Published in final edited form as:

Gene. 2013 May 1; 519(2): 251–259. doi:10.1016/j.gene.2013.02.021.

Distinct function of *P63* isoforms during embryonic skeletal development

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Abstract

P63 belongs to the P53 family of transcription factors. There are multiple P63 isoforms that play important functions both in cancer and development. The obvious limb defect in *p63* null mice and in human skeletal syndromes with *P63* mutations suggest its essential role in long bone development. However, how the different P63 isoforms function during long bone development is largely unknown. We have previously shown that *TAP63α*, the longest P63 isoform, plays a positive role in embryonic skeletal development, since targeting *TAP63α* expression in hypertrophic chondrocytes accelerates endochondral ossification at both E17.5 and P1 stages. Here, we report transgenic studies of $\Delta NP63\alpha$, another P63 isoform which lacks the N-terminal transactivation domain compared to *TAP63α*, using the same hypertrophic chondrocyte-specific *Col10a1* control element. No skeletal abnormalities were detected in these *Col10a1-ΔNP63α* transgenic mice at both E17.5 and P1 stages, suggesting less importance of $\Delta NP63\alpha$ during late embryonic skeletal development. To further investigate the function of P63 isoforms during early skeletal development, we have generated $\Delta NP63\alpha$ and *TAP63α* transgenic mice using a chondrocyte-specific *Col2a1* control element. Surprisingly, while no skeletal defect was shown in the *Col2a1-ΔNP63α* transgenic mice, reduced ossification was observed in the digit and tail bones of *Col2a1-TAP63α* transgenic mice at both E17.5 and P1 stages compared to their wild-type littermates. Expression profiling and immunohistochemical analysis detected upregulated expression of Sox9, a major negative regulator of endochondral ossification, in *Col2a1-TAP63α* transgenic mice. Taken together, our results suggest a distinct function of P63 isoforms, herein, $\Delta NP63\alpha$ and *TAP63α*, during endochondral ossification.

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Disclosures: All the authors state that they have no conflicts of interest.

Keywords

Col2a1; *Col10a1*; *P63* isoforms; Transgenic mice; Embryonic skeletal development

1. Introduction

P63 is a member of a well-known family of transcription factors that include also p53 and p73 tumor suppressors (Levrero et al., 2000). Given their structural similarity, p53, p63, and p73 genes are thought to have overlapping/redundant functions by regulating expression of similar downstream target genes (Levrero et al., 2000). However, extensive studies have demonstrated their unexpected functional diversity during tumor formation and development (Levrero et al 2000; Moll UM, Slade N 2004; Bergholz J, Xiao ZX 2012). Each of this family of genes is consisted of multiple isoforms due to alternative splicing and different promoter usage. There are also cross interactions among the different isoforms or family members. These facts cause the variety of functions, and also hinder the in vivo functional studies of this family of genes.

Unlike *P53*, *P63* has been predominantly implicated a role in development than in tumor formation, as *P63* is rarely mutated in human cancers, while *p63* null mice exhibit severe developmental abnormalities without increasing cancer susceptibility (Moll UM, Slade N 2004). P63 is known to play a fundamental role in epithelial development, as *p63*-deficient mice lack of epidermis and other epithelia (Koster et al., 2004; Candi et al., 2007; Romano et al., 2012). *p63* null mice also show severe embryonic cardiac defect suggesting its essential role in heart development (Rouleau et al., 2011). Notably, previous studies have shown that mice lacking *p63* are either absent or have truncated limbs (Mills et al., 1999; Yang et al., 1999), while human *P63* gene mutations are associated with the ectrodactyly, ectodermal dysplasia and facial clefts (EEC) syndrome and isolated split hand-split foot (SHSF) malformation, which show similar limb defect of *p63*-deficient mice (van Bokhoven et al., 2007; Levrero et al., 2000), suggesting a putative role of P63 during bone development.

Endochondral bone formation is a complex process that requires a cartilage intermediate during long bone development. It is characterized by production of collagen type II (COL2A1) at the early stage and collagen type X (COL10A1) at the late stage of chondrocyte differentiation, maturation or hypertrophy. The hypertrophic chondrocytes further undergo apoptosis and are eventually replaced by bone (Ornitz DM., Marie PJ., 2002). This is also a well-coordinated process regulated by an array of growth factors, transcription factors, and signaling pathways (de Crombrughe et al., 2001; Wu et al., 2008). The severe limb defect in *p63* null mice and in human skeletal syndromes with P63 mutations suggest that P63 may play essential roles in endochondral ossification during long bone development. However, P63 is divided into two subtypes: the TAP63 that contains an N-terminal transactivation domain, and the Δ NP63 that lacks this domain (Petitjean et al., 2007). Each subtype is also consisted of three transcript variants (*TAP63* α , $-\beta$, $-\gamma$ and Δ *NP63* α , $-\beta$, $-\gamma$) that differ only in the 3'-ends due to alternative splicing. How these P63 isoforms affect bone development remains elusive. We have recently performed transgenic studies by targeting *TAP63* α expression using the hypertrophic chondrocyte-specific *Col10a1* promoter elements. Accelerate ossification was observed in long bone, digit and tail bones of the *Col10a1-TAP63* α transgenic mice at both E17.5 and P1 stages, suggesting its positive role in late embryonic skeletal development (Li et al., 2012). In this manuscript, we report generation of multiple transgenic mouse lines in which *P63* transcript variants Δ *NP63* α and *TAP63* α were driven by either the hypertrophic chondrocyte-specific *Col10a1*-, or the chondrocyte-specific *Col2a1*-promoter elements. Diverse skeletal

phenotypes were observed in these *Col10a1-ΔNP63α*, *Col2a1-ΔNP63α*, and *Col2a1-TAP63α* transgenic mice, supporting a distinct function of *P63* variants during embryonic skeletal development.

