SHFM (Fig. 1A). Family 5 contains two affected individuals whose parents are first-degree cousins (Fig. 1A). The younger patient (II-6) exhibits severe hypotrichosis with a trichorrhexis nodosa-like hair shaft, weak eyesight and severe syndactyly. Trichorrhexis nodosa is a particular type of hair shaft anomaly characterized by nodular swelling of the hair due to its fragility. The elder patient (II-5) shows mild hypotrichosis with pili torti-like hair characterized by nodular swelling of the hair due to its fragility. The Trichorrhexis nodosa is a particular type of hair shaft anomaly characterized by nodular swelling of the hair due to its fragility. The Trichorrhexis nodosa-like hair shaft anomaly is characterized by nodular swelling of the hair due to its fragility. The Trichorrhexis nodosa-like hair shaft anomaly is characterized by nodular swelling of the hair due to its fragility.

Identification of three novel and one recurrent mutation in the CDH3 gene
Using genomic DNA from members of the five Pakistani families, we performed mutation analysis and identified pathogenic mutations in the CDH3 gene in all five families. First, we found a novel homozygous mutation 490insA in exon 5 of CDH3 in affected individuals of Family 1 (Fig. 1B). Mismatch allele-specific PCR showed that 50 unrelated healthy control individuals did not possess the mutation (data not shown). Next, affected individuals in both Families 2 and 3 were homozygous for a novel mutation IVS10-1G>T in CDH3 (Fig. 1C). This mutation abolished a BpmI restriction enzyme site, which was used to exclude the mutation from 50 control individuals (data not shown). Affected individuals in Family 4 were found to carry a novel homozygous substitution 535A>G in CDH3, which results in a substitution of glutamic acid by glycine at amino acid 118 (Fig. 1D). Multiple amino-acid sequence alignments showed that the glutamic acid at position 118 is completely conserved among P-cadherin of other species, as well as other human classical cadherins (Fig. 1D). In addition, the mutation generated a BstNI restriction enzyme site, which was used to exclude the possibility that it represents a common polymorphism (data not shown). Finally, patients in Family 5 possessed a homozygous transition 965A>T (N322I) (Fig. 1E), which was previously detected in a Danish family with EEM syndrome (Kjaer et al., 2005).

P-cadherin is expressed in the hair follicle placode and the AER of limb bud, overlapping with p63
In order to determine the precise expression pattern of P-cadherin in the developing HF, eye and limb bud, we performed immunostaining using cryosections of whole mouse embryos. At E15.5, P-cadherin was strongly expressed in the HF placode, whereas it was only weakly expressed at basal layer of the interfollicular epidermis (Fig. 2A). In addition, P-cadherin expression was detected in the RPE of the developing eye at E17.5 (Fig. 2B). These results are consistent with previous studies by others (Jamora et al., 2003; Xu et al., 2002). In forelimb bud at E9.5 (Fig. 2C) and E10.5 (Fig. 2D), P-cadherin was strongly expressed throughout the AER. The positive signal was also detected in dorsal and ventral surface ectoderm around the AER, but the expression was more prominent in the AER (Fig. 2C,D). At E15.5, P-cadherin was ubiquitously expressed at the epithelial cell junctions of the forelimb digits (Fig. 2E,F). We also performed whole-mount immunohistochemistry using E9.5 embryos, which precisely confirmed the expression of P-cadherin at the AER of forelimb buds (Fig. 2G,H).

Double immunostaining with antibodies for P-cadherin and p63 clearly demonstrated overlapping expression of the two proteins in the HF placode (Fig. 3A,B). In addition, p63 was strongly expressed throughout the nuclei of the forelimb bud epithelium, especially in the AER (Fig. 3C,D), which overlaps with P-cadherin expression (Fig. 3E-H).

