**Original Article**

**Mouse p63 variants and chondrogenesis**

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**Abstract:** As a critical member of the p53 family of transcription factors, p63 has been implicated a role in development than in tumor formation, because p63 is seldom mutated in human cancers, while p63 null mice exhibit severe developmental abnormalities without increasing cancer susceptibility. Notably, besides the major epithelial and cardiac defect, p63 deficient mice show severe limb and craniofacial abnormalities. In addition, humans with p63 mutations also show severe limb and digit defects, suggesting a putative role of p63 in skeletal development. There are eight p63 variants which encode for the TAp63 and ΔNp63 isoforms by alternative promoters. How these isoforms function during skeletal development is currently largely unknown. Our recent transgenic studies suggest a role of TAp63α, but not ΔNP63α, during embryonic long bone development. However, the moderate skeletal phenotypes in the TAP63α transgenic mice suggest requirement of additional p63 isoform(s) for the limb defects in p63 null mice. Here, we report analysis of mouse p63 variants in MCT and ATDC5 cells, two cell models undergo hypertrophic differentiation and mimic the process of endochondral bone formation upon growth arrest or induction. We detected increased level of p63 variants in hypertrophic MCT cells by regular RT-PCR analysis. Further analysis by qRT-PCR, we detected significantly upregulated level of γ variant (p<0.05), but not α or β variant (p>0.05), in hypertrophic MCT cells than in proliferative MCT cells. Moreover, we detected upregulated TAP63γ in ATDC5 cells undergoing hypertrophic differentiation. Our results suggest that TAp63γ plays a positive role during endochondral bone formation.

**Keywords:** Mouse chondrocytes, p63 variants, TAp63γ, qRT-PCR

**Introduction**

p63, also called transformation related protein 63 (Trp63), is an important member of the p53 family of transcription factors [1, 2]. Given their structural similarity, p63 was originally thought to have overlapping functions with p53 by regulating common down-stream target genes. However, unlike p53, p63 has been majorly implicated a role in development than in tumor formation, because not many p63 mutations have been found in human cancers, while severe developmental abnormalities were observed in p63 deficient mice that do not increase cancer susceptibility [3]. The predominant function of p63 is in epithelial development, as p63 null mice lack epidermis and other epithelia [4-6]. p63 may also be important for heart development given its cardiac defects in p63 null mouse embryos [7]. Notably, mice deficient for p63 also show severe skeletal defects including absent or truncated limbs and craniofacial skeletal abnormalities [1, 8]. The limb phenotype is majorly attributed to defect in apical ectodermal ridge (AER), which is a specialized epithelium at the limb bud directing its outgrowth along the axis [1, 9]. However, the shortened limb and craniofacial skeletal changes suggest that both endochondral and intramembranous ossifications are impaired in p63 null mice. In humans, P63 mutations are associated with EEC (ectrodactyly, ectodermal dysplasia, and cleft lip/palate) or SHFM (split hand-split foot malformation) syndrome, which also shows similar limb defects as seen in p63 null mice [10]. These observations suggest a role of p63 in long bone development, possibly by affecting endochondral bone formation that involves critical steps of chondrocyte differentiation and hypertrophy (or maturation).

p63 is generally divided into two major groups, TAP63 and ΔNP63, that are consisted of six
transcriptional variants encoding six different isoforms: TAP63α, -β, -γ and ΔNP63α, -β, -γ [11]. These p63 isoforms have been shown to play multiple functions during development and cancer formation [5]. However, the specific p63 isoforms that may play a role in bone and cartilage development is currently largely unknown. We have recently performed p63 gain-of-function studies using p63 variants TAP63α and ΔNP63α and the (hypertrophic) chondrocyte-specific Col2a1 or Col10a1 control elements. The results suggest an insignificant role of ΔNP63α in embryonic skeletal development, while TAP63α may play distinct functions during different skeletal developmental stages [12, 13]. However, the moderate skeletal phenotypes seen in TAP63α transgenic mice strongly suggest that additional p63 isoform(s) is required to be responsible for the severe skeletal defects seen in p63 null mice. In this manuscript, we report systematic analysis of p63 variants in two chondrogenic cell models: MCT and ATDC5 cells [14, 15]. We detected varied levels of p63 transcripts in these cells with the γ variants being more abundant. Moreover, TAP63γ is significantly upregulated both in hypertrophic MCT cells and in ATDC5 cells undergoing hypertrophic differentiation. Our results suggest that TAP63γ promotes chondrogenesis, and thereby, plays a positive role during endochondral bone formation.

