Locally produced estrogen promotes fetal rat metatarsal bone growth; an effect mediated through increased chondrocyte proliferation and decreased apoptosis

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Abstract

The importance of estrogens for the regulation of longitudinal bone growth is unequivocal. However, any local effect of estrogens in growth plate cartilage has been debated. Recently, several enzymes essential for estrogen synthesis were shown to be expressed in rat growth plate chondrocytes. Local production of 17β-estradiol (E2) has also been demonstrated in rat costal chondrocytes. We aimed to determine the functional role of locally produced estrogen in growth plate cartilage. The human chondrocyte-like cell line HCS-2/8 was used to study estrogen effects on cell proliferation (^{3}H-labeled thymidine uptake) and apoptosis (cell death detection ELISA kit). Chondrocyte production of E2 was measured by RIA and organ cultures of fetal rat metatarsal bones were used to study the effects of estrogen on longitudinal growth rate. We found that significant amounts of E2 were produced by HCS-2/8 chondrocytes ($64{\cdot}1 \pm 5{\cdot}3$ fmol/3 days/10^6cells). The aromatase inhibitor letrozole (1 μM) and the pure estrogen receptor antagonist ICI 182,780 (10 μM) inhibited proliferation of HCS-2/8 chondrocytes by 20% ($P<0{\cdot}01$) and almost 50% ($P<0{\cdot}001$), respectively. Treatment with ICI 182,780 (10 μM) increased apoptosis by 228% ($P<0{\cdot}05$). Co-treatment with either caspase-3 or pan-caspase inhibitors completely blocked ICI 182,780-induced apoptosis ($P<0{\cdot}001$ vs ICI 182,780 only). Moreover, both ICI 182,780 (10 μM) and letrozole (1 μM) decreased longitudinal growth of fetal rat metatarsal bones after 7 days of culture ($P<0{\cdot}01$). In conclusion, our data clearly show that chondrocytes endogenously produce E2 and that locally produced estrogen stimulates chondrocyte proliferation and protects from spontaneous apoptosis. In addition, longitudinal growth is promoted by estrogens locally produced within the epiphyseal growth plate.

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Introduction

Longitudinal bone growth occurs at the growth plate, a thin layer of chondrocytes between the epiphysis and the metaphysis of long bones, through a process called endochondral ossification, in which cartilage is first formed and then replaced by bone tissue.

The processes of chondrocyte proliferation and differentiation are regulated by many hormones and growth factors. Among these, sex steroids are of crucial importance, especially during puberty (Grumbach & Auchus 1999). A few rare individuals, one male with an inactivating mutation in the estrogen receptor α (ERα) (Smith *et al.* 1994) and a few males and females with aromatase enzyme deficiency (Morishima *et al.* 1995, Maffei *et al.* 2004), have clearly established that estrogens are important for the pubertal growth spurt and for epiphyseal closure. Similarly, experiments with estrogen receptor knockout mice have demonstrated the importance of both ERα and estrogen receptor-β (ERβ) in the regulation of longitudinal bone growth (Vidal *et al.* 2000, Chagin *et al.* 2004). In addition, both ERα and ERβ are expressed in growth plate cartilage from different species, including rat, rabbit and human (Kusec *et al.* 1998, Nilsson *et al.* 1999, 2002, 2003), suggesting that estrogens can act directly on growth plate chondrocytes. In spite of the well known growth promoting effects of estrogens in humans and animals *in vivo*, data from *in vitro* experiments are inconclusive. In fact, there are reports of estrogen stimulating (Somjen *et al.* 1989, 1991, Maor *et al.* 1999), inhibiting (Nasatzky *et al.* 1993, Schwartz *et al.* 1997) or having no effect (Rodd *et al.* 2004) on chondrocyte proliferation.