In addition to P-cadherin, we analyzed the expression of two other classical cadherin members in the developing mouse limb bud. At E9.5, E-cadherin was markedly expressed in the AER of forelimb bud (Fig. 4A), and its expression almost completely overlapped with that of P-cadherin (Fig. 4B,C). The strong expression of E-cadherin was similarly detected throughout the AER at E10.5 (Fig. 4D,E). N-cadherin expression was widely detected in the mesenchyme, but not in the overlying epithelium (Fig. 4F). In particular, the expression of N-cadherin was prominent just beneath the AER and never overlapped with E-cadherin expression (Fig. 4G,H).

p63 induces the expression of the P-cadherin (CDH3) gene
In order to investigate the role of p63 isoforms in regulating the expression of CDH3, we tested the ability of p63 to induce CDH3 expression. HEK293 cells were transfected with Tap63α,
TAp63γ, ΔNp63α, ΔNp63γ or empty vector, and the relative CDH3-transcript endogenous levels were examined by real-time PCR. Cells transfected with TAp63 isoforms showed significant increase in the expression of CDH3 (Fig. 5). In addition, cells transfected with ΔNp63 isoforms also exhibited a moderate, albeit statistically significant, increase (P<0.05) in CDH3 expression (Fig. 5).

Identification of two regions in the CDH3 promoter responsive to p63
Comparison of the CDH3 promoter sequences among different species using the whole genome rVISTA tool resulted in the identification of two distinct regions that were highly conserved between mouse and human (Fig. 6A). These are located at −2400/−2000 bp and +1/+500 bp of the CDH3 promoter, which we termed...
Region 1 and Region 2, respectively (Fig. 6A). Region 2 corresponds to exon 1 of the CDH3 that contains the start codon. In addition, analysis with the NCBI database (http://www.ncbi.nlm.nih.gov/) showed that both regions are included within CpG islands (Fig. 6A). Based on this information, we first cloned –2472 to +500 bp of the CDH3 promoter, which contained both regions, into the pGL3 basic plasmid, and tested whether the luciferase reporter gene activity is induced by TAp63/H9253 and H9004/Np63/H9253. As shown in Fig. 6B, both TAp63/H9253 and H9004/Np63/H9253 markedly upregulated luciferase activity (more than 25-fold relative to control).

Subsequently, we cloned four shorter fragments of the CDH3-promoter, –3366 to –2452 (low homology region upstream of Region 1), –2472 to –1833 (Region 1), –1944 to –1 (low homology region between Regions 1 and 2) and +1 to +500 (Region 2) into the pGL3 vector (Fig. 6A), and analyzed the promoter activity in the presence of p63. We observed that both Region 1 and Region 2 showed significant levels of transcription, as measured by relative luciferase activity (Fig. 6B). It has been well established that the binding sequence of p63 is similar to the canonical responsive element of p53 (RRRCWWGYYY), with minor differences (Osada et al., 2005). We analyzed the sequence of Regions 1 and 2 and found five and two sites, respectively, that show high homology (7/10 to 9/10) with the sequence ‘RRRCWWGYYY’, which could be regarded as potential p63-binding sites (Fig. 6C,D). Indeed, both regions were sufficient to show a significant increase in transcription by p63 when they were truncated to ~230 bp fragments containing these potential p63-binding sites (Fig. 6E). By contrast, we noted that p53 did not induce transactivation of the CDH3 in any of the constructs analyzed (data not shown). Of the five potential p63-binding sites in Region 1, the downstream two sites partially overlapped with each other at –2088/–2070 (Fig. 6C). We focused on these successive binding sites and further analyzed them in detail.