2. Materials and Methods

2.1. Cloning of transgenic constructs containing P63 variants

Three transgenic constructs containing *P63* transcript variants *ΔNP63α* or *TAP63α* were generated using the cell-specific *Col2a1* or *Col10a1* promoter elements (Zhou et al., 1995; Zheng et al., 2009). Specifically, for the *Col10a1-ΔNP63α* construct, HA- and Flag-tagged human *ΔNP63α* cDNA was released from pcDNA3.1(–) by *Bam*HI and *Xho*I digestion (Fig. 1A). This fragment was blunted and cloned into the *Hind*III site of the pcDNA3.1(–) downstream of the hypertrophic chondrocyte-specific *Col10a1* promoter element, which contains four copies of the 300-bp *Col10a1* cis-enhancer and a short basal promoter (265 bp) as previously described (Zheng et al., 2009; Li et al., 2012). The junction sequence and the insert orientation of *Col10a1-ΔNP63α* were confirmed by sequencing using *ΔNP63α*-specific primers. For the *Col2a1-ΔNP63α* (Fig. 2A) and *Col2a1-TAP63α* (Fig. 4A) constructs, full length human *ΔNP63α* or *TAP63α* cDNA in-frame with a 5'-HA and a 3'-Flag followed by a polyA sequence was released from pcDNA 3.1(–) by *Sal*I and *Bsr*bI digestion. After blunted, these fragments were cloned into the *Swa*I site downstream of a 6kb chondrocyte-specific *Col2a1* promoter and intron sequence in a modified pBluescript II SK vector (Stratagene, La Jolla, CA.). This vector contains a 5'-tyrosinase expression (TYR, coat color) cassette, a 3'-WPRE element (the woodchuck hepatitis virus posttranscriptional regulatory element) and a chicken β-globin HS4 insulator (Zufferey et al., 1999; Hsiao et al., 2004). The junction sequence and the insert orientation of *Col2a1-ΔNP63α* or *Col2a1-TAP63α* were also confirmed by sequencing using *ΔNP63α* or *TAP63α*-specific primers.

2.2. Generation of *Col10a1-ΔNP63α*, *Col2a1-ΔNP63α* and *Col2a1-TAP63α* transgenic mice

Three transgenic mouse lines were generated. Briefly, to generate *Col10a1-ΔNP63α* transgenic mice, a ~3.2 kb fragment was released from the *Col10a1-ΔNP63α* transgenic construct by *Bsr*bI digestion (Fig. 1A). The 3.2 kb *Bsr*bI fragment contains the hypertrophic chondrocyte-specific *Col10a1* promoter element, followed by the HA- and Flag-tagged *ΔNP63α* cDNA and a polyA sequence. To generate *Col2a1-ΔNP63α* and *Col2a1-TAP63α* transgenic mice, a ~14 kb fragment was released from above *Col2a1-ΔNP63α* or *Col2a1-TAP63α* transgenic construct by *Pac*I digestion (Fig. 2A and 4A). This fragment contains the whole transgene expression cassette which includes the tyrosinase expression cassette, the chondrocyte-specific *Col2a1* promoter, the HA- and Flag-tagged *ΔNP63α* or *TAP63α* cDNA with polyA, and the WPRE element and HS4 insulator. The above three DNA fragments containing either *ΔNP63α* or *TAP63α* expression cassette driven by cell-specific *Col2a1* or *Col10a1* promoter elements were purified and subjected to DNA microinjection. The purified DNAs were injected into pronuclei of FVB/N/J mouse zygotes and transplanted into pseudopregnant ICR mice by the University of Illinois at Chicago Transgenic Production Service (UIC-TPS) core facility. Transgenic founders or offspring were identified by PCR genotyping or by direct visual screening of the eye color (newborn mice) as described below.