Besides the gonads, several other tissues can produce estrogens, for instance adipose tissue (Folkerd *et al.* 1982),

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osteoblasts (Bruch *et al.* 1992), vascular endothelial cells and aortic smooth muscle cells (Sasano *et al.* 1999) as well as several sites in the brain (Naftolin *et al.* 1975). The process of local estrogen synthesis and action within the cells of peripheral target tissues is often referred to as intracrinology. In this process, active synthesis of sex steroids occur from inactive precursor steroids, such as dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S), secreted in large amounts by the adrenal glands (Labrie *et al.* 1988). In fact, plasma DHEA-S levels in adult men and women are 1000 to 10 000 times higher than those of estradiol, thus providing a large reservoir for conversion into estrogens in peripheral tissues. There are several enzymes involved in this conversion. The most important are aromatase p450, type I and II 17β-hydoxysteroid dehydrogenase (17β-HSD) and steroid sulfatase (STS), for review see Simpson (2003). Intriguingly, all enzymes needed for the conversion of DHEA to estrogen, such as p450 aromatase, type I and II 17β-HSD and STS, have recently been demonstrated in the rat tibia growth plate (Van Der Eerden *et al.* 2002). These findings open the possibility that locally produced estrogen could play a role in the regulation of chondrocyte proliferation, differentiation and/or apoptosis.

This study was designed to determine the role of locally produced estrogen in the regulation of growth plate cartilage. For this purpose, we chose two different experimental models, a human chondrocyte cell line HCS-2/8 and an organ culture model of fetal rat metatarsal bones.

Materials and Methods

Materials

Recombinant human IGF-1 was kindly supplied by Pharmacia Corp. 17β-estradiol (E2), dexamethasone, BSA and β-glycerophosphate were purchased from Sigma. Letrozole was kindly provided by Novartis AB. ICI 182,780 was purchased from Tocris Inc. (Bristol, UK). Anti IGF-I (H-70), IGF-II (H-103), p450 aromatase CYP 19 (C-16) and IGF-I receptor beta subunit (IGF-IRβ; C-20) antibodies were all purchased from Santa Cruz Biotech. Inc (Santa Cruz, CA, USA). Anti ERβ antibody was purchased from Upstate (Lake Placid, NY, USA). For immunohistochemistry, we used anti-ERα (MC-20) antibody from Santa Cruz Biotech. Inc. and for Western blot analysis, ERα antibody from StressGen Biotech Inc (Victoria, BC, Canada). PBS, DMEM/F12 medium, FBS, Trypsin-EDTA solution and gentamycin were all from Invitrogen Inc. Charcoal treated fetal bovine serum (CTS) was from HyClone (Logan, UT, USA). Ascorbic acid was from ICN Corp. (Aurora, OH, USA).

Cell culture

The human chondrocyte-like cell line HCS-2/8, which is of male origin (Takigawa *et al.* 1989) was used as a model of human growth plate chondrocytes. The cells have several markers of growth plate chondrocytes, such as expression of collagen type II, and have the ability to form columns and to differentiate into hypertrophic chondrocytes expressing alkaline phosphatase and collagen type X (Takigawa *et al.* 1989). The cells were cultured in DMEM/F12 mixture medium supplemented with 20% fetal bovine serum (FBS) and 20 μg/ml gentamycin at 37 °C under 5% CO_2 in humidified atmosphere. Before experiments, cells were trypsinized, counted and plated in 96-well plates (20 000 cells per well, for experiments shown in Fig. 2 and 3; 2×10^6 cells per 25 cm^2 flask for detection of E2 in the culture medium). After 72 h of recovery, cells were washed with PBS and then cultured in DMEM/F12 medium (no phenol red) with different concentrations of serum, steroids and/or IGF-I added.

Metatarsal organ culture

Fetal rat metatarsal rudiments were collected from 20-day embryos (De Luca *et al.* 2001). The three middle metatarsals were dissected out from each hind leg. After dissection, bones were transferred to 24-well plates, one bone per well and cultured in 1 ml phenol red-free DMEM/F12 medium supplemented with 0·2% endotoxin-free fraction V BSA, 1 mM β-glycerophosphate, 0·05 mg/ml ascorbic acid and 20 μg/ml gentamycin at 37 °C under 5% CO_2 in humidified atmosphere. Medium was changed every 2–3 days. Bones were measured as previously described (Martensson *et al.* 2004) at days 0, 2, 5, 7, 12 and 19 of culture. Metatarsal growth is expressed as a percentage of the length at the day of dissection (day 0=baseline). Treatment with IGF-I and steroids was started 4–6 h after dissection. All isolated bones (usually 30 to 40 per experiment) were pooled and then randomly divided into the different treatment groups (3 to 5 bones per group). Each experiment was repeated at least 3 times. The study was approval by the local ethical committee at Karolinska Institute (Stockholm, Sweden).

