

# RAPID COMMUNICATION

## Expression of aromatase in the human growth plate

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### ABSTRACT

Aromatase catalyzes the synthesis of estrogen from its androgen precursors. Estrogen is known to be important in regulating long bone growth and epiphyseal plate closure. To assess whether there may be growth plate-specific production of estrogen, we performed reverse transcriptase polymerase chain reaction (RT-PCR) to determine whether aromatase transcripts are present in the human growth plate. Immunohistochemistry was also employed to identify the specific sites of expression. Growth plates were obtained from an adolescent male and female undergoing epiphysectomy to

counter premature growth plate closure in the opposite leg. Aromatase transcripts were detected in RNA preparations from both growth plates. The aromatase protein was mainly expressed in the zone of maturation and the hypertrophic zone, with greatest expression in the latter. Since estrogen receptors are known to be expressed in chondrocytes, this data is consistent with a role for local estrogen production in the autocrine/paracrine control of long bone growth and growth plate maturation.

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### INTRODUCTION

Estrogens are known to have a major role in regulating bone growth in the human skeleton. Its effects on epiphyseal growth are thought to be biphasic in that low serum levels correlate with periods of accelerated growth during puberty while high levels lead to epiphyseal maturation and closure (Cutler 1997). The growth plate as a target tissue of estrogen is supported by the demonstration of ER $\alpha$  in prehypertrophic and hypertrophic chondrocytes and ER $\beta$  in hypertrophic chondrocytes (Kusec *et al.* 1998, Nilsson *et al.* 1999). The recent observations of lack of epiphyseal closure and absence of pubertal growth spurt in males with either estrogen receptor mutation (estrogen resistance) or aromatase mutation (estrogen deficiency) point to a major role for estrogen in skeletal growth and maturation in males as well as females (Smith *et al.* 1994, Bilezikian *et al.* 1998). However, because circulating estradiol concentrations are exceedingly

low in males, the question arises as to the source of estrogens in male subjects.

Aromatase catalyzes the synthesis of estrogens from their C19 steroid precursors. It is expressed in human ovaries, adipose, placenta, muscle and brain. More recently, it has been shown to be expressed in osteoblasts, lining cells, chondrocytes of articular cartilage, and adipocytes adjacent to trabeculae in bone derived from middle-aged or older adults (Sasano *et al.* 1997). Although its expression in the human growth plate has not been reported, there are reasons to suspect such expression. First of all, local growth plate production of estrogen would provide an explanation for the apparent estrogen-dependent effects on long bone growth and plate closure observed for males, even though serum levels are always low. Secondly, the fact that the onset of the pubertal growth spurt in girls precedes breast budding, a period of relatively increased estrogen levels, by up to 6 months, suggests that locally produced

estrogen as opposed to circulating estrogen might play a role in the pubertal growth spurt.

To determine if estrogen might be synthesized locally in the growth plate by conversion of its precursors we have used RT-PCR to determine whether aromatase transcripts are present in the human growth plate and immunohistochemistry to locate the protein within the growth plate and primary spongiosa.

## MATERIALS AND METHODS

### Patients and tissue preparation

Tissue samples of human growth plate were obtained during curettage of the growth plate from a 14 y/o male and 11 y/o female undergoing epiphyseodesis to induce arrest of leg growth. The tissue was obtained from the normal side. Only tissues that otherwise would have been discarded were used. Fragments of tissue were either rapidly frozen in liquid nitrogen for RNA preparation or immediately fixed in ice-cold 4% (w/v) paraformaldehyde (pH 7.4, 24 h), followed by decalcification in 15% EDTA for 7 days, dehydrated, and then embedded in paraffin.

