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Original Article

Epithelial-mesenchymal transition and mesenchymal-epithelial transition response during differentiation of growth-plate chondrocytes in endochondral ossification

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Abstract: For linear longitudinal bone elongation, the stem-like progenitor chondrocytes distributed in resting zone (RZ) of growth plate have a capacity to differentiate towards the spindle chondrocytes in proliferative zone (PZ), then towards the columnar and tightly adjacent chondrocytes in hypertrophic zone (HZ). We hypothesized this process of endochondral ossification with cells morphological change was occurred along with the inter-conversion between epithelial to mesenchymal cell types. Consistent with this hypothesis, our study demonstrated the chondrocytes highly expressed mesenchymal-like biomarkers and loss of epithelial surface markers in PZ, while converse in RZ and HZ of the growth plate in mice distal tibia *in vivo*. To further determine these process and correlation regulatory pathway, the 4-week old male and female mice were treated with estradiol cypionate or oxandrolone, then investigated the response of epithelial- and mesenchymal biomarkers, and demonstrated that estrogen blocked the EMT process from RZ to PZ while androgen promoted MET from PZ to HZ. Our observations supported the hypotheses that the growth plate firstly go through EMT from RZ to PZ, then MET process from PZ to HZ during the epiphyseal fusion. Our results could interpret the different roles of estrogen and androgen in growth plate cartilage when endochondral ossification.

Keywords: Growth plate, cartilage, EMT, MET, estrogen, androgen

Introduction

Multiple tissues differentiation and organs formation in embryonic development arise from a series of conversion from epithelial to mesenchymal cells, through epithelial to mesenchymal transition (EMT) or mesenchymal to epithelial transition (MET). In primary EMT process, the primitive epithelia lose their characterization of rounded shape, sequential arrangement and compact junctions to convert a population of spindle, loosely organized but motile mesenchymal cells for gastrulation formation and neural crest migration. Then, after a transient epithelial structure condensation through MET, these population in notochord, somites, somatopleure and splanchnopleure derived from mesoderm generate mesenchymal cells which have ability to differentiate into specific cells types of diverse tissues via the secondary EMT [1, 2].

With regard to chondrogenesis and osteogenesis during embryonic development, the neural crest cells migrate to somites of mesoderm following stereotyped pathways and undergo a secondary EMT to generate mesenchymal condensation. These mesenchymal cells differentiate into osteoprogenitors for intramembranous ossification and chondrocytes for endochondral ossification. The radial elongation of longitudinal bone occurs via endochondral ossification at the growth plate cartilage [3]. The growth plate consists of three histologically and func-

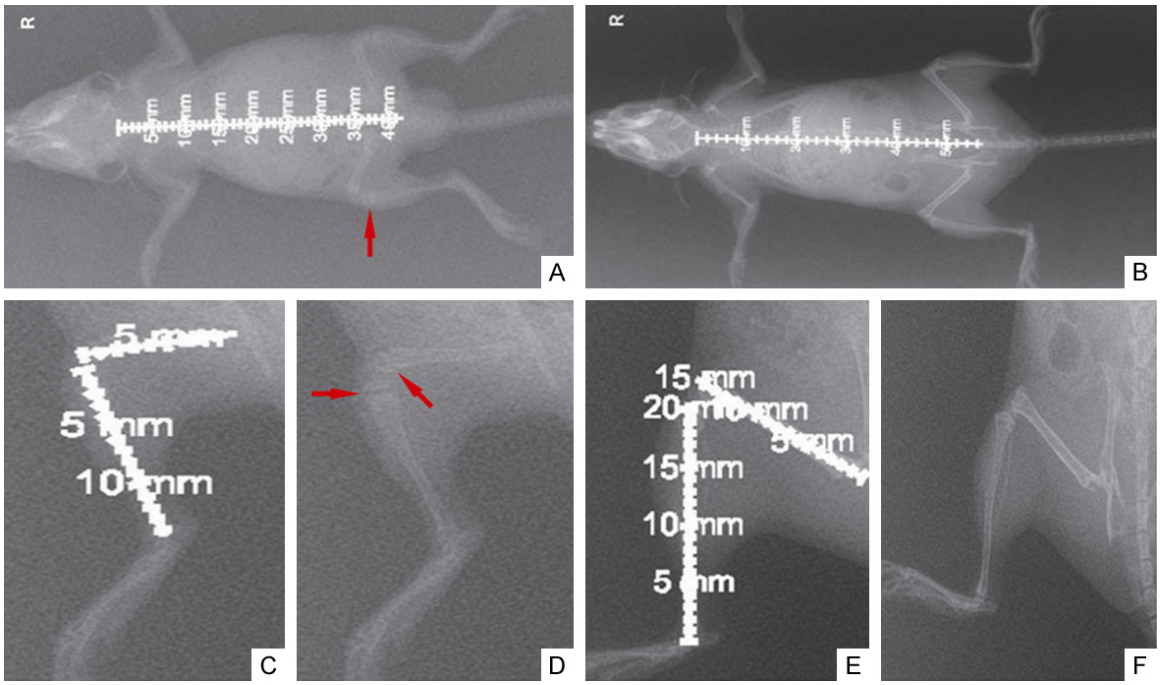

Figure 1. X-ray detection of mouse skeleton. The left growth plate at the distal tibia (C-F) with the 4-(A) and 16-(B) week old. The red arrows showing the gap between knee and tibia or femur means cartilage of growth plate.

tionally multilayer, resting zone (RZ), containing stem-like cells capable of differentiating into proliferative chondrocytes [4]; proliferative zone (PZ), a population of spindle, loosened arranged and rapidly dividing chondrocytes [5]; and hypertrophic zone (HZ), the columnar distributed and tightly adjacent chondrocytes with stromal vesicles having a controversy that program to terminally differentiation or preceding osteogenesis [3].