^{3}H-thymidine incorporation

Cells in 96-well plates were labeled for the last 2·5 h of culture with ^{3}H-thymidine (Amersham Pharmacia Biotech), 2·5 μCi per ml, and then harvested (Soder *et al.* 1992). Incorporated radioactivity was measured by a Beckman scintillation spectrophotometer (Beckman Instruments, Fullerton, CA, USA) as counts per min (c.p.m.). For each treatment, triplicate or quadruplicate cell cultures were used and each experiment was repeated 3–8 times.

Analysis of E2 production

The cell culture medium was changed 3 days prior to assay. Additionally, the same medium was added to cell-free dishes as a control. At the day of assay, conditioned medium was collected and cells were counted. E2 in 20% FBS-supplemented medium was measured by RIA without prior extraction (PerkinElmer, Turku, Finland). The amount of E2 in the blank (cell free, 20% FBS or 1% CTS supplemented medium) was subtracted. The amount of E2 produced was normalized per 3 days and per number of cells. The 1% CTS-supplemented medium was first partially vacuum dried, then precipitated with ethylene acetate, dried and then dissolved in ethanol in order to reach detectable concentrations. The concentration of E2 was measured by RIA (PerkinElmer, Turku, Finland). The limit of detection for the estradiol assay was 4·8 pmol/l and the intra-assay coefficient of variation was 4%. The cross reactivity (at the 50% displacement level) with estrone was 0·75%, estriol 0·40%, testosterone <0·01%, and for DHEA-S <0·001%.

Immunohistochemistry

Fetal rat metatarsal bones were cultured for 7 days, fixed in 4% formaldehyde, paraffin embedded and 5 μm-thick sections were obtained. Immunohistochemistry was performed exactly as previously described (Nilsson *et al.* 2002). Dilution of primary antibodies was as follows: anti-ERα antibody 1:500 (MC-20, Santa Cruz), anti-ERβ antibody 1:1000 (Upstate), anti-CYP19 aromatase P450 1:100 (C-16, Santa Cruz), anti-IGF-I antibody 1:300 (H-70, Santa Cruz), anti IGF-II antibody 1:100 (H-103, Santa Cruz), anti-IGF-IRβ antibody 1:150 (C-20, Santa Cruz). As a control, antibodies were pre-incubated for 1 hour at room temperature with 10 × (w/w) excess of the corresponding blocking peptide (MC-20, C-16 and C-20 antibodies) or IGF-I (H-70 and H-103 antibodies).

Reverse transcription-PCR

Extraction of total RNA from HCS-2/8 proliferative chondrocytes was performed using the Qiagen RNeasy mini kit. The following pairs of primers were used to generate the respective cDNAs: human IGF-I, 5′-AAATCAGCAGTCTTCCAACC-3′ and 5′-CTTCTG GGTCTTGGGCATGT-3′ (396 bp), human IGF-II 5′-CTGGAGACGTACTGTGCTAC-3′ and 5′-GGTGTT TAAAGCCAATCGAT-3′ (547 bp), human type X collagen, 5′-AGCCAGGGTTGCCAGGACCA-3′ and 5′-TTTTCCCACTCCAGGAGGGC-3′ (387 bp). The β-actin gene, 5′-CACACTGTGCCCATCTACGA-3′ and 5′-GTTTCATGGATGCCACAGGA-3′ (349 bp), was used as a control to normalize the data. The annealing temperature was 56 °C for IGF-I (35 cycles), 58 °C for IGF-II (30 cycles), 56 °C for collagen type X (35 cycles) and 58 °C for β-actin (25 cycles). The PCR-products were separated by electrophoresis in a 2% agarose gel with ethidium bromide (15 μg/10 ml). RNA from HCS-2/8 chondrocytes, cultured for 18 days under differentiation conditions, was used as a positive control for type X collagen.