In order to test each binding site, we compared the activities of the CDH3-2106/-2004-Wild, CDH3-2106/-2004-M1 (both sites were mutated), CDH3-2106/-2004-M2 (only the upstream site was mutated), and CDH3-2106/-2004-M3 (only the downstream site was mutated) constructs in the presence of TAp63γ (Fig. 6F). All three mutated
The strong expression of E-cadherin throughout the AER. (red in D,E. Scale bars: 100
patterns (H). Counterstaining with DAPI is shown in blue in C and in
merged image of F and G clearly demonstrates their distinct expression
whereas E-cadherin is prominently expressed in the AER (G). The
cadherin is strongly expressed at mesenchyme just beneath the AER (F),

required for the transactivation by TA-p63
we also introduced mutations into the putative binding site at +31/40

CDH3+/+124-Mutant construct, indicating that both sites are
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The mutation Ivs10-1G>T found in Families 2 and 3 with
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p63 binds directly to two regions in the CDH3
promoter
We investigated the ability of p63 to bind to the CDH3 promoter
using ChIP assays. First, we overexpressed both TAp63α and
TAp63γ isoforms in HEK293 cells, and precipitated the DNA-
protein complexes with anti-p63 4A4 antibody. The precipitated
DNA fragments were PCR-amplified. In the cells in which
TAp63α and TAp63γ were induced, anti-p63 antibody clearly
immunoprecipitated both Region 1 and Region 2 of the CDH3
promoter, which contain putative p63-binding sites, whereas it did
not immunoprecipitate the CDH3 coding region (Fig. 8A). Finally,
we performed in vivo ChIP using HaCaT cells that predominantly
express ΔNp63α isoform (Vigano et al., 2006). Similarly, both
Region 1 and Region 2 were PCR-amplified from the sample
precipitated with anti-p63 antibody (Fig. 8B). These results
demonstrate that both TAp63 and ΔNp63 isoforms directly bind to
two distinct regions within the CDH3 promoter.

DISCUSSION
P-cadherin mutations cause HJMD or EEM syndrome in humans
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are caused by mutations in the CDH3 gene, encoding P-cadherin
(Sprecher et al., 2001; Kjaer et al., 2005). In this study, we identified
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Region 2 and compared the induction of transcription by TA-p63γ
between CDH3+/+/124-Wild and CDH3+/+/124-Mutant constructs
(Fig. 6G). Significant reduction in luciferase activity was detected
using the CDH3+/+124-Mutant construct (Fig. 6G).

TAp63γ (R280C) mutant fails to transactivate
CDH3 expression

Previous studies have shown that mutations in the DNA-binding
domain of p63 result in SHFM (Celli et al., 1999; Ianakiev et al.,
2000). In particular, missense mutations at codon 279 or 280 have
been shown to cause SHFM at a high frequency (Rinne et al., 2006).
Therefore, we generated a construct expressing TAp63γ with the
common SHFM mutation R280C, and tested whether this mutation
affects the CDH3 expression. The mutant TAp63γ did not activate
the reporter activity in either Region 1 (Fig. 7A) or Region 2 (Fig.
7B). Furthermore, in both regions, wild-type TAp63γ-induced
transactivation of the CDH3 reporter was markedly inhibited by the
mutant TAp63γ in a dose-dependent manner (Fig. 7A,B).

Fig. 4. Expression of classical cadherins in the forelimb bud of
mouse. (A-C) Double immunostaining of a longitudinal section of the
forelimb bud at E9.5 shows that the expression of E-cadherin (green, A)
and P-cadherin (red, B) overlaps along the AER (yellow in merged
image, C). (D, E) Transverse section of the forelimb bud at E10.5 shows
the strong expression of E-cadherin throughout the AER. (red in D,E. Scale bars: 100
μm in A-D,F-H; 20 μm in E.

Fig. 5. CDH3 expression is upregulated by p63 in 293 cells.
HEK293 cells were transfected with TAp63α, TAp63γ, ΔNp63α,
ΔNp63γ or empty vector. At 24 hours post-transfection, the CDH3
expression levels were measured by real-time PCR. All four p63
isoforms upregulated CDH3 expression. Asterisks indicate a statistically
significant difference (P<0.05) compared with the empty vector.