**Materials and methods**

**Analysis of p63 variants**

Based on literature review and the gene records in NCBI (National Center for Biotechnology Information) database, we performed detailed sequence analysis of the multiple mouse p63 variants with the most recently updated information. The gene structure of p63 variants was drawn based on previous studies and modified with updated information [16].

**Cell culture, total RNA extraction and cDNA synthesis**

Mouse chondrocytes (MCT cells) were cultured at 32 °C in standard DMEM with 8% FBS (Gibco
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Figure 1. Structure of mouse p63 variants. There are 8 mouse p63 variants (V) encoding two subtypes of isoforms TAp63 and ΔNP63. V1 is the longest p63 mRNA transcript encoding the longest isoform a--TAP63α, which includes transactivation (TA), DNA-binding, Oligomerization (Oligo), Sterile alpha motif (SAM), and Transcription inhibition (TID) domains as illustrated. Compared to V1 (or isoform a), V2 lacks a 3' exon and encodes isoform b--TAp63β, with a shorter and different C-terminus; V3 lacks an internal 12 nt and several 3' exons (an alternate 3' exon) and encodes isoform c--TAp63γ, with a shorter and different C-terminus; V4 (and V5, lacks an internal 12 nt) lacks several 5' exons (an alternate 5' exon) and encodes isoform d (and e)--ΔNP63α with a shorter and different N-terminus; V6 lacks several 5' exons (an alternate 5' exon) and an internal 3' exon and encodes isoform f--ΔNP63β with shorter and different N- and C-termini; V7 (and V8, lacks an internal 12 nt) lacks several 5' and 3' exons, (with alternate 5' and 3' exons) and encodes isoform g (and h)--ΔNP63γ with shorter and different N- and C-termini.

BRL) and 8% CO₂ as per published protocol [14, 15]. After grown until sub-confluence, these MCT cells were further cultured at either 32 °C (proliferative) or 37 °C for additional 3 days (become hypertrophic) before harvest. Total RNAs from both proliferative and hypertrophic MCT cells were isolated and reversely transcribed using Trizol reagent and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) to synthesize the first strand cDNA. ATDC5 cells were maintained in a mixed DMEM/F-12 (1:1) medium (Invitrogen) with 5% FBS and 1% human insulin, transferrin, and sodium selenite (ITS, Sigma) at 37 °C and 5% CO₂ [17]. Cells were then harvested at days 0, 4, 7, 10, 14, and 21 and subjected to RNA extraction and cDNA synthesis respectively as described above.

Expression analysis of genes using real-time/qRT-PCR

The RT product was subjected to real-time or quantitative polymerase chain reaction (qRT-PCR) to show the relative mRNA levels of genes of interest. These genes include hypertrophic chondrocyte-specific Col10a1, Runx2, as well as p63 variants and the endogenous control gene Gapdh for normalization of the RNA quality and quantity. For qRT-PCR, the cDNA templates were amplified with relevant gene- or p63 variants-specific primers (listed in Table 1) using the Bio-Rad iQ™ SYBR Green supermix and the MyiQ Real-Time PCR Detection System (Bio-Rad Hercules, CA). Relative mRNA changes were analyzed by manufacturer provided MyiQ Optical System Software. The mean threshold cycle number (CT values) of target genes was normalized to endogenous Gapdh and calculated using 2⁻ΔΔCt and student t-test [18, 19]. Data is collected from multiple runs of real-time PCR with duplicate templates and the relative mRNA level was compared between proliferative and hypertrophic MCT cells and between day 0 and days 4, 7, 10, 14, and 21 respectively. p<0.05 was considered statistically significant fold change of mRNA level between samples.