Western blot

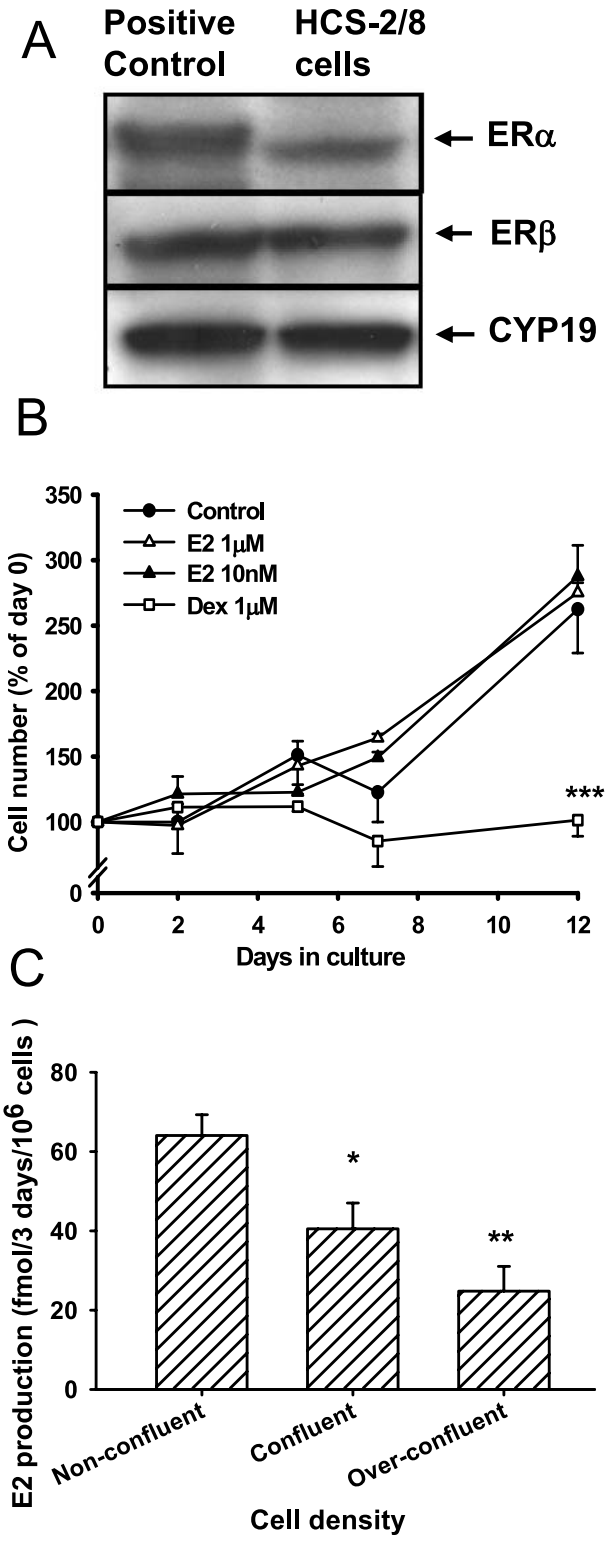
Cells, growing in logarithmic phase of growth were washed twice with ice-cold PBS, scraped in 2 × loading buffer (250 mM Tris pH 6·8, 4% SDS, 10% glycerol, 0·006% bromophenol blue, 2% β-mercaptoethanol), boiled for 5 min and sonicated on ice. Twenty μg of protein per lane was subjected to SDS–PAGE and than transferred to an Immobilon-P membrane (Amersham Pharmacia Biotech). The filters were blocked in 5% blocking agent (Amersham Pharmacia Biotech) and subsequently incubated with the first antibody (anti-ERα, 1:4000 dilution; anti-ERβ, 1:1000 dilution; anti-CYP19 aromatase P450, 1:500 dilution; anti-IGF-IRβ, 1:1000 dilution) and then the appropriate secondary HRP-conjugated antibody. Detection was performed with ECL+Plus (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Protein concentrations were measured by spectrophotometer using a Protein Assay Kit (Bio-Rad Laboratories) before gel electrophoresis. Equal loading of total proteins was confirmed by Coomasie Blue staining after the experiment.