### RNA Preparation and RT-PCR

Fragments of growth plate were weighed and homogenized in TRI Reagent according to the manufacturer's recommendations (Sigma Chemical Company, St Louis, MO, USA). RNA was isolated from the aqueous phase by ethanol precipitation followed by DNase I treatment. An aliquot of the 1 µg of total RNA was subjected to reverse transcriptase using the MuLV reverse transcriptase and oligo DT primed synthesis (PE Applied Biosystems, Branchburg, NJ, USA) according to the manufacturer's suggested protocol. The resulting cDNA preparations were utilized for PCR. Forward primer, GTG ACA GAG ACA TAA AGA TCG, and reverse primer, GTA AAT TCA TTG GGC TTA GGG (IDT, Inc. Coralville, IA), derived from aromatase exon 9, were utilized for amplification of aromatase exon 9 as a means to detect aromatase transcripts in the preparation. The amplification protocol consisted of initial denaturation at 95 °C for 5' followed by 35 cycles of 94 °C for 45", 55 °C for 30", 72 °C for 45", and final elongation at 72 °C for 5'. The final Mg concentration was 3 mM. As a negative control for aromatase transcripts we used cDNA prepared from aromatase deficient mouse. A plasmid containing exon 9 of the *cyp19* gene served as a positive control for the PCR reaction.

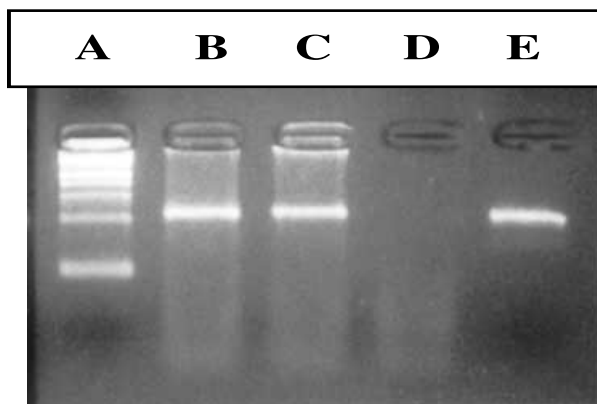


FIGURE 1. Amplification of aromatase transcripts in growth plate cDNA. A. Standards B. cDNA from male patient. C. cDNA from female patient D. Negative control cDNA from an aromatase deficient mouse E. Positive control, plasmid DNA containing aromatase exon 9.

### Immunohistochemistry

Rabbit anti-aromatase antibody was prepared by immunizing with the peptide CMDFATDLIF AERRGDTLTK-KLH conjugate. This peptide was chosen because of its immunogenic potential and strong homology between the mouse and human proteins. The preparation was done at Bethyl Laboratories (Montgomery, TX, USA) on a fee for service basis. Serum IgG was purified on a Cibacron Blue DEAE column (Bio-Rad Hercules, CA, USA) or peptide affinity column in the case of preimmune and immune serum respectively. Human placenta served as a positive control in initial immunohistochemistry experiments. Near term human placenta was fixed overnight in 10% neutral buffered formalin. Subsequently, the fixed tissue was dehydrated in a graded series of alcohols and paraffin embedded. Five micron thick sections were cut and mounted onto glass slides. The slides were then processed for immunohistochemistry as described below for the growth plate specimens. Four to five micron thick sections of demineralized growth plate were mounted onto glass slides and processed for immunohistochemistry. Endogenous peroxidase activity was neutralized by incubating the sections for 5 min with 3% H<sub>2</sub>O<sub>2</sub>. After rinsing these sections in buffer (phosphate buffered saline, pH 7.5), the slides were blocked with normal goat serum for 20 min at room temperature. After blotting excess serum from the sections, the slides were then incubated with avidin D blocking solution (Vector Laboratories, Burlingame, CA, USA) for 15 min at room temperature. Sections were then briefly rinsed with PBS and incubated in

