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All-trans-retinoid acid (ATRA) suppresses chondrogenesis of rat primary hind limb bud mesenchymal cells by downregulating p63 and cartilage-specific molecules

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ABSTRACT

P63 null mice have no or truncated limbs and mutations in human *p63* cause several skeletal syndromes that also show limb and digit abnormalities, suggesting its essential role in bone development. In the current study, we investigated the effect of ATRA on chondrogenesis using mesenchymal cells from rat hind limb bud and further examined the mRNA and protein expression of Sox9 and Col2a1 and p63 in rat hind limb bud cells. Limb buds were isolated from embryos from euthanized female rats. Growth of hind limb bud mesenchymal cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays. Formation of cartilage nodules was examined by Alcian blue-nuclear fast red staining. The expression of Sox9, Col2al and p63 was determined by Real-time RT-PCR and immunoblotting assays, respectively. Our MTT assays revealed that ATRA at 1 and 10 μM significantly suppressed the growth of mesenchymal cells from rat hind limb bud at 24 and 48 h ($P < 0.01$ *vs.* controls). Alcian blue staining further showed that ATRA caused a significant dose-dependent reduction in the area of cartilage nodules ($P < 0.05$ in all *vs.* controls). At 1 μM ATRA, the area of cartilage nodules from hind limb bud cells was reduced to 0.05 ± 0.03 mm from 0.15 ± 0.01 mm in controls. Real-time RT-PCR assays further indicated that 1 and 10 μM ATRA markedly reduced the mRNA expression of Sox9, Col2al and p63 in hind limb bud cells ($P < 0.05$ in all *vs.* controls). In addition, ATRA time-dependently inhibits the mRNA expression of p63, Sox9 and Col2al. Western blotting assays additionally showed that ATRA dose-dependently reduced the expression of Sox9, Col2al and p63 ($P < 0.05$ in all *vs.* controls). Together, our results suggest that ATRA suppresses chondrogenesis by modulating the expression of Sox9, Col2al and p63 in primary hind limb bud mesenchymal cells.

1. Introduction

P63, a member of the *p53* gene family, is located on chromosome 3q27-29 in humans; it shows a high homology to the

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tumor suppressor *p53* in sequence and structure (Mills, 2006). Although numerous studies have shown that p63 plays critical roles in regulating cell cycle checkpoint, DNA damage repair and apoptosis of tumor cells, the protein has generally been recognized as an essential transcription factor for the development and maintenance of normal epithelial structures and functions since its discovery more than a decade ago (Dietz et al., 2002; Wu et al., 2003, 2005; Perez and Pietenpol, 2007). Interestingly, p63 has also been suggested to be crucial for embryonic ontogenesis, mostly in the development of limbs and other ectodermal derived tissues (Rouleau et al., 2011; Li et al., 2012; Lu et al., 2013; Gu et al., 2013). It was previously reported that, apart from severe defects of epithelial development, limbs are either absent or truncated in mice lacking *p63* (Mills et al., 1999; Yang et al., 1999). Meanwhile, *p63* mutations are responsible for a group of human ectodermal dysplasia syndromes such as the EEC syndrome, LMS syndrome, SHSM syndrome and ADULT syndrome (Rinne et al., 2007; Reisler et al., 2006; Kouwenhoven et al., 2010; Tadini et al., 2013), underlining the key role of p63 in the development of ectoderm-derived tissues. Despite the craniofacial involvement in these syndromes, severe limb deformities in *p63* null mice and limb and digit abnormalities in p63-related diseases strongly suggest p63 may be a candidate that plays a critical role during limb development and the progression of limb diseases.

Formation of limb bones requires a cartilage intermediate. The process of forming cartilage called chondrogenesis can be divided into two main impartible stages: condensation and differentiation (Ahrens et al., 1977; Goldring et al., 2006). This process is well coordinated and is regulated by multiple transcription factors and signaling pathways (de Crombrugghe et al., 2001). However, currently, there is scant data on the effects of p63 on chondrogenesis.

Sex determining region Y box gene 9 (Sox9) is a key chondrogenesis-related transcription factor. While type II collagen (Col2a1), synthesized by cartilage cells, is the principal constituent of extracellular cartilage matrix. Col2a1 Given the findings that *p63* null mice have no or truncated limbs and mutations in human *p63* cause several skeletal syndromes that also show limb and digit abnormalities, we hypothesized that retinoid acid (RA), a potent teratogen responsible for a variety of abnormalities of organs (such as limb) and tissues in both humans and animals (Santos-Alvarez et al., 2003), may impact on chondrogenesis by modulating the expression of cartilage-specific molecules such as Sox9 and Col2a1 and p63. In the current study, we investigated the effect of ATRA upon chondrogenesis using mesenchymal cells from rat hind limb bud from rats and further examined the mRNA and protein expression of Sox9 and Col2a1 and p63 in rat hind limb bud cells.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats (the Animal Experiment Center, Shantou University Medical College, Shantou, Guangzhou, China) weighing 220–250 g each were used. All animals were housed in environmentally controlled conditions (22 °C, a 12 h light/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) with *ad libitum* access to standard laboratory chow and water. Female rats were mated with male rats overnight. Gestational age was designated as day 0 when a vaginal plug was detected after morning cohabitation. Pregnant rats were euthanized by cervical dislocation on embryonic day 12.5 (E12.5).

The study protocol was approved by the Institutional Animal Care and Use Committee of Shantou University Medical College. Care of rats in this investigation conformed to the Guide for the Care of Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and animal study was carried out in accordance with the National Regulations for Animal Protection of China.

2.2. Isolation and culture of mesenchymal cells from rat hind limb bud

Embryos were obtained from the euthanized female rats and put in phosphate buffered saline (PBS). Then, limb buds were isolated under a dissecting microscope and digested with trypsin (0.1%) for 10 min at 37 °C with slight agitation with a plastic pipette. Cells were filtered through a pre-wetted 40-μm cell strainer to remove clumps followed by centrifugation at 1000 rpm for 5 min. Then, the cell pellets were resuspended in DMEM/F12 (Hyclone, USA) containing 10% fetal bovine serum (FBS) (GIBCO, USA), 1% penicillin and 1% streptomycin (Sigma, St. Louis, MO) and cultured at 37^0C in a humidified atmosphere containing 5% CO_2. After serum starvation for 24 h, cells were treated with 0.01–10 μM ATRA (Sigma) in dimethyl sulfoxide (DMSO) at a final concentration lower than 0.1%.

2.3. The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assays

Hind limb bud mesenchymal cells were plated into 96-well plates at a density of 5000 cells/well in DMEM/F12 with 10% FBS. After serum starvation for 24 h, cells were treated with appropriate concentrations of ATRA as indicated. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide colorimetric assays as instructed by the manufacturer (Takara, Dalian, China) at 0, 12, 24, and 48 h. Absorbance was measured using a microplate reader (Thermo Scientific, Beijing, China) at 490 nm. Cell proliferation was expressed as optical density (OD) value.

2.4. Alcian blue staining

Alcian blue-nuclear fast red staining was utilized to examine cartilage nodules using the modified method as depicted previously (Jiang et al., 1995; Andrea et al., 2000). The cells were fixed in 4% (w/v) paraformaldehyde (Sigma), pH 7.4, for 30 min, followed by staining with alcian blue (Sigma) for 5 min. Then, the cells were dehydrated with 95% ethylalcohol. The images were acquired by a Nikon TE2000-S inverted microscope (Nikon, Japan). All pictures were then imported into the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). The threshold was standardized to detect the area of