The growth plate undergoes a program from differentiation with the developing stage, proliferation to calcification, ultimately causing epiphyseal fusion and bone elongation termination. The whole process is governed locally or indirectly by one complicated network of endocrine signals or extracellular matrix, while the complete regulatory mechanism is still obscure. However, the alteration of cell morphology occurs in growth plate is reminiscent of the feature of EMT and MET conversion. Given the histoembryology and development biology, EMT is broadly recognized as the differentiation process, while MET in contrast, comes along with the somatic cell reprogramming [6]. Pluripotent stem cells exhibit epithelial characteristics, down-regulate the epithelial markers such as Cdh1, Cldn6, Epcam and enhance the mesenchymal markers including Snai1/2, Zeb1, CtnnbIP1 [7-11] regulated by TGF-beta, BMP signaling pathways which were also determined to play a key role in chondrocytes differentiation and epiphyseal fusion [12, 13]. Thus, we attempt to demonstrate the hypothesis that the endochondral ossification with morphological change of cells was occurred along with EMT or MET, thereby to gain insight into the regulatory mechanism by which hormones effects in growth plate and screening the new drug targets for epiphyseal premature fusion when precocious puberty. Herein, the wide type mice were used for this study to validate cell type conversion of the growth plate *in vivo* and to investigate the endocrine regulatory roles that estrogen and androgen play in when puberty initiation.

Materials and methods

Animal study and chondrocytes isolation

All the procedure was approved by the Institutional Animal Care and Use Committee of Shanghai, China. The outbred ICR mice (JAX laboratory) were used in this study. Animals were fed with food and water *ad libitum* freely. Protocols were conducted to minimize pain and

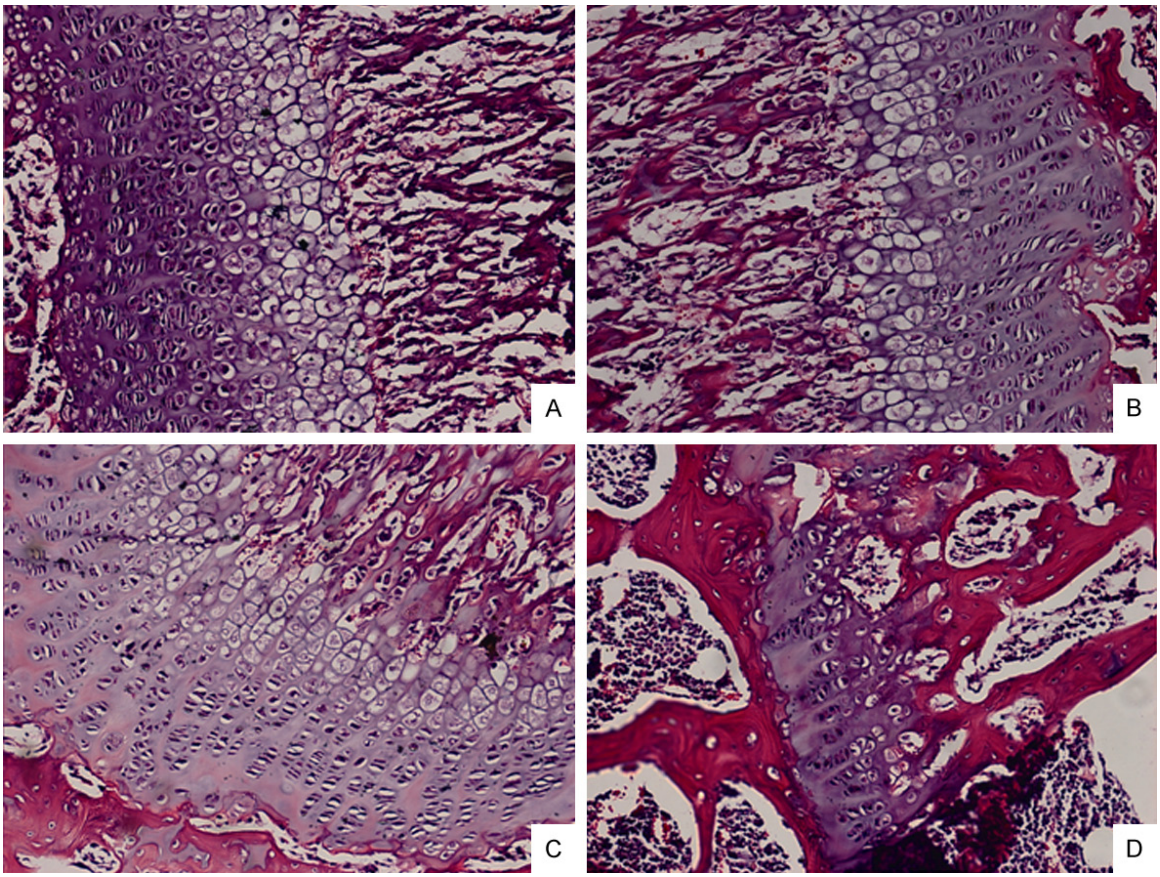

Figure 2. HE-staining of the growth plate with 2-(A), 4-(B), 8-(C), 16-(D) week old mice.