Sequence analysis of the RT-PCR product

Regular RT-PCR was performed using standard protocol to amplify the cDNA templates from
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ATDC5 cells with designated primes as listed in Table 2. The RT-PCR products were subjected to electrophoresis in agarose gel and ethidium bromide (EB) staining. Selected PCR product was confirmed by sequencing at the University of Illinois at Chicago’s Center for Genomic Research–DNA Services Facility.

Results

Mouse P63 variants in NCBI database

Based on the most recently updated NCBI database (October 2013), mouse p63 gene is consisted of eight transcriptional variants (variants 1-8) that encode eight p63 isoforms a-h. Among these, isoform a, also called TAp63α, is the longest isoform encoded by variant1. Compared to variant1, some variants lack several 5’-exons due to the alternative use of their 5’-promoters and result in different N-termini, while some other variants lack some 3’-exons due to alternative splicing and thereby, lead to different C-termini. There are also variants that lack 12 nucleotides within internal exons and thus, lead to an isoform lacking an internal 4 aa. The structure of each of the eight variants is as illustrated (Figure 1).

Hypertrophic differentiation of MCT and ATDC5 cells

Total RNAs from MCT cells growing at 32 °C and 37 °C (for 3 days) were reversely transcribed and the cDNA templates were used for real-time PCR to examine the relative mRNA levels of Col10a1 and Runx2, two marker genes of chondrocyte hypertrophy or maturation.

After normalization to Gapdh, both Col10a1 (8.8 fold, p=0.009) and Runx2 (4.0 fold, p=0.04) show significant upregulation in hypertrophic MCT cells compared to that in proliferative MCT cells grown for 3 days at 37 °C. Similarly, total RNAs from ATDC5 cells cultured with or without ITS induction were also reversely transcribed and subjected to real-time PCR analysis of Col10a1. The results show that Col10a1 is significantly upregulated in cells maintained in ITS medium and grown for 7, 10, 14, and 21 days (but not 4 days) respectively compared to cells in day 0 without ITS induction (Figure 2B). Among these, day 14 shows the highest level of Col10a1 upregulation (5 fold, p<0.001). These results demonstrated the successful induction of hypertrophic differentiation of the corresponding ATDC5 cells.

p63 variants expression in MCT and ATDC5 cells

To examine the expression of p63 variants in MCT cells, semi-quantitative regular RT-PCR was performed using cDNA templates from both proliferative and hypertrophic MCT cells with ΔNP63, TAp63 and variant-specific primers (listed in Table 1). The results suggest more abundant of mRNA transcripts that encode isoforms α and γ than isoform β (data not shown). qRT-PCR was then performed using the same

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primers and cDNA templates generated from MCT cells. The result showed that both $\Delta NP63$ (1.8 fold, $p=0.04$) and TAp63 (2.6 fold, $p=0.002$) are significantly upregulated in hypertrophic MCT cells compared to proliferative MCT cells. Using variant-specific primers, we detected significantly upregulated $\gamma$ variant (4.6 fold, $p=0.002$), but not $\alpha$ or $\beta$ variant ($p>0.05$), in hypertrophic MCT cells. B: p63 variants were examined in Day 14 ATDC5 cells after ITS induction and compared to that in Day 0 ATDC5 cells. While the mRNA level of $\Delta NP63$, TAp63, and variants $\alpha$ and $\beta$ did not show significant change ($p>0.05$), $P63-\gamma$ variants show significant upregulation after treatment with ITS for 14 days (2.3 fold, $p=0.04$) compared to that in Day 0 ATDC5 cells without ITS induction.