2.3 Genotyping and skeletal phenotypic analysis

PCR genotyping was performed for all the *Col10a1-ΔNP63α*, *Col2a1-ΔNP63α* and *Col2a1-TAP63α* transgenic mice using DNAs prepared from skins, snipped tails, or limb tissues. The specific primer pairs HA-Forward1 (5'-GTA CCT GAC TAT GCA TAT CCG-3') and human *P63*-Reverse1 (5'-CAG TGG AAT ACG TCC AGG TG-3') were used for genotyping of *Col10a1-ΔNP63α* mice. Primers HA-Forward2 (5'-TCC GTA CGA

TGT TCC AGA CTA TGC T-3') and human *P63*-Reverse2 (5'-CGT CGA AAC TGT GCG GGC CT-3') were used for *Col2a1-ΔNP63α* and *Col2a1-TAP63α* mice genotyping. Transgenic construct *Col2a1-ΔNP63α* or *Col2a1-TAP63α* each has a tyrosinase expression cassette. This allows direct visual screening of transgenic mice based on their coat color or color of the naked eyes (black eyes), as these mice are on a FVB/N albino (white) background (Zufferey et al., 1999; Hsiao et al., 2004). After genotyping, transgenic (*TG*) mice or their wild-type (*WT*) littermates at embryonic day 15.5 (E15.5), 17.5 (E17.5), and postnatal day 1 (P1) stages were subject to skeletal phenotypic analysis. Mouse skeletons were prepared and stained with Alcian blue (8GX, Sigma, St. Louis, MO) and Alizarin red (Sigma, St. Louis, MO) according to published protocol and as previously described (Ovchinnikov D, 2009, Li et al., 2012). The signal intensity of the Alizarin red staining indicating ossification status were evaluated for mouse limb, digit, and tail bones (ossified caudal vertebrae numbers). At least three lines of *TG* and *WT* littermates from each of the *Col10a1-ΔNP63α*, *Col2a1-ΔNP63α* and *Col2a1-TAP63α* mouse lines were analyzed. The animal studies were approved by the animal care and oversight committees at the University of Illinois at Chicago and Rush University Medical Center.

2.4. Total RNA extraction, RT-PCR and qRT-PCR

Total RNAs from limb tissues of mice at E17.5 and P1 stages from above three transgenic mouse lines were prepared using the Trizol reagent (Invitrogen, Carlsbad, CA). These total RNAs were then reversely transcribed (RT) using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) to synthesize the first strand cDNA. The RT products were subjected to regular or real-time polymerase chain reaction (RT-PCR or qRT-PCR) to examine the mRNA levels of the transgenes (HA- and Flag-tagged *ΔNP63α* or *TAP63α*) and related marker genes. These genes include *Bax*, *Bcl-2*, *Col2a1*, *Col10a1*, *Runx2*, and *Sox9* that are related to chondrocyte differentiation, maturation, apoptosis, and/or bone formation. The endogenous *Gapdh* gene was used as a control for normalization of RNA quantity. The primer sequence and amplicon size were as listed (Table1). For regular RT-PCR, standard protocol was used to amplify the cDNA templates from both transgenic and littermate controls. For qRT-PCR, same amount of cDNA templates were amplified using the Bio-Rad iQ™ SYBR Green supermix with specific primers. The MyiQ Real-Time PCR detection system (Bio-Rad Hercules, CA) was used to calculate the mean threshold cycle number (CT value) of target genes and normalized with endogenous *Gapdh*. Data is collected from multiple runs of real-time PCR with duplicate templates. $P < 0.05$ indicate significant fold-change of mRNA.

2.5. Hematoxylin/eosin staining, and IHC analysis

Mouse limbs at E17.5 or P1 stages were collected and fixed in 10% formalin and stored in 70% ethanol. These mouse limbs were then subjected to dehydration, paraffin embedding and sectioning without decalcification. H & E staining was performed using standard protocol and as previously described (Ding et al., 2012). At least 30 longitudinal (sagittal) sections (5-μm-thick) of the limb growth plate from both *TG* and *WT* littermates were collected and analyzed with the microscope (Nikon Eclipse 80i, Nikon Instruments Inc., Melville, NY USA) and the Qcapture Suite software (version, 2.95.0, Quantitative Imaging Corp., USA). For immunohistochemical (IHC) analysis, detailed protocol using paraffin-embedded sections and Sox9 antibody (H-90, sc-20095, Santa Cruz) has been described previously (Li et al., 2012). The concentration used for the primary anti-Sox9 antibody was at 1:3000 dilution. Non-immune mouse IgG was used as a negative control. Tissue sections were further incubated with biotinylated secondary antibody (anti-rabbit IgG, Santa Cruz). Detection using the ABC kit (Elite PK-6200 Universal, VECTOR laboratories) and microscopic analysis was as previously described (Li et al., 2012).

2.6. Statistical Analysis

One-way ANOVA and student *t*-tests were used to assess the significance across experimental groups (*TG* vs *WT*). Values (ossified caudal vertebrae numbers) are presented as means $\pm$ SEM (standard error of mean). Real-time RT-PCR data was analyzed using $2^{-\Delta\Delta C_t}$ and student *t*-test (Livak KJ, Schmittgen TD, 2001; Pfaffl MW, 2001). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Insignificant skeletal consequence of *Col10a1-ΔNP63α* transgenic mice

To gain insight of the putative function of $\Delta NP63\alpha$, a *P63* transcript variant, during embryonic skeletal development, we have generated *Col10a1-ΔNP63α* transgenic mice using similar strategy (Li et al., 2012) and as described above (Fig. 1A). PCR genotyping using the HA- and human *P63*-specific primers indicated that we have successfully generated three *Col10a1-ΔNP63α* transgenic founders (Fig. 1B, left, lanes 2, 3, and 8). Transgene expression was also confirmed in the offspring of each of above transgenic founders by RT-PCR analysis using the same HA- and human *P63*-specific primers. The representative transgene expression in transgenic offspring of line 2 is shown (Fig. 1B, right, lanes 1, 3, 6, and 7).