discomfort to the animals. 6 mice in one group randomly according to gender and age and weekly intraperitoneal injected 70 ug/kg estradiol cypionate [14], 15 mg/kg oxandrolone [15] and 2.5 mg/kg SIS3 [16] respectively for 4 weeks. The mice were sedated to assess the status of skeleton using X-ray assay (Philips Digital Diagnost, 50 kV, 2 mA·s for the whole body and 45 kV, 1.5 mA·s for the feature on leg), then sacrificed by cervical dislocation. The growth plates between proximal femur and distal tibia were separated and cut into pieces of 1 mm×1 mm in cold PBS with 1% antibiotic under integrated microscope, and digested by 0.25% trypsin for 10 min and 0.1% type II collagenase overnight in 37°C, then sieved 70-μm filter [17]. The cells pellet was fixed by 1% PFA for 30 min at room temperature, then 90% methanol for 30 min at 4°C, after washing by PBS twice, incubated with 10 μl Col10a1 and 15 μl Bmp3 (Santa Cruz, USA) antibodies [18] diluted by PBS with 0.5% BSA and 0.1% Triton X-100 for 1 h at room temperature, washed by PBS 3 times, spin down with 800 rpm, then incubated with 1:2000 Alexa Fluor 488 nm anti-goat IgG and 1:2000 Alexa Fluor 594 nm anti-rabbit IgG (Invitrogen, USA) in dark place for 30 min in 37°C, washed by PBS 3 times and spin down with 800 rpm again. The cells pellet was resuspended by 300 μl PBS for fluorescent sorting using BD FACSAria III. The flow cytometric analysis showed the chondrocytes were divided into 3 groups, in which cell population of $Bmp3^+$ and $Col10a1^+$ represent RZ and HZ respectively, and the double negative is PZ (Figure S1).

HE staining

The fresh proximal femur and distal tibia washed by PBS, was fixed within 4% formalin 1 h at room temperature, decalcified in 10% HCl 72 h, orderly dehydrated in the ethanol of 50%, 75%, 85%, 95%, 100% and 100%, displaced the ethanol out using xylene of 50%, 100% and 100%, incubated in paraffin of 50%, 100% and 100% at 50°C to remove the additional xylene, then embedded within the 60°C wax box 1 h,

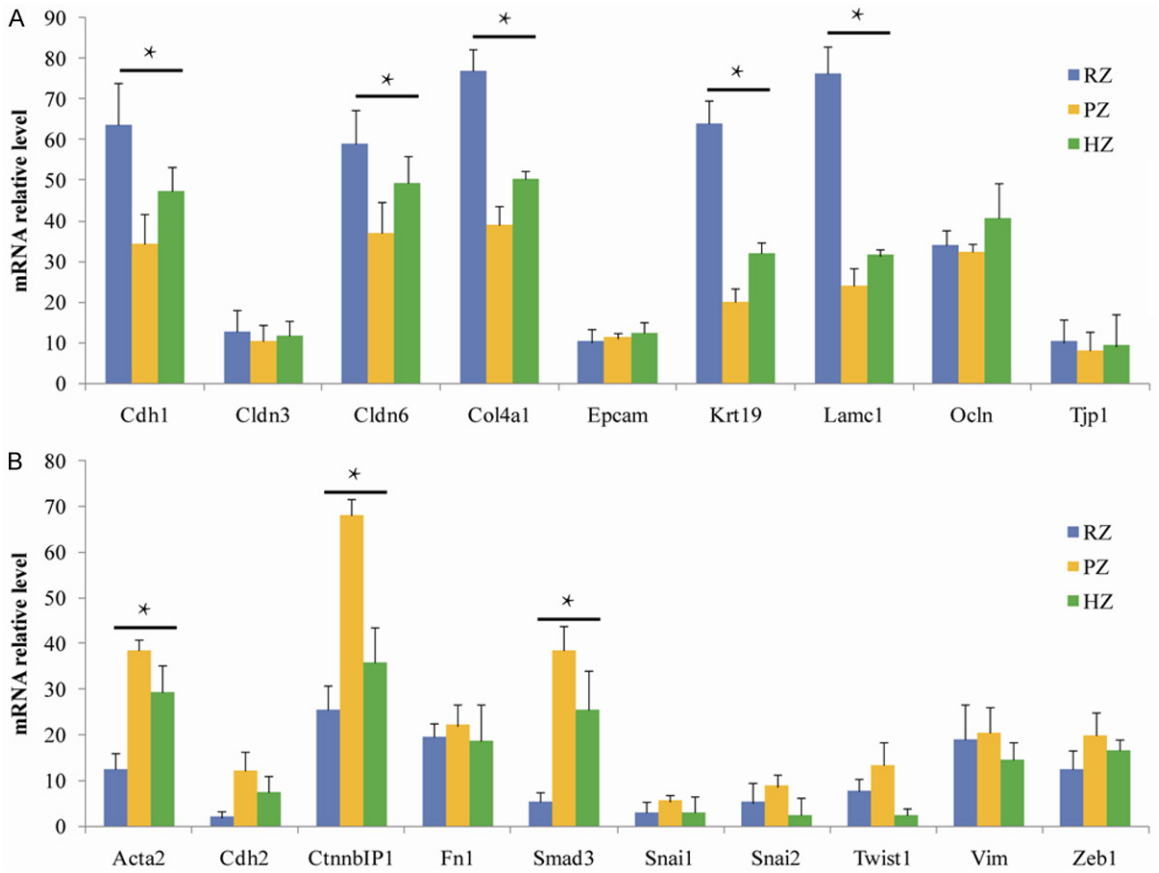

Figure 3. The mRNA expression of epithelial-(A) and mesenchymal-(B) like genes within each zone of the growth plate in 8-week old mouse.

cooled down in water 4 h, and sectioned into 7-10 μm paralleled the tibia. The slices were dewaxed followed the reversed steps of previous paraffin preparation, then incubated in the hematine 15 min and 1% hydrochloric acid alcohol 10 s and washed 30 min, and in 0.5% eosin 15 min, then followed the reversed protocol of dehydration, and enveloped for observation.

Real-time PCR

The total RNA of cells isolated was extracted using trizol. cDNA was produced from 100 ng of total RNA by Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) according to the manufacturer's protocol. Quantitative real-time PCR was performed using the SYBR Premix ExTaq Kit (Roche, Switzerland). The PCR protocol consisted of 95°C for 30 s, 40 cycles of 5 s at 95°C, 30 s at 60°C. Mouse Gapdh transcript served as an internal reference gene. The values were analyzed using the comparative CT method. The primers used in this study were listed in Table S1.