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HJMD is a novel insertion mutation that results in a frameshift at
codon 164 and premature termination codon (PTC) at codon 171
(Fig. 1B). The mutation Ivs10-1G>T found in Families 2 and 3 with
HJMD is a novel splice site mutation that is predicted to cause out
frame skipping of exon, 11, 146 bp in size, and generate a PTC
within exon 12 (Fig. 1C). It is possible that truncated proteins
lacking most of the essential domains could be generated from both
the 490insA and the Ivs10-1G>T mutant alleles. Alternatively, the
Fig. 6. Identification of two distinct regions in the CDH3 promoter that are responsive to p63. (A) Schematic of the CDH3 promoter. Regions 1 and 2 that show high homology with mouse sequences are boxed. CpG islands are indicated by shaded boxes. Five templates that were cloned into the pGL3 basic vector are shown at the bottom. (B) Reporter gene assay in HeLa cells. The longest construct (−2472/+500), which contains both Regions 1 and 2, showed more than 25-fold transactivation of the reporter gene by TAp63γ and ΔNp63γ. Furthermore, shorter constructs that contain either Region 1 (−2472/−1833) or Region 2 (+1/+500) resulted in a much greater activation of luciferase expression by p63. (C–D) Potential p63-binding sites in Region 1 (C) and Region 2 (D) are shaded with the canonical p53-binding sequence shown either above or below. Asterisks show nucleotide residues that correspond to the ‘RRRCWWGYYY’ sequence. The positions where primers for ChIP assay were designated are underlined. (E) Both Regions 1 and 2 showed further increase of the reporter gene activity by p63, when they were truncated to about 230 bp fragments with potential p63-binding sites. (F) Sequence of the two partially overlapping p63-binding sites in Region 1 (−2088 to −2070). Sequences of the three mutated constructs (M1-M3) are also shown. All three mutated constructs led to a marked reduction of luciferase activity by TAp63γ. (G) Sequence of a p63-binding site in Region 2 (+31 to +40). Sequence of the mutated construct is also shown. TAp63γ showed only weak transactivation of the reporter gene in the mutated construct.
aberrant CDH3-transcripts containing the PTC might be largely degraded due to nonsense-mediated messenger RNA decay (Maquat, 1996; Frischmeyer and Dietz, 1999). E118G is a novel missense mutation detected in Family 4 with EEM syndrome showing severe SHFM (Fig. 1D). The glutamic acid at position 118 (E118), which corresponds to E11 in the mature protein, is a highly conserved amino acid residue within the EC1 domain of P-cadherin (Fig. 1D, Fig. 9). The side chain of E11 coordinates calcium in the EC1-EC2 calcium-binding site (Fig. 9), and without this side chain, the structure of the calcium-binding site will be compromised. Calcium binding rigidifies cadherin ectodomains, which is thought to geometrically constrain cadherins so as to interact preferentially with cadherins from apposing cells rather than the same cell (Boggon et al., 2002; Patel et al., 2006). The mutation E118G may thus enable the interaction of mutant proteins on the same, rather than opposing, cell surfaces, thereby negating adhesive function. The mutation N322I found in Family 5 with EEM syndrome is a non-conservative amino acid change in the EC2-EC3 calcium-binding site (Fig. 9), which was previously identified in a Danish family with EEM syndrome (Kjaer et al., 2005). It is noteworthy that the affected individuals in the Danish family exhibited more severe hand involvement (Kjaer et al., 2005) than those in Family 5 (Fig. 1A). Similarly, in EEM families, all affected individuals show not only hair and eye phenotype but also hand/foot involvement (Kjaer et al., 2005). An obvious genotype-phenotype correlation between CDH3 mutations has yet to emerge.