We have performed skeletal phenotypic analysis of these *Col10a1-ΔNP63α* transgenic mouse lines. The ossification signals indicated by alizarin red staining were evaluated. No signal difference was observed between the limbs, digits and tail bones of *TG* and *WT* littermates at both E17.5 and P1 stages (Fig. 1C1–4 and data not shown). We specifically calculated the ossified caudal vertebrae for all three *Col10a1-ΔNP63α* transgenic lines at the P1 stage. No statistical significance was detected between *TG* and *WT* littermates (Fig. 1C5, $p > 0.05$), suggesting an insignificant role of $\Delta NP63\alpha$ during late embryonic skeletal development.

3.2. Insignificant skeletal consequence of *Col2a1-ΔNP63α* transgenic mice

To further investigate the role of $\Delta NP63\alpha$ during early embryonic skeletal development, we performed transgenic studies by targeting $\Delta NP63\alpha$ expression in chondrocytes using the chondrocyte-specific *Col2a1* regulatory elements (Fig. 2A). Given its tryrosinase expression cassette, *Col2a1-ΔNP63α* transgenic founders show black eyes as illustrated in Fig. 2B, (left). PCR genotyping using *P63* and HA-specific primers confirmed that we have successfully generated three *Col2a1-ΔNP63α* transgenic founders (Fig. 2C, top, lines 4, 11, and 12). The representative transgene expression in transgenic offspring of line 4 is shown (Fig. 2C, bottom, lanes 1, 2, 3, 4, and 6). The results indicated that we have successfully established *Col2a1-ΔNP63α* transgenic mouse lines.

We then performed skeletal staining of all three lines of *Col2a1-ΔNP63α* mice at E15.5, E17.5, and P1 stages as described above. No obvious skeletal difference was observed between *TG* and *WT* littermates at both E15.5 and E17.5 stages (data not shown). Skeletal staining of mice from representative line 4 at the P1 stage was shown. *TG* mice are similar in size as shown by whole skeleton preparation (Fig. 3, A1). The ossification signals in *TG* mice were similar to that of *WT* controls as indicated by alizarin red staining in mouse fore- and hind-limb digits and the tail bones (Fig. 3, A2–A4). The ossified caudal vertebrae showed no difference between *TG* and *WT* littermates of lines 4, 11 and 12 ($p > 0.05$, Fig. 3B). The results also suggested an insignificant role of $\Delta NP63\alpha$ during early embryonic skeletal development stages.

3.3. Delayed ossification in *Col2a1-TAP63α* transgenic mice

We have performed similar transgenic studies by targeting *TAP63α* expression in chondrocytes using the same chondrocyte-specific *Col2a1* regulatory elements (Fig. 4A). Direct visual and PCR genotyping indicated that we have generated four *Col2a1-TAP63α* transgenic founders (Fig. 4B, 4C top, lines 1, 4, 8, and 9). The representative transgene expression in transgenic offspring of line 1 is shown (Fig. 4C, bottom, lanes 3, 4, 5, 6, and 7). The results demonstrated that we have successfully established *Col2a1-TAP63α* transgenic mouse lines.

Skeletal phenotypes of *Col2a1-TAP63α* mice were also analyzed at E15.5, E17.5, and P1 stages using similar strategy as described above. No obvious skeletal difference was observed between *TG* and *WT* littermates at E15.5 based on whole skeletal staining of all four *Col2a1-TAP63α* transgenic mouse lines (data not shown). At E17.5, the representative *TG* mouse embryos from line 1 are generally smaller in size than the *WT* controls (Fig. 5, A1). These *TG* embryos show delayed ossification as less or no alizarin red staining was observed in the fore and hind limb digits and the tail bones (Fig. 5, A2–4, red arrows) compared to *WT* littermates. For mice at the P1 stage, *TG* mice are also smaller in size as shown by whole skeleton preparation (Fig. 5, B1). Faint ossification signals were observed in fore- and hind-limb digits of *WT* mice (Fig. 5, B2–3, black arrows) but not in *TG* mice (Fig. 5, B2–3, red arrows). Ossification signals can be observed up to the 12th caudal vertebra in *WT* mouse tail compared to the 10th caudal vertebra in the *TG* mouse tail (Fig. 5, B4). The ossified caudal vertebrae of all four lines of *Col2a1-TAP63α* mice at the P1 stage were calculated and subjected to statistical analysis. While no difference was observed between *TG* and *WT* littermates of line 8 ($p=0.08$), *TG* mice of lines 1, 4, and 9 have significant less ossified caudal vertebrae than their *WT* littermates (Fig. 5C, $p<0.05$, $p<0.01$, and $p<0.02$ respectively), suggesting a negative role of *TAP63α* at early embryonic skeletal developmental stage.