Western blot

In brief, the proteome was harvested using RIPA buffer, denatured with 1× SDS buffer at 95°C for 10 min. 30 μg protein was separated by 8% polyacrylamide gel subjected to 90 V constant voltage SDS electrophoresis, and transferred onto NC membrane subjected to 100 V constant voltage, then blocked by 5% nonfat milk, and incubated with the following antibodies AR, Col4a1, ER-alpha, ER-beta, Gapdh, Krt19, Smad3, Tgf-beta1, Tgf-beta2, anti-mouse, anti-rabbit, anti-goat (Santa Cruz, USA) and finally determined by ECL enhancing solution of chemiluminescence agents.

Statistical analysis

The Student's *t*-test was used to analyze values of measurement data. All analyses were pro-

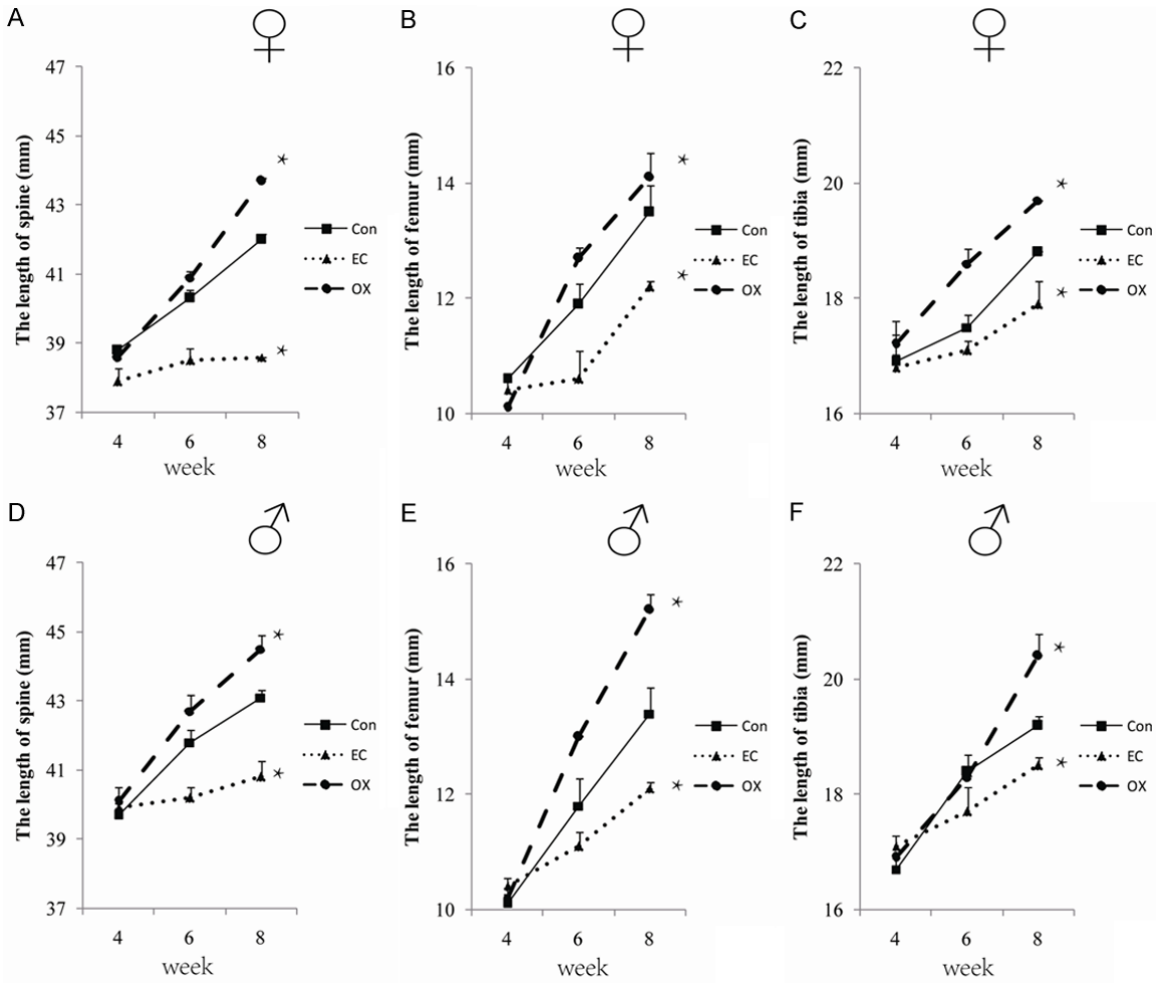

Figure 4. The length assay of spine (A, D), femur (B, E) and tibia (C, F) in male and female mice treated by estrogen and androgen. Con, EC, OX represent untreatment, estradiol cypionate and oxandrolone resectively.

cessed by SPSS 20 software. Values of $P<0.05$ were regarded as statistical significance.

Results

To verify the histological and morphological alteration of endochondral ossification during longitudinal bone development, the growth plate in distal tibia harvested from 2, 4, 8 and 16-week old ICR mice were detected using X-ray and HE staining assay. A notable presence of growth plate was observed in distal tibia of the 4-week but not in 16-week old mice (**Figure 1**). Moreover, the cells in RZ and PZ were randomly distributed and displayed vague boundary in 2- and 4-week old mice; and then emerged the distinct difference in 8-week old mice, which the linear arrangement was found in the cells in PZ; and finally were largely exhausted in 16-week old mice. Whereas, the HZ gradually spread across the growth plate and tightly stacked from 2- to 8-week old and then disappear in 16-week old mice (**Figure 2**). Consistent with the X-ray assay, the total size of growth plate declined during the time course of bone elongation. Taken together, the observation of histological patterns of growth plate in mice confirmed the differentiation process of cells within growth plate cartilage when epiphyseal fusion and determined the significant time points for further study.