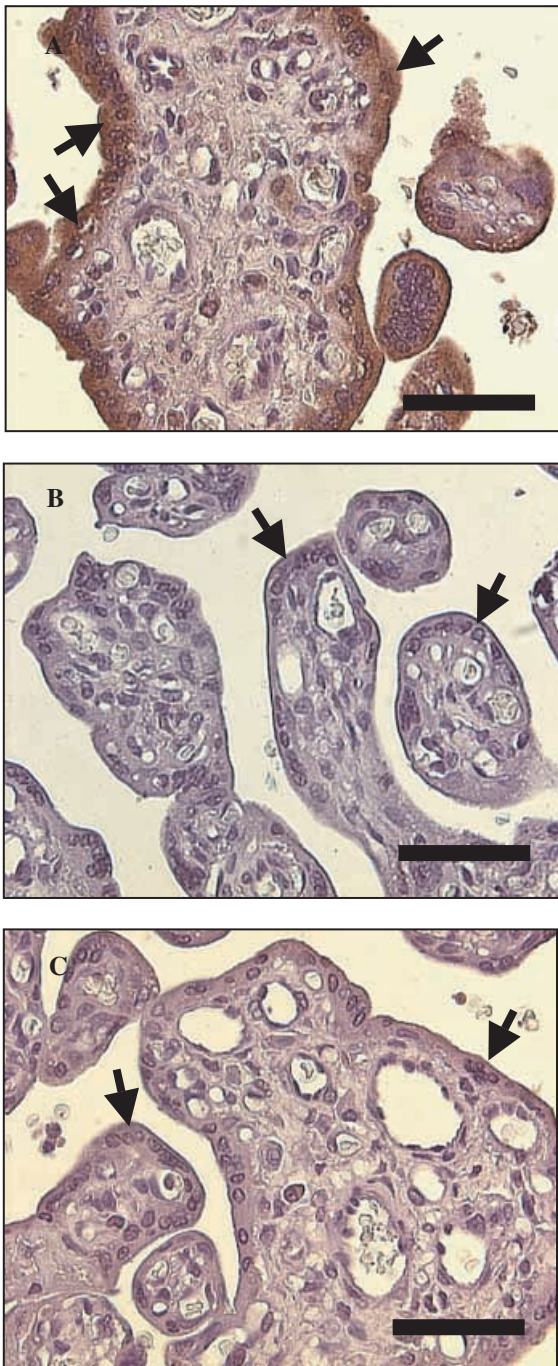


FIGURE 2. Validation of anti-aromatase antiserum by immunohistochemistry. Sections of human placenta were subjected to immunohistochemistry using rabbit anti-human aromatase. A. Placenta incubated with affinity purified anti-aromatase IgG. B. Negative control, placenta incubated with purified preimmune IgG. C. Placenta incubated with affinity purified anti-aromatase IgG preincubated with immunizing peptide. Arrows identify syncytiotrophoblasts. Bar=50  $\mu$ m.

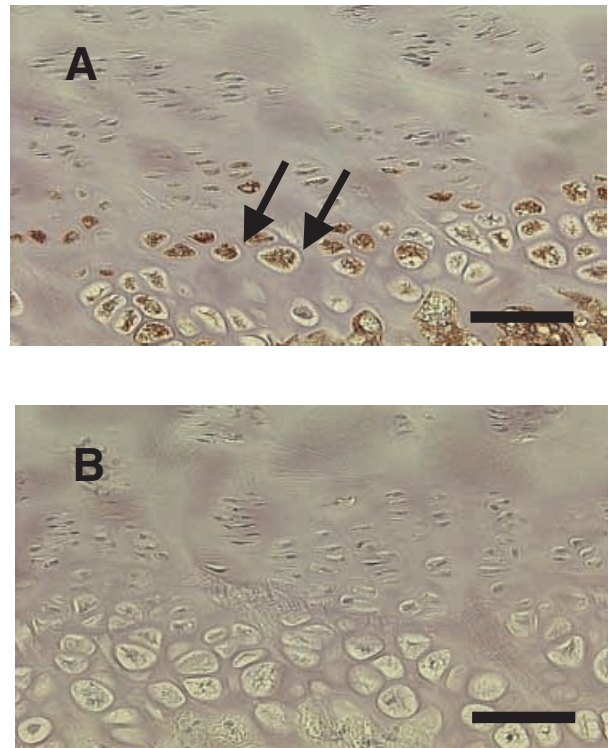


FIGURE 3. Immunohistochemical staining of aromatase in the human female femoral growth plate. A. Growth plate incubated with affinity purified anti-aromatase IgG. B. Negative control, growth plate incubated with purified preimmune IgG. The arrows point to examples of positively stained retracted cytoplasm. Bar=100  $\mu$ m.

an identical fashion with biotin blocking solution. After rinsing the sections, the slides were then incubated overnight at 4 °C with affinity purified IgG at a final concentration of 1  $\mu$ g/ $\mu$ l. The primary antibody was detected in the tissue sections using a VectaStain Elite kit specific for rabbit antiserum (Vector Labs, Burlingame, CA, USA). Final color development was obtained through the use of DAB for 3 min on each section. Following rinsing in tap water, the sections were then counterstained in Mayer's hematoxylin.