Bromodeoxyuridine labeling (BrdU labeling)

BrdU-labeling was performed as previously described (Martensson *et al.* 2004). Briefly, fetal rat metatarsal bones were cultured for 7 days with/without 10 μM ICI 182,780. Two and a half hours prior to fixation, the DMEM/F12 medium was changed to MEM medium, supplemented with BrdU labeling reagent (1:200 dilution of labeling reagent, Cell Proliferation Kit RPN20; Amersham Biosciences). The bones were then fixed and 5 μm-thick paraffin-embedded sections were obtained. Detection of BrdU-positive cells were performed as recently described (Martensson *et al.* 2004). The level of proliferation was expressed as number of BrdU-positive cells per mm^2 surface area.

TUNEL

Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) immunohistochemistry according to instructions for the TdT-FragEL DNA fragmentation kit, (Oncogene Research, Boston, MA, USA) with the modification that sections were treated with 5 μg/ml proteinase K for 10 min (Martensson *et al.* 2004). Sections



were counterstained with Alcian Blue, dehydrated, and mounted. For each group, the number of apoptotic cells was determined in five metatarsals, and at least 2 sections were assessed for each bone. The number of apoptotic chondrocytes was counted and expressed per mm^2 surface area. After pre-treatment of sections with distilled water instead of TdT, all cells were negative, whereas treatment with DNase labeled all cells.

Cell death detection ELISA

Apoptosis was studied by measuring the cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleasomes) by photometric enzyme-immunoassay (cell death detection ELISA; Roche Diagnostics GmbH) exactly as recently described (Chrysis *et al.* 2005). Three samples were analyzed for each experiment, which was repeated 4 times.

Statistical analysis

One-way ANOVA followed by Student–Newman–Keuls method was applied for all statistical analysis if not otherwise stated. *P*-values<0·05 were considered statistically significant.

Results

Role of estrogen in HCS-2/8 human chondrocytes

HCS-2/8 human chondrocytes expressed ER-α, ERβ and aromatase P450 (Fig. 1A). To investigate any effect of exogenous estrogen on cell proliferation, HCS-2/8 chondrocytes were cultured for 12 days with E2 added at

Figure 1 Local production of estrogen by HCS-2/8 chondrocytes and expression of estrogen receptors, which lack response to exogenous E2. (A) HCS-2/8 cells express ERα, ERβ and aromatase P450 (CYP19) as detected by Western immunoblotting (see Material and Methods). MCF-7 breast cancer cells were used as positive controls for ERα and rat testis for ERβ and aromatase P450. (B) HCS-2/8 cells did not respond to E2 (10 nM and 1 μM), while dexamethasone (1 μM) decreased cell proliferation. Cells were cultured in the presence of 5% CTS and counted after 0, 2, 5, 7 and 12 days of culture. Data represent the mean ± S.E.M. for at least 3 independent experiments (***P<0·001 vs control). (C) HCS-2/8 cells locally synthesized E2. Cultures of HCS-2/8 chondrocytes reaching three different densities (non-confluent – 3 days in culture confluent – 10 days in culture and over-confluent – 17 days in culture) were rinsed with PBS and then fresh culture medium supplemented with 20% FBS was applied for 3 days. The medium was then collected and the levels of E2 were analyzed by RIA (see Material and Methods). The concentration of E2 in culture medium without cells (20% FBS, DMEM/F12) was analyzed in parallel (22·4 ± 3·3 pmol/l) and subtracted from the conditioned medium. The production of E2 was normalized per cell number. Data represent the mean ± S.E.M. for 3 independent experiments. *P<0·05; **P<0·01 vs non-confluent cells.

different concentrations in culture media containing no serum or charcoal treated serum (CTS; 0·2%, 1% or 5%). Independent of what culture media used, cell number (data for media containing 5% CTS and 10 nM and 1 μM E2 are shown in Fig. 1B) and thymidine incorporation were unaffected by exogenous E2 at concentrations ranging between 0·1 nM and 1 μM (data not shown, see also Fig 2B for 100 pM E2 in 1% CTS). IGF-I (100 ng/ml) increased cell proliferation 3- to 4-fold, an effect which was unchanged if the cells were co-treated with IGF-I (100 ng/ml) plus E2 (1 nM – 1 μM) (data not shown). Dexamethasone (1 μM) significantly decreased cell proliferation (Fig. 1B).