Since this cell morphological alteration was coincident with a conversion between epithelial and mesenchymal cell types, thus the expression of epithelial- and mesenchymal-like biomarkers in different zones harvested from 8-week old mice were further investigated to

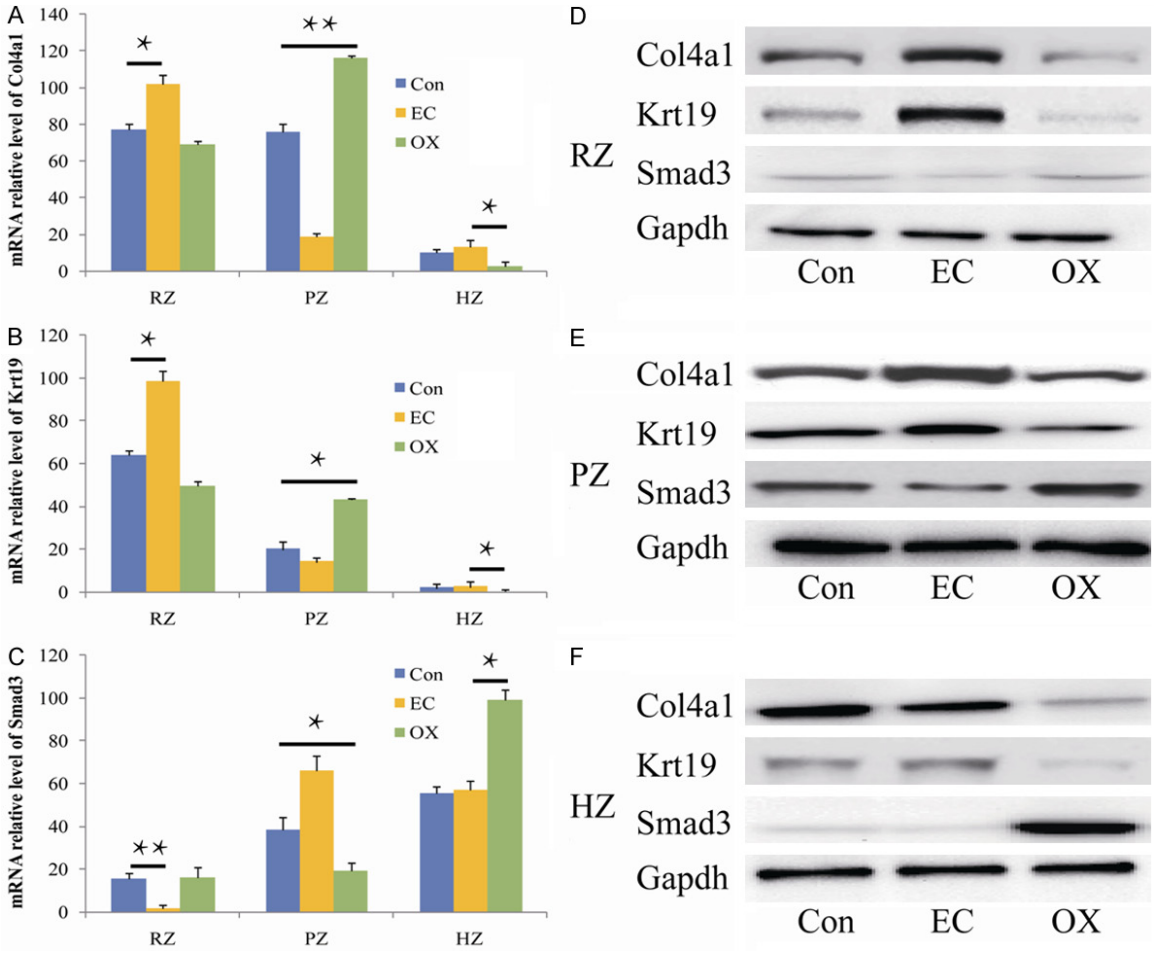

Figure 5. The mRNA (A-C) and protein (D-F) level of Col4a1, Krt19, Smad3 in female mice treated by estradiol cypionate and oxandrolone.

validate the hypothesis. The mRNA level of epithelial markers including Cdh1, Cldn6, Col4a1, Krt19, Lamc1 expressed in RZ and HZ were significantly higher than in PZ while the mesenchymal markers such as Acta2, Ctnnb1, Smad3, displayed the converse tendency (**Figure 3**). The results suggested that a process of EMT occurred in the programming of RZ towards PZ and MET in PZ towards HZ.

To further determine the EMT or MET process and the potential regulatory mechanism, 4-week old mice were weekly treated with 70 ug/kg estradiol cypionate and 15 mg/kg oxandrolone respectively for 4 weeks. The length of spine, femur and tibia was investigated using X-ray assay to show that estrogen inhibited while androgen enhanced the skeleton radial growth both in male and female mice (**Figure 4**). And the level of epithelial- and mesenchymal-like biomarkers expressed in different zones of treated growth plates were detected using real-time PCR and western blot. Interestingly, the expression of Col4a1, Krt19 and Smad3 showed the different response to estrogen or androgen stimulation both in female (**Figure 5**) and male mice (**Figure 6**). Collectively, our observation demonstrated that the chondrocytes differentiation in growth plate cartilage affected by sexual hormones *in vivo* came along with EMT-like process.