3.4. Relevant marker gene expression in *Col2a1-TAP63α* transgenic mice

We have examined the relative mRNA levels of certain marker genes in mouse limbs for all four *Col2a1-TAP63α* transgenic mouse lines at both E17.5 and P1 stages. The results showed that, *Col2a1*, *Col10a1*, *Sox9*, and *p63* genes are significantly upregulated in *TG* mouse embryos at E17.5 compared to *WT* littermates, while no obvious fold-changes was detected for *Bax*, *Bcl-2*, and *Runx2* genes (Fig. 6A, left). For mice at the P1 stage, only *p63* show significantly increased level of mRNA transcript in *TG* mice compared to the *WT* controls, while no notable difference was observed between *TG* and *WT* mice for genes *Bax*, *Bcl-2*, *Col2a1*, *Col10a1*, *Runx2*, and *Sox9* (Fig. 6A, right). To confirm the increased *Sox9* expression in *TG* mice at E17.5, we have performed immunohistochemical staining with or without anti-*Sox9* antibody on mouse limb (humerus) sections from line 1 at E17.5 (Fig. 6B). Much stronger brown staining indicating increased *Sox9* expression was observed in the resting and proliferative zones of *TG* mice (Fig. 6B, middle panels, red rectangles and arrows) compared to *WT* controls (Fig. 6B, left panels, black rectangles and arrows).

4. Discussion

It's generally accepted that *P63* gene has six transcript variants that encode multiple protein isoforms. Given the severe skeletal phenotypes in mice lacking *p63* and in human skeletal syndromes with *P63* mutations, *P63* apparently plays important function during skeletal development. However, how these *P63* isoforms function during bone formation remains unknown. *TAP63α* is the longest *P63* isoform that differs with Δ NP63α only in the N-terminus transactivation domain. We have recently performed *TAP63α* gain of function studies using the cell-specific *Col10a1* promoter elements. The results demonstrate that over

expressing *TAP63α* in hypertrophic chondrocytes, the terminal stage of chondrocyte differentiation, promotes late embryonic bone formation (Li et al., 2012).

Δ NP63 α has been majorly implicated a role in carcinogenesis, however, multiple studies have shown that Δ NP63 α is also a key player in epithelial and thymic development (Wu et al., 2003; Wu et al., 2005; Candi et al., 2007; Romano et al., 2012). To investigate if Δ NP63 α plays a role in bone development, we targeted Δ NP63 α expression in hypertrophic chondrocytes using the same *Col10a1* promoter elements (Zheng et al., 2009). Similar ossification signals were observed in the limb, digit and tail bones of *TG* and *WT* littermates at both E17.5 and P1 stages, suggesting an insignificant role of Δ NP63 α at the late stage of embryonic skeletal development (Fig. 1 and data not shown).

The above *Col10a1* promoter element was previously shown to drive transgene or reporter expression specifically in hypertrophic chondrocytes (Zheng et al., 2009; Li et al., 2012), the late cell stage of chondrocyte differentiation during endochondral bone formation. To further investigate the putative function of Δ NP63 α and *TAP63α* during early embryonic skeletal development, we have generated two additional transgenic mouse lines (*Col2a1-ΔNP63α* and *Col2a1-TAP63α*) using the chondrocyte specific *Col2a1* promoter element. This 6-kb *Col2a1* promoter-intron element was able to drive reporter expression specifically in chondrocytes, an early stage of cartilage development during endochondral ossification (Zhou et al., 1995). Skeletal phenotypic analysis revealed no difference between *TG* and *WT* littermates of *Col2a1-ΔNP63α* mice at E15.5, E17.5, and P1 stages (Fig. 3 and data not shown). This result suggests an insignificant role of Δ NP63 α at early stage of embryonic skeletal development. Surprisingly, while no obvious skeletal phenotypes were shown in *Col2a1-TAP63α TG* mice at E15.5, moderately delayed ossification was observed in the digit and tail bones of *Col2a1-TAP63α TG* mice at both E17.5 and P1 stages (Fig. 5). This result together with the accelerate skeletal phenotypes observed in *Col10a1-TAP63α TG* mice (Li et al., 2012) support a diverse function of *TAP63α* during different embryonic skeletal developmental stages.

To investigate the potential mechanism that leads to the delayed ossification in *Col2a1-TAP63α TG* mice, we have performed expression profiling of relevant genes. We detected significantly increased *p63* mRNA transcripts in *Col2a1-TAP63α TG* mice at both E17.5 and P1 stages, as we used specific primers that were able to amplify both human and mouse *p63* genes (Table 1). Interestingly, we also detected significantly upregulated levels of *Col2a1*, *Col10a1*, and *Sox9* transcripts in *TG* mice at E17.5 but not at P1 stage (Fig. 5A). The increased *Sox9* expression in *TG* mice at the E17.5 stage was also confirmed by IHC analysis using mouse limb sections and anti-*Sox9* antibody (Fig. 5B). The type II and type X collagen genes are known specific markers for chondrocyte differentiation and maturation, two critical steps for endochondral ossification. While it is not clear how altered expression of *Col2a1* and *Col10a1* impacts bone formation, mutations in *COL2A1* and *COL10A1* genes have previously been associated with multiple chondrodysplasias (Bateman et al., 2009). Most importantly, as an essential transcription factor for cartilage development, *Sox9* also acts as a major negative regulator for endochondral ossification (Bi et al., 2001; Akiyama et al., 2002; Wu et al., 2008; Hattori et al., 2010). It was previously shown that *SOX9* dominates and determines the transactivity of *RUNX2*, an essential transcription factor for osteoblast differentiation during bone formation (Otto et al., 1999; Zhou et al., 2006; Cheng et al., 2010). While it is not clear if *Sox9* is a direct transcriptional target of *TAP63α*, the delayed ossification in *Col2a1-TAP63α TG* mice may be partially attributed to increased *Sox9* expression.