## RESULTS

### Identification of aromatase transcripts in human growth plate

Figure 1 shows a representative amplification of a portion of aromatase exon 9 from human growth plate cDNA. Clearly, both male and female growth plates contain aromatase transcripts.

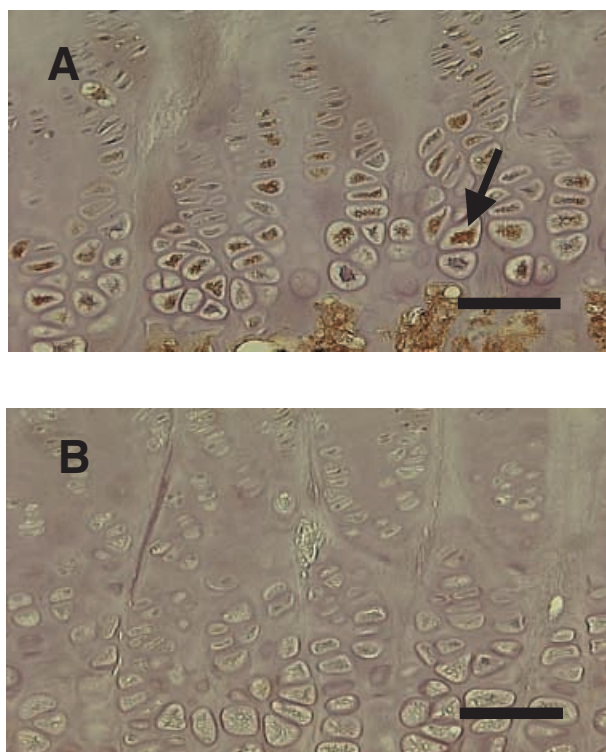


FIGURE 4. Immunohistochemical staining of aromatase in the human male femoral growth plate. A. Growth plate incubated with affinity purified anti-aromatase IgG. B. Negative control, growth plate incubated with purified preimmune IgG. The arrow points to an example of positively stained retracted cytoplasm. Bar=100  $\mu$ m.

### Immunolocalization of aromatase in human femoral epiphyseal plate

Prior to utilization in bone sections, the rabbit polyclonal antiserum was validated on a 28–29 week human placenta. These sections show aromatase expression in syncytiotrophoblasts (depicted by arrows) (Fig. 2A). The staining was specific since preimmune IgG (Fig. 2B) or preincubation of the antiaromatase IgG with the immunogen resulted in no staining (Fig. 2C).

Figures 3 and 4 show a representative section from the human female and male growth plate, respectively. The cytoplasm of many chondrocytes appears retracted. The retracted cytoplasm should not be confused with the nucleus. Note the intense staining of the chondrocytes in the maturation and hypertrophic zones. The most intense staining was observed in the hypertrophic chondrocytes. Staining intensity appeared to diminish in the younger, less differentiated chondrocytes with least staining observed in the reserve zone chondrocytes. Intense staining was also observed in the