Finally, since the Smad3 was demonstrated to be differentially regulated by estrogen or androgen within growth plate especially in RZ, the underlying mechanism of Smad3-mediated TGF-beta pathway was investigated. 4-week old male and female mice were intraperitoneal injected weekly 2.5 mg/kg SIS3, Smad3 phosphorylation inhibitor for 4 weeks. The results showed that the level of Smad3 phosphorylation in RZ obviously decreased compared with

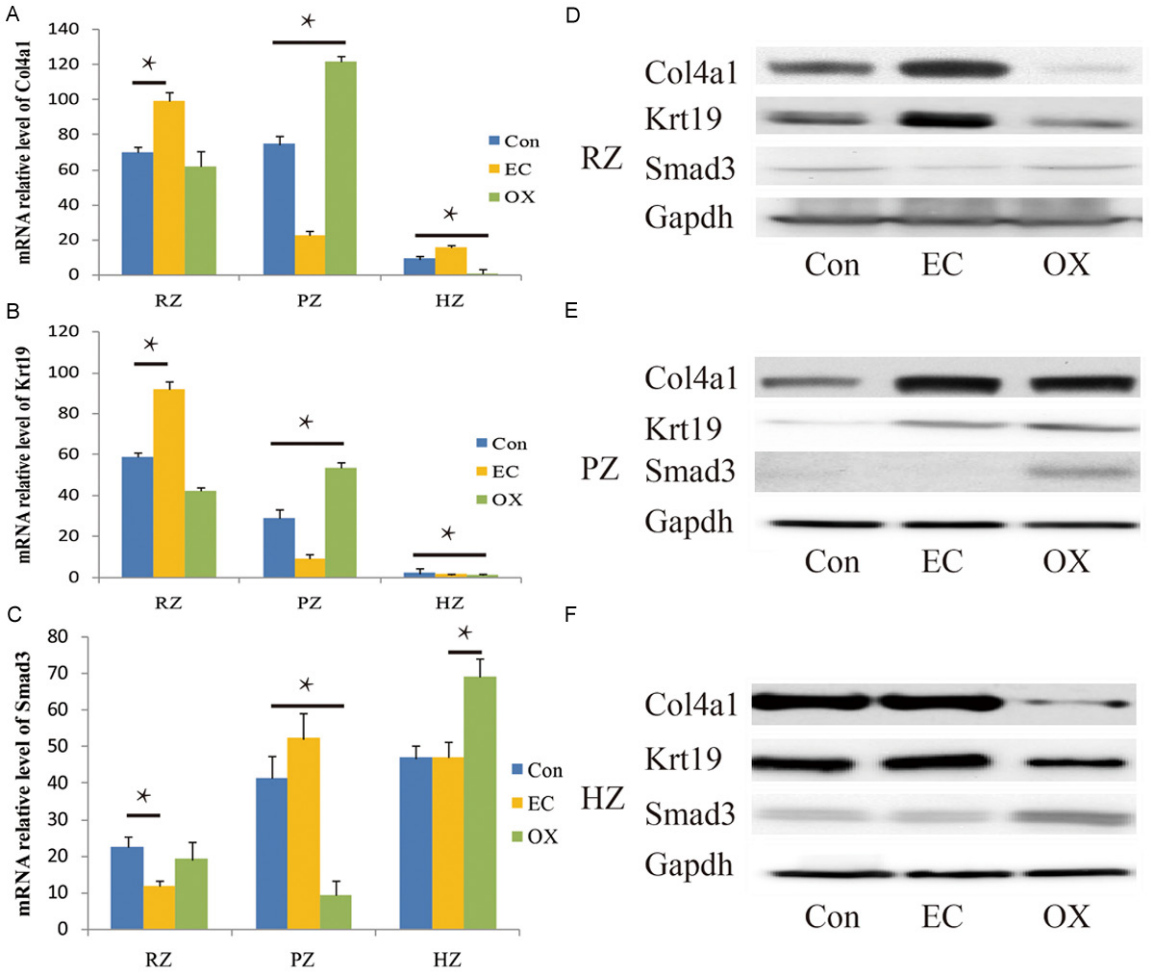

Figure 6. The mRNA (A-C) and protein (D-F) level of Col4a1, Krt19, Smad3 in male mice treated by estradiol cypionate and oxandrolone.

the control group. Meanwhile, the expression of TGF-beta1 and 2, estrogen receptor-(ER) alpha and beta but not androgen receptor increased both in female and male mice (**Figure 7**). Taken together, Smad3 blocking modulate TGF-beta signaling pathway for chondrocytes differentiation in growth plate.

Discussion

In mammalian, epiphyseal growth plate the remarkable fusion regulated by endocrine signals arises in puberty. Here, our system of 2-, 4-, 8- and 16- old mice model display an obviously different pattern of layers in growth plate during endochondral ossification (**Figures 1** and **2**), which is consistent with previous studies that skeletal maturation and epiphyseal fusion occur at the postnatal age of 8-week old and finish at 16-week old [19, 20]. Thus, the endocrine stimulation and Smad3 inhibitor in our study were both treated in 8 and analyzed in 16-week old stage.

EMT and MET are termed as a transition between epithelial and mesenchymal type in embryo development, fibrosis, and tumor metastasis, in which epithelial cells are mainly involved in pluripotent or undifferentiated cells. The morphology transformation process of chondrocytes in growth plate, from a group of epithelial stem-like cells in RZ to spindle shaped cells in PZ, then to tightly adherent and columnar distributed cells in HZ, however, is never reported associated with EMT or MET. We validated the chondrocytes in growth plate undergo an analogous process (**Figure 2**) and detected the relevant genetic patterns. Consistent with other gene profiles in growth plate [21, 22], a portion of epithelial-like genes down-reg-