In summary, we have investigated the in vivo function of *p63* variants, here in Δ NP63 α and *TAP63α*, during endochondral bone formation. Our results suggest a putative role of

TAP63 α , but not Δ NP63 α , during embryonic skeletal development. Given the diverse skeletal phenotypes in *Col2a1-TAP63 α* and *Col10a1-TAP63 α* TG mice, TAP63 α may play distinct function during different embryonic skeletal developmental stages (Li et al., 2012). However, we notice that both the *Col2a1-TAP63 α* and the *Col10a1-TAP63 α* TG mice only show moderate skeletal phenotypes. These results together may suggest a requirement of other alternative P63 variants (such as TAP63 β/γ and/or Δ NP63 β/γ) during bone development. Interestingly, two new C-terminus p63 variants, namely p63 δ and ϵ , were recently identified and increased the number of the p63 isoforms from 6 to 10. These p63 variants all show a tissue/cell-type-specific nature and are therefore, related to their different cellular functions (Mangiulli et al., 2009). Further characterization of these P63 variants during chondrocyte and osteoblast differentiation will provide novel insights of P63 (isoforms) upon skeletal development and disease progression.

Acknowledgments

We are grateful to Dr. Kotaro Sena and Mr. David Karwo for technical help on histological analysis of the transgenic mice at the Rush soft tissue and decalcified hard tissue histology sub-core. All transgenic mice were initially generated within the Transgenic Production Service core facility (directed by Dr. Roberta Franks) at the University of Illinois at Chicago. This work was supported by the 2008 Rush University Pilot Project (Q.Z.), the Bear Necessities Pediatric Cancer Foundation (Q.Z.), the grant from the American Cancer Society, Illinois Division, Inc (#254598, Q.Z.), the NIH/NCI grant (R21CA161461, Q.Z.), and the NSFC grant (31271399, Q.Z., J.G.).

Abbreviations

RT-PCR	Reverse transcription polymerase chain reaction
q-(RT-)PCR	quantitative or real-time (RT-) polymerase chain reaction
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
Col10α1	type X collagen, alpha 1
mTrp63	murine transformation related protein 63
Bax	BCL-2-associated X protein
Bcl-2	B-cell leukemia (lymphoma) 2
Runx2	runt-related transcription factor 2
Sox9	SRY-box containing gene 9
H & E staining	Hematoxylin/Eosin
BGHPA	Bovine growth hormone polyA
XBP	<i>Col10a1</i> basal promoter
WT	Wild-type
TG	Transgenic
E15.5	Embryonic day 15.5
E17.5	Embryonic day 17.5
P1	Postnatal day one
ANOVA	Analysis of variance between groups

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Highlights

Transgenic mice expressing P63 variants were generated using *Col2a1/Col10a1* elements. No skeletal abnormalities were observed in *Col2a1-* or *Col10a1-ΔNP63α* transgenic mice. Delayed ossification and increased Sox9 expression was shown in *Col10a1-ΔNP63α* mice. Conclusion: *P63* variants play distinct function during embryonic skeletal development.

Figure 1. *Col10a1-ΔNP63α* transgenic mice

(A). Schematic map of *Col10a1-ΔNP63α* transgenic construct. The hypertrophic chondrocyte-specific *Col10a1* cis elements (300-bp cis-enhancer and 265-bp basal promoter) were illustrated and have been described previously (Li et al., 2012). The HA and Flag tagged human *ΔNP63α* cDNA were cloned into pcDNA3.1(-) upstream of the bovine growth hormone polyA (BGHpA). *BsrB1* and *SalI*: restriction enzymes. XBP: *Col10a1* basal promoter; ATG: start codon; TAG: stop codon. (B). *Col10a1-ΔNP63α* transgenic mouse lines. The 3.2 kb *BsrB1* fragment containing the transgene were purified and subjected to microinjection. PCR genotyping using mouse tail genome DNA and human *P63*- and *HA*-specific primers showed that we have generated three transgenic founders (left, lanes 2, 3, and 8). Transgenic mouse lines were established by breeding transgenic founders with wild-type mice and as confirmed by PCR genotyping of the offspring (right). (C). Skeletal phenotype of *Col10a1-ΔNP63α* mice. The *TG* and *WT* controls at the P1 stage were subjected to skeletal preparation and stained by alcian blue and alizarin red. No difference of alizarin red staining (ossification signals) was observed between *TG* and *WT* mice for the whole skeleton (C1), fore limb phalanges (C2), hind limb metatarsals (C3), and tail bones (C4). Ossified caudal vertebrae were statistically analyzed for all mice at the P1 stage (C5). Line2: N=9, Cwt=10.0±0.4, Ctg=9.8±0.2, $p>0.05$; Line3: N=7, Cwt=11.3±0.3, Ctg=9.3±0.9, $p>0.05$; and Line8: N=13, Cwt=9.0±0.5, Ctg=9.6±0.5, $p>0.05$.