Balanced expression of classical cadherin members might be crucial for limb bud development
To our knowledge, the expression of P-cadherin in the limb bud has not been previously reported. Here, we clearly demonstrated that P-cadherin expression is upregulated in the AER of the limb bud in the developing mouse embryo (Fig. 2C-H). Our data suggest that P-cadherin plays an important role in outgrowth of limb bud, perhaps akin to its role in the downgrowth of the HF placode. In addition to P-cadherin, we also examined the expression of N- and E-cadherins in the limb bud. Like previous reports (Yajima et al., 2002), we showed that N-cadherin expression is detected in mesenchyme underlying the AER (Fig. 4F-H). Surprisingly, E-cadherin was strongly expressed in the AER, overlapping with P-cadherin (Fig. 4A-E), suggesting that the E-cadherin to P-cadherin switch does not occur in the AER of the developing mouse embryo as it does in the HF placode.

The identification of mutations in the human CDH3 gene underscores the involvement of P-cadherin in the human HF, eye and limb development. Despite these findings in humans, it is
somewhat surprising that P-cadherin-knockout mice, which are on a C57BL/6 background, show only precocious mammary gland development, whereas hair and limb anomalies are not observed in these animals (Radice et al., 1997). These differences may be due to the influence of some modifier genes and/or genetic background in the animal models. The strong expression of E-cadherin in the AER raises the possibility that E-cadherin could play a role as a modifier and compensate for the P-cadherin deficiency in the mouse model. In addition, as it is known that the sensitivity to teratogen-induced limb malformations is variable among different mouse strains (Shimizu et al., 2007), there is the possibility that P-cadherin deficiency might cause more severe anomalies, including hair and limb phenotypes on different mouse strains.

**P-cadherin is a direct transcriptional target gene of p63**

Double immunostainings with anti-P-cadherin and anti-p63 antibodies clearly showed that the expression patterns of p63 and P-cadherin overlap in the HF placode (Fig. 3A,B) and in the AER of the limb bud (Fig. 3E-H). The transcription factor p63 has been known to be involved in the HF and limb development because p63 mutations result in developmental defects of hair and limb in both human (Celli et al., 1999) and mouse (Mills et al., 1999; Yang et al., 1999). In particular, SHFM caused by human p63 mutations is phenotypically similar to that in affected individuals with CDH3 mutations. Our expression studies, as well as the phenotypic similarity, prompted us to postulate that P-cadherin could be a direct transcriptional target of p63. In order to test this hypothesis, we first examined whether P-cadherin expression is affected when p63 is overexpressed in 293 cells. We found that both TAp63 and ΔNP63 isoforms upregulated the expression of the CDH3 mRNA (Fig. 5).

Osada et al. have recently shown that a total of 129 genes were activated more than four-fold when TAp63 was ectopically expressed in 293 cells, and CDH3 was found among these genes (8.1-fold upregulation) (Osada et al., 2005).

Subsequently, we focused on p63 isoforms and performed promoter assays using the luciferase reporter gene. We identified two distinct regions (Regions 1 and 2), which were significantly responsive in reporter gene expression analysis by both TAp63 and ΔNP63 (Fig. 6A,B,E). The reporter gene activity in the longest construct containing both regions was lower than that of a shorter construct with either region (Fig. 6B). A suppressor element might be present in the region between Regions 1 and 2, thereby repressing the expression of P-cadherin. Furthermore, a mutant (R280C) TAp63 inhibited the transactivation by the wild-type TAp63 in a dose-dependent manner in both regions (Fig. 7A,B), indicating that the mutant TAp63 would cause a dominant-negative effect to the wild-type TAp63 and thereby reduce P-cadherin expression. Regions 1 and 2 have a total of seven potential p63-binding sites (Fig. 6C,D). Even though they show high homology with the canonical p53-binding sequence (RRRCWWGYYY), p53 did not activate the luciferase expression in either region (data not shown), indicating that these binding sites are specific to p63. Four of seven binding sites possess a CCTG core sequence (RRRCWWGYYY), p53 did not activate the luciferase expression in either region (data not shown), indicating that these binding sites are specific to p63. Four of seven binding sites possess a CCTG core sequence (Fig. 6C,D), which has been found in the promoter regions of recently identified p63-target genes as well (Yan and Chen, 2006; Romano et al., 2006). We generated mutated constructs in which mutations were introduced to abolish the core sequence, and showed significant reduction of transactivation compared with wild-type constructs (Fig. 6F,G). These data indicate that the CCTG core sequence represents a p63-specific element in addition to the recently identified CGTG core sequence (Fig. 8A). Furthermore, we also performed in vivo ChIP assay using HEK293 cells in which either TAp63α or TAp63 was induced, and demonstrated that TAp63 isoforms bind to both Regions 1 and 2 of the CDH3 promoter containing potential p63-binding sites (Fig. 8A). Furthermore, we also performed in vivo ChIP assay using HaCaT cells in which endogenous ΔNP63α is strongly expressed, and clearly showed that p63, especially ΔNP63α, binds to the CDH3 promoter in vivo (Fig. 8B). Collectively, our data indicate that P-cadherin is a direct transcriptional target of p63.