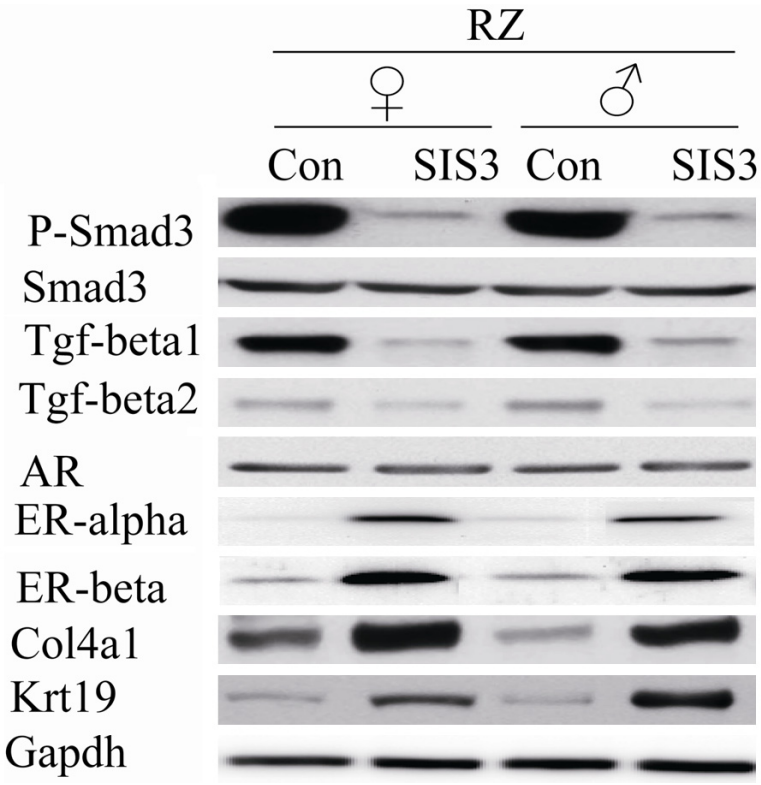

Figure 7. The expression of Smad3 and the other associated genes in RZ of male and female mice treated by SIS3.

ulate in PZ, then up-regulate in HZ compared to RZ, while mesenchymal-like genes such as Smad3 display an opposite tendency (**Figure 3**), which suggests that RZ transform to PZ by EMT, then to HZ by MET.

In human, the epiphyseal fusion launch in pubertal growth spurt with sexual hormone exposure. Estrogen and androgen are considered as the important endocrine factors to affect growth plate. Premature estrogen accelerates cell depletion in growth plate and epithyseal fusion [23]. In mice, estrogen plays a role in growth suppression, an effect seen only at high doses in human [24]. In contrast, androgen per se without conversion to estrogen by aromatase [25] contributes to longitudinal bone stimulation [26, 27]. Thus, the observations of reverse action to chondrocytes by estrogen and androgen [28] were all demonstrated in our study (**Figure 4**). Interestingly, in our study, estrogen administration maintains the epithelial type genes expression in growth plate particularly in RZ (**Figure 5D**) implies that estrogen appears to block EMT process. Not like human or rabbit, the expression of estrogen receptors within HZ of growth plate in mice and rat was extremely low until at the last time point prior to epiphyseal fusion [29], which also reflects the less effect of estradiol cypionate to in the HZ in our study (**Figures 5F**, **6F**). Conversely, Androgen effectively promotes EMT for chondrocytes differentiation (**Figures 5**, **6**). Taken together, the EMT or MET hypothesis in our study illuminates the roles of estrogen and androgen play in growth plate.

In the further study to investigate the underlying mechanism of endocrine regulation in growth plate, Smad3 attracted our attention. The estrogen is reported to restrain the TGF-beta/Smad3 signaling pathway in recent studies [30, 31], which is also consistent with our observation in growth plate (**Figures 5**, **6**). Furthermore, Smad2/3 exert as key mediators of the inhibitory effect of upstream TGF-beta on chondrocyte maturation [32, 33]. Here, Smad3 inhibitor mimic the similar effect with estradiol cypionate, simultaneously fed back to estrogen receptors enhancement in our study (**Figure 7**). Thus, we can speculate that estrogen may interdict TGF-beta, then further repress Smad3 expression, so that postpone chondrocytes differentiation via EMT blocking. Although estrogen prevents RZ from differentiation, there is no epiphyseal fusion at the time of sexual maturation in mice and we cannot figure out the reason of chondrocytes depletion or apoptosis regulated by estrogen in our study.

Androgen is determined to promote EMT for differentiation. However, androgen improves Smad3 expression but appears to have no response to SIS3, which indicates that androgen may participate in other pathways rather than TGF-beta/Smad3. The potential mechanism may be involved in advanced calcium and phosphorus absorption mediated by androgen for chondrocytes maturation and calcification [34]. Referred to this point, with regard to the

controversial fate of hypertrophic chondrocytes, the type 2 EMT, a physiological response for organ fibrosis [2] may be an analogous process occurring when HZ finally turn to the calcified cartilage zone.

Collectively, our results demonstrated EMT and MET conduct the chondrocytes differentiation and cartilage epiphyseal fusion in growth plate, and revealed the different roles that estrogen and androgen played in growth plate cartilage via the transition between epithelial and mesenchymal types.

Acknowledgements

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Disclosure of conflict of interest

None.