Figure 2. Generation of *Col2a1-ΔNP63α* transgenic mice

(A). *Col2a1-ΔNP63α* transgenic construct: HA- and Flag-tagged human $\Delta NP63\alpha$ cDNA and the polyA sequence were cloned into the *Swa1* site downstream of the chondrocyte-specific *Col2a1* cis-element in a modified coat-colored vector containing a TYR cassette, a WPRE element, and a HS4 insulator sequence (Hsiao et al., 2004). *Pac1* and *Swa1*: restriction enzymes. (B). Visual genotyping: The *Pac1* fragment containing transgene $\Delta NP63\alpha$ was purified and subjected to microinjection. Representative *Col2a1-ΔNP63α* TG founder mouse was identified by its eye color at the P1 stage. Black eyes indicate tyrosinase expression (left, black arrow) on the FVB/N white background. (C). PCR genotyping and transgene expression: PCR genotyping with mouse tail genome DNA and human *P63* and *HA* fragment-specific primers confirmed that we have generated three *Col2a1-ΔNP63α* transgenic founders (top, lanes 4, 11 and 12). Transgenic mouse lines were established as illustrated by the RT-PCR result showing transgene (HA-tagged $\Delta NP63\alpha$) expression in the limbs of offspring of *Col2a1-ΔNP63α* line 4 (bottom, lanes 1–4 and 6).

Figure 3. Skeletal phenotypes of *Col2a1-ΔNP63α* transgenic mice

(A). A1: Whole skeletal staining of mice of *Col2a1-ΔNP63α* transgenic mouse lines at the P1 stage did not observe any difference between *TG* and *WT* littermates. B2: Fore limbs, the phalange and digit bones of both *TG* and *WT* controls showed similar ossification signals (alizarin red staining). B3: Hind limbs, the metatarsal and digit bones of *TG* and *WT* littermates also showed similar ossification signals. B4: Tail bones, alizarin red staining can be seen up to the 10th caudal vertebra of both *TG* and *WT* mouse tails. Black arrow: wild-type, Red arrow: transgenic. (B). Illustrated are statistical analysis of the ossified caudal vertebrae of all three lines of *Col2a1-ΔNP63α* transgenic mice at the P1 stage. Line4: N=9, $C_{wt} = 11.4 \pm 0.9$, $C_{TG} = 11.8 \pm 0.5$, $p > 0.05$; Line11: N=11, $C_{wt} = 10.2 \pm 0.8$, $C_{TG} = 10.3 \pm 0.5$, $p > 0.05$; and Line12: N=11, $C_{wt} = 10.4 \pm 1.1$, $C_{TG} = 10.0 \pm 0.6$, $p > 0.05$.

Figure 4. Generation of *Col2a1-TAP63α* transgenic mice

(A). *Col2a1-TAP63α* transgenic construct. HA- and Flag-tagged human *TAP63α* cDNA and the polyA sequence were cloned into the *Swa1* site downstream of the chondrocyte-specific *Col2a1* cis-element in the same modified coat-colored vector (Hsiao et al., 2004) as described above. *Pac1* and *Swa1*: restriction enzymes. (B). Visual genotyping: The *Pac1* fragment containing transgene *TAP63α* was purified for microinjection. The *Col2a1-TAP63α* TG founder mice were also identified by their eye color showing tyrosinase expression on the FVB/N white background at the P1 stage (left, black arrow). (C). PCR genotyping and transgene expression: PCR genotyping using mouse tail genome DNA and human *P63* and *HA* fragment-specific primers indicated that we have generated four *Col2a1-TAP63α* transgenic founders (top, lanes 1, 4, 8, and 9). The representative RT-PCR result shows transgene (HA-tagged *TAP63α*) expression in the limbs of offspring of *Col2a1-TAP63α* mouse line 1 (bottom, lines 3–7). “–” and “+” are negative and positive PCR control.