As HCS-2/8 chondrocytes did not respond to exogenous E2, we hypothesized that the cells might synthesize estrogen by themselves and that this could be of physiological importance. Indeed, we found that HCS-2/8 cells produced 64·1 ± 5·3 fmol/3 days/10^6 cells of E2 (Fig. 1C). Interestingly, the production of estrogen was dependent on the stage of chondrocyte differentiation, being high in proliferative cells (non-confluent) and gradually decreasing in more differentiated confluent and over-confluent cell cultures ($P<0·01$ between non-confluent and over-confluent cultures, Fig. 1C). The chondrocyte differentiation in over-confluent cell cultures was confirmed by detection of collagen type X by RT-PCR (data not shown). The aromatase inhibitor letrozole (10 μM) decreased E2 production by HCS-2/8 chondrocytes from 64·1 ± 5·3 fmol to 24·5 ± 6·7 fmol/3 days/10^6 cells ($P<0·01$ by *t*-test). HCS-2/8 chondrocytes still produced small amounts of E2 (0·234 ± 0·071 fmol/3 days/10^6 cells) when cultured under steroid depleted conditions (1% CTS, phenol red free medium).

Role of locally produced estrogen in HCS-2/8 chondrocytes

To study any physiological relevance of endogenously produced estrogen, HCS-2/8 chondrocytes were treated with an aromatase inhibitor, letrozole, or with a pure non-selective anti-estrogen, ICI 182,780. Cell proliferation was significantly decreased in HCS-2/8 cells treated with either letrozole or ICI 182,780 (Fig 2A). Moreover, E2 (100 pM) was able to restore cell proliferation when added in combination with letrozole (completely for 1μM and partially for 10 μM letrozole; Fig 2B). Treatment with ICI 182,780 not only inhibited cell proliferation but also induced apoptosis in a dose-dependent way when cultured for 48 h (Fig. 3). Further studies showed that ICI 182,780 (10 μM) induced apoptosis already after 24 h (130 ± 10%), being maximal after 48 h (228 ± 30%, $P<0·05$ vs control) and then slightly lower after 72 h of treatment (170 ± 19%, $P<0·05$ vs control). To further support these results and also to understand the apoptotic pathways involved in ICI 182,780-induced apoptosis, caspase inhibition experiments were performed. Both caspase-3 and pan-caspase inhibitors completely blocked ICI 182,780-induced apoptosis (Fig 3), showing that it is a caspase dependent apoptosis. As another evidence of caspase involvement, we found that cleavage of the proteins PARP and fodrin occurred after treatment of HCS-2/8 cells with 10 μM ICI 182,780 for 72 h (data not shown).