**Figure 3.** Expression of p63 variants in MCT and ATDC5 cells. A: qRT-PCR was performed to examine p63 variants in MCT cells. As illustrated, both $\Delta NP63$ (1.8 fold, $p=0.04$) and TAp63 (2.6 fold, $p=0.002$) are significantly upregulated in hypertrophic MCT cells compared to proliferative MCT cells. Using variant-specific primers, we detected significantly upregulated $\gamma$ variant (4.6 fold, $p=0.002$), but not $\alpha$ or $\beta$ variant ($p>0.05$), in hypertrophic MCT cells. B: p63 variants were examined in Day 14 ATDC5 cells after ITS induction and compared to that in Day 0 ATDC5 cells. While the mRNA level of $\Delta NP63$, TAp63, and variants $\alpha$ and $\beta$ did not show significant change ($p>0.05$), $P63-\gamma$ variants show significant upregulation after treatment with ITS for 14 days (2.3 fold, $p=0.04$) compared to that in Day 0 ATDC5 cells without ITS induction.

**Figure 4.** TAP63$\gamma$ in hypertrophic ATDC5 cells. Regular RT-PCR using TAp63, $\Delta NP63$, and $\gamma$ variants-specific primers detected mRNA transcripts of TAp63 and $\gamma$ variants, but not $\Delta NP63$ variants, both in Day 0 ATDC5 cells and in Day 14 ATDC5 cells with ITS induction (lanes 1, 1', 3, and 3'). TAP63$\gamma$ is expressed in Day 14 ATDC5 cells (lane 2') but not in Day 0 ATDC5 cells (Lane 2). Sequence analysis of the PCR product (lane 2') confirmed that it contains sequence from exon 9 of TAP63$\gamma$.

**Table 2.** Similar to the findings in MCT cells, we detected mRNA transcripts of TAp63 and $\gamma$ variants (lanes 1, 1', 3, and 3'), but not $\Delta NP63$ variants (lanes 4, 4', 5, and 5'), both in Day 0 ATDC5 cells and in Day 14 ATDC5 cells with ITS induction. In addition, we detected TAP63$\gamma$ in Day 14 ATDC5 cells (lane 2') but not in Day 0 ATDC5 cells (Lane 2, Figure 4A). Sequence analysis confirmed that the PCR product contains sequence from exon 9 of TAP63$\gamma$ (Figure 4A).

**TAP63$\gamma$ is upregulated in hypertrophic ATDC5 cells**

To further determine the specific p63 variants that are expressed in ATDC5 cells, we performed regular RT-PCR using TAp63, $\Delta NP63$, and $\gamma$ variants-specific primers as listed in Table 2. Similar to the findings in MCT cells, we detected mRNA transcripts of TAp63 and $\gamma$ variants (lanes 1, 1', 3, and 3'), but not $\Delta NP63$ variants (lanes 4, 4', 5, and 5'), both in Day 0 ATDC5 cells and in Day 14 ATDC5 cells with ITS induction. In addition, we detected TAP63$\gamma$ in Day 14 ATDC5 cells (lane 2') but not in Day 0 ATDC5 cells (Lane 2, Figure 4A). Sequence analysis confirmed that the PCR product contains sequence from exon 9 of TAP63$\gamma$ (Figure 4A).
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Figure 3

Figure 2

Figure 4

These results suggest a role of TAP63γ in ATDC5 cells undergoing hypertrophic differentiation.

Discussion

Most of our skeleton develops through a pathway called endochondral ossification. This involves multiple stages of chondrocyte differentiation and maturation characterized by expression of chondrocyte- and hypertrophic chondrocyte-specific genes Col2a1 and Col10a1 [20]. Multiple transcription factors and signaling pathways have been shown to control the process of endochondral bone formation [21, 22]. The severe limb and digit defects in p63 null mice and in humans with P63 mutations suggest a role of p63 during skeletal development, possibly by affecting the endochondral bone formation pathway [1, 8, 10, 23, 24]. p63 is consisted of multiple transcriptional variants encoding two types of p63 isoforms TAp63 and ΔNP63 that each has three subtypes α, β, and γ. These six p63 isoforms have been extensively studied and are known to play distinct functions during development and cancer formation [5]. However, how p63 and its variants function during chondrocyte differentiation and maturation, steps critical for endochondral bone formation during long bone development, is currently largely unknown.