Figure 5. Skeletal Phenotypes of *Col2a1-TAP63a* transgenic mice

(A). Skeletal staining of mouse embryos at E17.5 stage. A1: Whole skeletal staining showed smaller skeletal size of *TG* mice with less ossification signal (alizarin red staining) in metatarsal bones compared to *WT* littermates. A2: Fore limb, ossification signal can be seen in the middle phalanges of *WT* mouse embryo but not in the *TG* mouse (one of the fore limbs was used for histological analysis). A3: Hind limb, the metatarsal bones show faint ossification signs in *WT* but not *TG* embryos (one of the hind limbs was used for RNA extraction). A4: Tail bones, ossification signs were clearly shown in the seventh caudal vertebra of *WT* mouse embryo compared to the faint signal in the sixth caudal vertebra of *TG* mouse embryo. (B). Skeletal staining of mice at P1 stage. B1: Whole skeletal staining showed smaller size of the *Col2a1-TAP63a* *TG* mouse than its *WT* littermate. B2: Fore limbs, faint ossification signal can be seen in the digit of *WT* but not *TG* mouse. B3: Hind limbs, more intensive ossification signals can be seen in the calcaneus and digits of *WT* than in *TG* mouse. B4: Tail bones, alizarin red staining showed clear ossification signal up to the 12th caudal vertebra in *WT* mouse tail compared to the faint signal in the 10th caudal vertebra of *TG* mouse tail. Black arrow: wild-type, Red arrow: transgenic. (C). Illustrated are statistical analysis of the ossified caudal vertebrae from four *Col2a1-TAP63a* transgenic

mouse lines at the P1 stage. Line1: N=12, Cwt=10.3±0.8, Ctg=9.2±1.0, p<0.05; Line4: N=11, Cwt=11.9±0.4, Ctg=10.8±0.5, p<0.01; Line8: N=11, Cwt=11.6±0.5, Ctg=10.5±1.3, p=0.08; and Line9: N=12, Cwt=10.8±0.4, Ctg=9.8±0.8, p<0.02.

Figure 6. Relevant marker gene expression in *Col2a1-TAP63a* transgenic mice

(A). Total RNAs from mouse hind limbs at E17.5 (left) and P1 (right) stages were prepared and reversely transcribed for expression analysis using qRT-PCR approach. Illustrated are the relative mRNA levels of indicated marker genes from representative line 4. *p63* is significantly upregulated (3–5 folds) in *TG* mice at both E17.5 (*WT* vs. *TG*: 1.00 ± 0.34 vs. 4.81 ± 0.92 , $N = 10$, $p < 0.05$) and P1 (*WT* vs. *TG*: 1.00 ± 0.08 vs. 2.79 ± 0.44 , $N = 10$, $p < 0.05$) stages, while *Col2a1*, *Col10a1*, and *Sox9* are upregulated (2–3 folds) only at the E17.5 stage (*Col2a1*: *WT* vs. *TG*: 1.00 ± 0.21 vs. 1.78 ± 0.32 ; *Col10a1*: *WT* vs. *TG*: 1.00 ± 0.23 vs. 2.11 ± 0.24 ; and *Sox9*: *WT* vs. *TG*: 1.00 ± 0.19 vs. 2.05 ± 0.59 ; $N = 10$, $p < 0.05$). The mRNA levels of *Bax*, *Bcl2*, and *Runx2* in *TG* mice are similar to that of *WT* controls in both stages. (B). Sagittal sections of proximal humerus from both *TG* and *WT* mouse limbs at the E17.5 stage were subjected to immunohistochemical analysis using anti-*Sox9* antibody. More brown staining indicating upregulated *Sox9* expression was observed in the proliferative, and hypertrophic zone of *TG* mice (top, middle) compared to the *WT* (top, left) or no antibody control (top, right), 20x magnification. Bottom panels highlight the corresponding IHC results from the proliferative zone of both *TG* and *WT* mice as indicated (arrowed rectangle). Much stronger immunostaining signals were observed in the nuclear area of *TG* mice (bottom, middle, red arrows) compared to the littermate control (bottom, left, black arrows).

Table 1

Primers designed for real-time PCR

Name	RefSeqID	Sense Primer (5'-3')	Antisense Primer (5'-3')	Size (bp)
<i>Gapdh</i>	NM_008084	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC	171
<i>Bax</i>	NM_007527	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTAG	173
<i>Bcl-2</i>	NM_009741	CTGGCATCTTCTCCTTCCAG	GACGGTAGCGACGAGAGAAG	183
<i>Col2a1</i>	NM_031163.3	CACACTGGTAAGTGGGGCAAGACCG	GGATTGTGTTGTTTCAGGGTTCGGG	173
<i>Col10a1</i>	NM_009925	AAAGCTTACCCAGCAGTAGG	ACGTACTCAGAGGAGTAGAG	319
<i>Runx2</i>	NM_001145920	ACCCAGCCACCTTTACCTAC	TATGGAGTGCTGCTGGTCTG	150
<i>Sox9</i>	NM_011448	TTCATGAAGATGACCGACGA	ATGCACACGGGGAACCTATC	200
<i>mTrp63</i>	NM_001127259	TCAGCCACCTGGACGTATTC	TCATTGAACTCACGGCTCAG	202

Gapdh: glyceraldehydes-3-phosphate dehydrogenase; **Bax**: BCL-2-associated X protein; **Bcl-2**: B-cell leukemia (lymphoma) 2; **Col2a1**: type II collagen, alpha 1; **Col10a1**: type X collagen, alpha 1; **Runx2**: runt-related transcription factor 2; **Sox9**: SRY-box containing gene 9; **mTrp63**: murine transformation related protein 63