**The interaction between p63 and P-cadherin gene is crucial for HF and limb bud development in humans**

Using large-scale genomic approaches, a number of potential p63-target genes have been predicted by several groups (Carroll et al., 2006; Barbieri et al., 2006; Vigano et al., 2006; Yang et al., 2006; Testoni et al., 2006). Of these, Carroll et al. showed the upregulation...
of CDH3 expression when TAp63α was overexpressed in MCF-10A (normal human breast epithelial) cells (Carroll et al., 2006). In addition, Yang et al. performed ChIP using an anti-p63 antibody and ME180 (human cervical carcinoma) cells, which was followed by microarray studies (Yang et al., 2006). As a result, approximately 5800 p63-target sites were identified in the whole genome. One of these sites corresponded to intron 2 of the CDH3, which is not conserved between human and mouse, and is distant from the transcription start site. These data, together with Osada et al. (Osada et al., 2005), suggested a potential relationship between p63 and the CDH3. For the first time, definitive evidence to prove that the CDH3 is a direct target of p63 is provided in this study, and our results are consistent with the recent report that implicates p63 as a key regulator of broader cell adhesion gene expression programs (Carroll et al., 2006). Recently, Senoo et al. showed that one role of p63 is to control programmed cell death and maintain the proliferative potential of embryonic and adult stem cells (Senoo et al., 2007). p63 might prevent cell death through regulating the expression of some adhesion molecules that are essential for epithelial integrity and maintenance of proliferation (Blanpain and Fuchs, 2007). P-cadherin might be involved in this mechanism.

During mouse embryogenesis, TAp63 isoforms have been shown to be expressed much more strongly than ΔNp63 isoforms (Koster et al., 2004; Radoja et al., 2007), whereas ΔNp63 isoforms are predominantly expressed in the basal layer of the epidermis and govern basal-epidermal gene expression (Laurikkaka et al., 2006; Candì et al., 2006). Recently, a TAp63-specific knockout mouse, in which ΔNp63 isoforms are expressed normally, has been reported to show neither hair nor limb anomalies (Suh et al., 2006). In our study, we showed that not only TAp63, but also ΔNp63 isoforms, bind to the CDH3 promoter and activate CDH3 expression. We conclude that TAp63 isoforms, together with ΔNp63, play an essential role in maintaining the P-cadherin expression in the AER during limb bud growth, as well as in the HF placode. As p63 is more broadly expressed than P-cadherin, not only p63 but also other factors are likely to play a role in regulating P-cadherin expression in the HF placode and the AER. Nevertheless, the phenotypic similarities caused by p63 and P-cadherin mutations, as well as our results, strongly suggest that the interaction between p63 and P-cadherin is crucial for HF and limb bud development in humans, whereas it does not appear to be essential for either in mice. We have defined a functional relationship between p63 and P-cadherin, and also determined their role in failure of the HF placode and the AER that can lead to sparse hair and SHFM when either gene is mutated in humans.

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