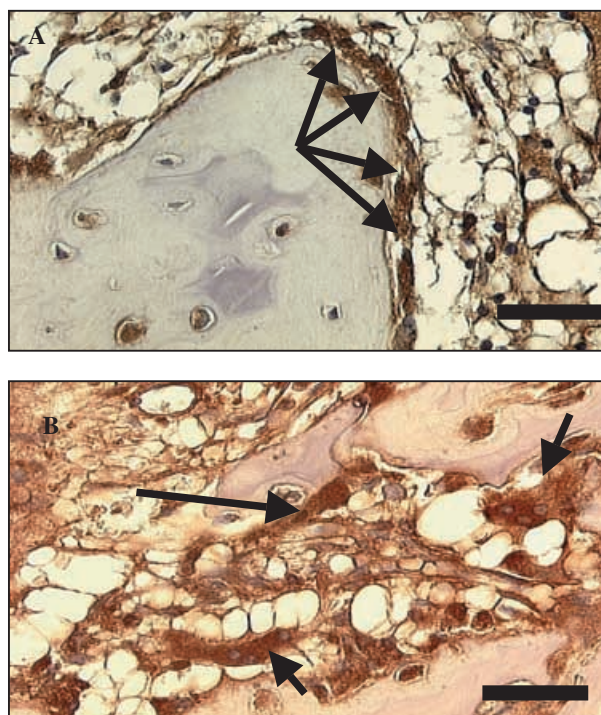


FIGURE 5. Immunohistochemical staining of aromatase in the primary spongiosa. A. Active osteoblasts on a trabecular surface stain intensely (arrows). B. Multinucleated osteoclasts on a trabecular surface or very near the surface have intense cytoplasmic staining. Nuclei appear a pale blue and do not stain. Bar=50  $\mu$ m.

osteoblasts and osteoclasts of the primary spongiosum (Figs 5 and 6); however, some osteoclasts, those not near or on trabecular surfaces, were aromatase negative.

### DISCUSSION

The present study is the first report of aromatase expression in human growth plate cartilage. Taken together with the demonstration of estrogen receptor expression in the growth plate, the data suggest an autocrine/paracrine network with a direct action of estrogen on chondrocytes in the regulation of long bone growth. It is currently not known what specific role(s) estrogen may subserve during the pubertal growth spurt in humans. In addition there is now the intriguing possibility that local estrogens may be important for growth plate closure in both males and females.

Smith *et al.* (1994) described a man with normal testosterone levels, but with a null mutation of his estrogen receptor in whom there appeared to be no fusion of the epiphyseal plate, and he continued to

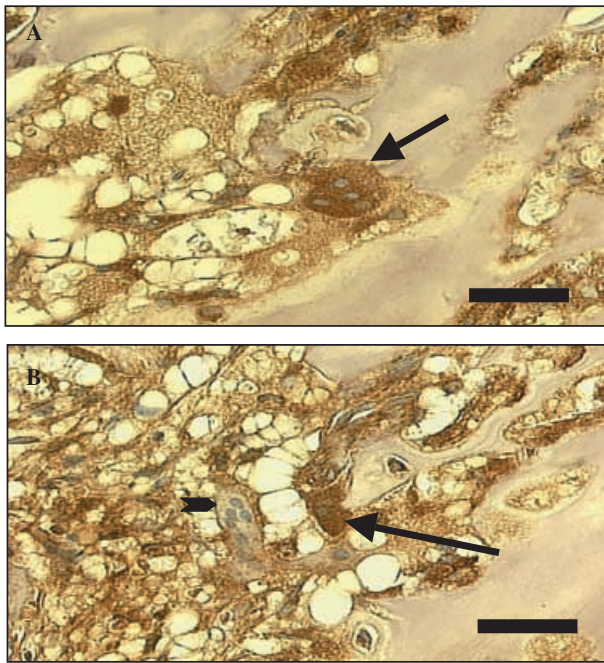


FIGURE 6. Immunohistochemical staining of aromatase positive and negative osteoclasts in the primary spongiosa. A. An active osteoclast on a trabecular surface stains intensely (arrow). B. Aromatase negative (arrowhead) and aromatase positive (arrow) multinucleated osteoclasts are present in the field. Nuclei appear a pale blue and do not stain. Bar=50  $\mu\text{m}$ .

grow linearly in adult life. A similar phenotype has also been described by Bilezikian *et al.* (1998) for a young man with a mutation of the aromatase enzyme. Furthermore, estrogen appears to be important for skeletal maturation since inhibition of aromatase in male rats results in reduced bone mineral density and impaired skeletal modeling (Vanderschueren *et al.* 1997). Similarly, aromatase deficiency in male mice, created by homologous recombination, also results in the development of an osteopenic phenotype with shortened long bones (Öz *et al.* 2000, ÖK Öz and JE Zerwekh, unpublished observations). These observations in conjunction with the current demonstration of aromatase expression in the growth plate indicate that estrogens may play a much more significant role in the development of the mature skeletal

phenotype for both genders than previously believed. The demonstration of aromatase in chondrocytes, osteoblasts, and osteoclasts is consistent with a role for estrogens in a paracrine/autocrine regulatory network within the growth plate and primary spongiosa.

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