In this manuscript, we report analysis of mouse p63 variants by literature review and by referring to the most updated NCBI database. p63 is generally known to consist of 6 isoforms, however, more human P63 transcript variants that may encode additional P63 isoforms have recently been reported [25, 26]. In mouse, based on the latest NCBI database, there are two more p63 transcript variants which make functional studies of p63 more complicated (Figure 1). To study the potential function of p63 during endochondral bone formation, we analyzed its expression in MCT and ATDC5 cells, two cell models that undergo chondrocyte hypertrophic differentiation either by growth arrest or by induction [14, 15]. The hypertrophic MCT or ATDC5 cells show significant upregulation of Col10a1 and Runx2 (Figure 2) and, therefore, act as an in vitro cell model mimicking the process of endochondral ossification [14, 15]. We have previously reported detection of p63 in MCT cells. Notably, p63 showed ~3 fold upregulation in hypertrophic MCT cells compared to proliferative MCT cells [12]. To examine the specific p63 variants and their expression level within the MCT cells, we performed qRT-PCR using p63 and variant-specific primers. In accordance with previous observation, we detected significantly upregulated ΔNP63 and TAP63 in hypertrophic MCT cells. Interestingly, while the p63-α and p63-β variants did not show significant change, the γ variant(s) is significantly higher in hypertrophic MCT cells. In addition, we also detected significantly higher level of γ variant(s) in Day 14 hypertrophic ATDC5 cells compared to Day 0 ATDC5 cells, while the mRNA levels of ΔNP63, TAP63, p63-α, and p63-β variants did not show significant change (Figure 3). Moreover, our regular RT-PCR detected TAp63y variant, which was confirmed by sequencing, in ATDC5 cells undergoing hypertrophic differentiation, but not in Day 0 ATDC5 cells (Figure 4), suggesting a potential role of γ variants, especially TAp63y during chondrocyte maturation.

It has been shown that p63 isoforms have overlapping and distinct functions during development and cancer formation [2]. Generally, the TAP63 isoforms act as transcription factors, while the ΔNP63 isoforms lack the main transactivation (TA) domain and act as dominant-negative inhibitors of TA isoforms. Interestingly, p63 has been implicated a role in bone development for over a decade, however, not many studies have been reported thereafter that aim to delineate p63’s function during skeletal development. This is at least partially due to the multiple isoforms of p63 and their complex functions during development. We have recently performed p63 gain-of-function studies by generating a series of transgenic mice using chondrocyte or hypertrophic chondrocyte-specific Col2a1 or Col10a1 control elements to drive ΔNP63α or TAP63α variant. Phenotypic analyses of these Col2a1-ΔNP63α/TAP63α and Col10a1-ΔNP63α/TAP63α transgenic mice suggest an insignificant role of ΔNP63α during embryonic skeletal development, while TAP63α may play a distinct function during different skeletal developmental stages [12, 13]. However, as mentioned before, the skeletal phenotypes in TAP63α transgenic mice are moderate, suggesting a potential importance of other p63 isoforms in controlling bone formation. The fact that TAp63y is expressed in proliferative chondrocytes and upregulated
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upon hypertrophic differentiation suggests a potential involvement of TAp63γ in chondrocyte differentiation and maturation during endochondral bone formation. In addition, TAp63γ has been indicated as the most active form showing strong transcriptional activities toward target genes [11]. These findings make TAp63γ an interesting target and worth further investigation regarding its function during skeletal development.

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Disclosure of conflict of interest

All authors have no conflict of Interest.

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