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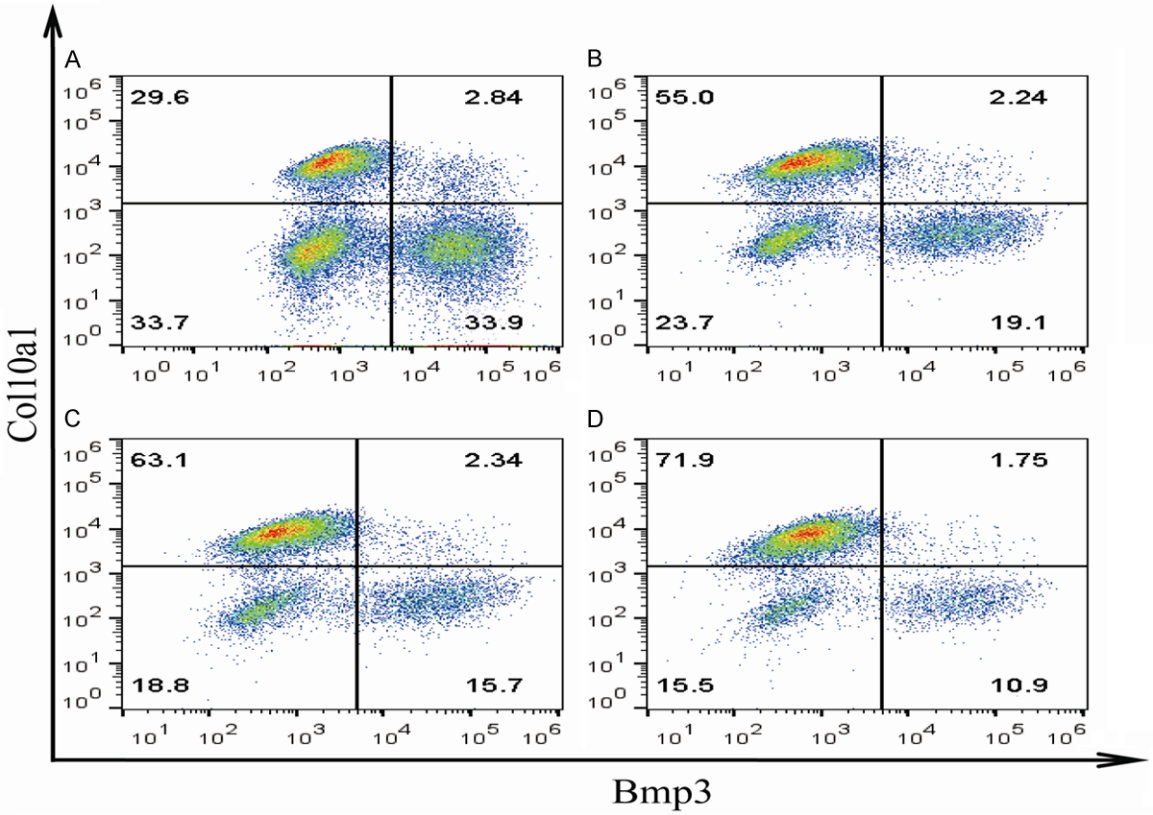

Figure S1. The flow cytometric analysis of growth plate sorted by Bmp3 and Col10a1 in 2-(A), 4-(B), 8-(C) and 16-(D) week old mice.

Table S1. The primers used in this study

Gene symbols	Primers sequence	Length (bp)	Tm (°C)
Acta2	F: 5'-GCTCTGCCTCTAGCACACAA-3'	229	60
	R: 5'-ATTCCTGACCACTAGAGGGGG-3'		
Cdh1	F: 5'-TACACAGGCGGTGAGACCTA-3'	159	60
	R: 5'-CGGCCAGCATTTTCTGTAGC-3'		
Cdh2	F: 5'-GCGGGATAAAGAGCG-3'	319	55
	R: 5'-GGAGTCATACGGTGGC-3'		
Cldn3	F: 5'-AGACCGTACCGTCACCACTA-3'	289	55
	R: 5'-TGCCCACTGTGAGCTTCTG-3'		
Cldn6	F: 5'-ACTGGACGTCCGCAGACAAA-3'	172	55
	R: 5'-GATGCTGTTGCCGATGAAGG-3'		
Col4a1	F: 5'-TTCTTGCTTCTGCTCTGCGT-3'	232	60
	R: 5'-CATAGTGGTCCGAGTCTGCC-3'		
CtnnbIP1	F: 5'-GGCTGTAGACACCTGGACAC-3'	152	60
	R: 5'-ACGCGGACCTTCTGTTGAAT-3'		
Epcam	F: 5'-GCAGGTCCAGTGTGGTACTC-3'	351	60
	R: 5'-GGAAGCGCTAACCCTCCTAC-3'		
Fn1	F: 5'-GAGCCGGACAACTTCTGGTC-3'	357	58
	R: 5'-CCTAGGTAGGTCCGTTCCCA-3'		
Gapdh	F: 5'-CCCTTAAGAGGGATGCTGCC-3'	263	60
	R: 5'-ACTGTGCCGTTGAATTTGCC-3'		
Krt19	F: 5'-CAGTCCCAGCTCAGCATGAA-3'	288	58
	R: 5'-AGTCTCGCTGGTAGCTCAGA-3'		
Lamc1	F: 5'-AGCACCTTCACTGTCTGACG-3'	334	60
	R: 5'-ACAAAAGGCCTCGGAGGATG-3'		
Ocln	F: 5'-CCGGCCGCCAAGGTTC-3'	376	60
	R: 5'-TCCAAAGAGCCCTGTCCCAT-3'		
Smad3	F: 5'-GCGGCACCCAAACAGCTA-3'	228	60
	R: 5'-TCGCCCGAACTTCGCTTTTA-3'		
Snai1	F: 5'-CCAGCTGTAACCATGCCTCA-3'	177	58
	R: 5'-CCACTTGGCCCCTAACAAGT-3'		
Snai2	F: 5'-TTTACTGACAGCTAGATTGAAAGGA-3'	452	55
	R: 5'-ACACGCACCAGGAATGTTTG-3'		
Tjp1	F: 5'-GGAGCAGGCTTTGGAGGAG-3'	163	60
	R: 5'-TGGGACAAAAGTCCGGGAAG-3'		
Twist1	F: 5'-GGACAGAGATTCCCAGAGGG-3'	102	56
	R: 5'- TCGTCAAAAAGTGGGGTGGG-3'		
Vim	F: 5'-AGCGCTCCTACGATTCACAG-3'	341	60
	R: 5'-GTCCACCGAGTCTTGAAGCA-3'		
Zeb1	F: 5'-AGTTGGCCCAGCCATAACTC-3'	235	60
	R: 5'-AAGAACGACAGCAGGTCAGG-3'		