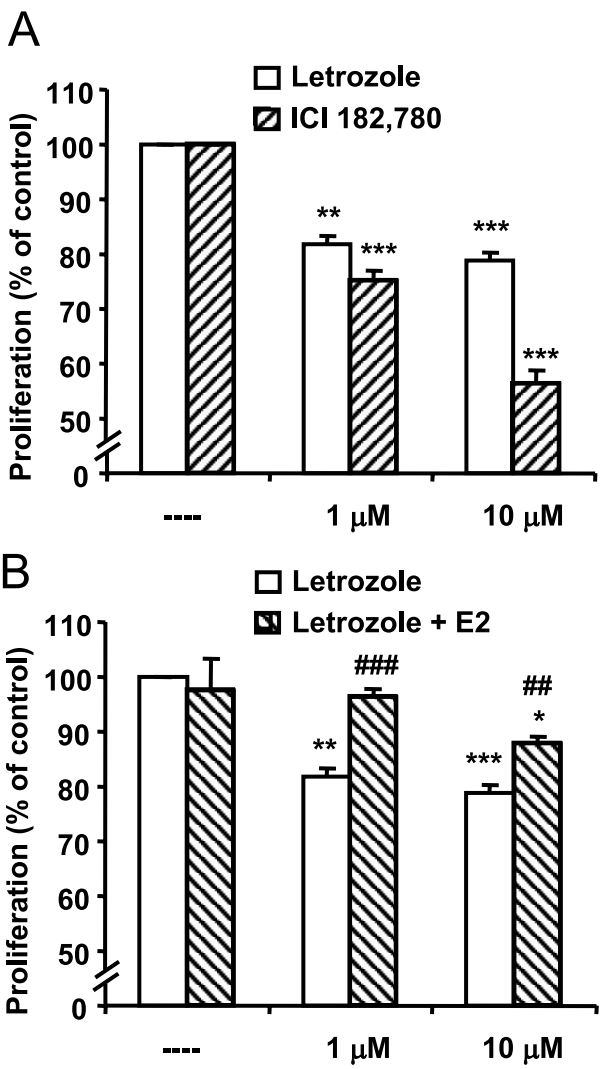


Figure 2 Locally synthesized E2 promotes HCS-2/8 cell proliferation. (A) Both letrozole (aromatase blocker) and ICI 182,780 (non-selective ER-blocker) inhibited HCS-2/8 cell proliferation when added for 48 h in the presence of 1% charcoal treated serum (CTS). (B) E2 (100 pM) restored letrozole-mediated inhibition of HCS-2/8 cell proliferation, when added in combination with letrozole (1 μM and 10 μM) for 48 h in the presence of 1% CTS. Cell proliferation was analyzed by ^{3}H-thymidine incorporation (see Material and Methods). Data represent the mean ± S.E.M. for at least 3 independent experiments. *$P<0·05$, **$P<0·01$, ***$P<0·001$ vs untreated cells; $^{\#\#}P<0·01$, $^{\#\#\#}P<0·001$ vs Letrozole alone.

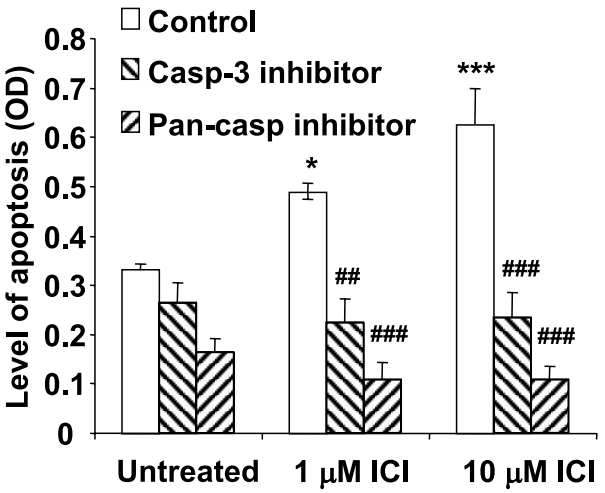


Figure 3 Caspase-dependent apoptosis induced by ICI 182,780. HCS-2/8 cells were treated for 48 h with ICI 182,780 (1 μM or 10 μM) with/without caspase-3 (50 μM) or pan-caspase (50 μM) inhibitors added (1% CTS in all medium). The level of apoptosis was analyzed by a cell death detection ELISA Kit (see Material and Methods). Data represent the mean ± S.E.M. for at least 3 independent experiments. $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$ vs untreated control; $^{\#\#}P<0{\cdot}01$, $^{\#\#\#}P<0{\cdot}001$ vs ICI 182,780 alone.

Locally produced estrogen maintains fetal rat metatarsal bone growth

Fetal rat metatarsal bones expressed ERα, ERβ and aromatase P450 (Fig. 4, upper, middle and lower panels, respectively). The expression of ERα and ERβ was widely distributed and mainly associated with resting and proliferative chondrocytes (Fig. 4, upper and middle panels). However, the expression of aromatase P450 was associated with hypertrophic chondrocytes (Fig. 4, lower panels).

Treatment with E2 at 1 μM (Fig. 5A) or 10 nM (data not shown) did not affect longitudinal growth at any time point when cultured in serum free medium for up to 19 days. When analyzed separately, neither female nor male metatarsals responded to E2 (data not shown). However, metatarsal bone growth was increased by IGF-I (100 ng/ml; Fig. 5A) and decreased by dexamethasone (1 μM; Fig. 5A).

Longitudinal growth of cultured fetal rat metatarsal bones was significantly inhibited when cultured in the presence of the aromatase inhibitor letrozole (1 μM),

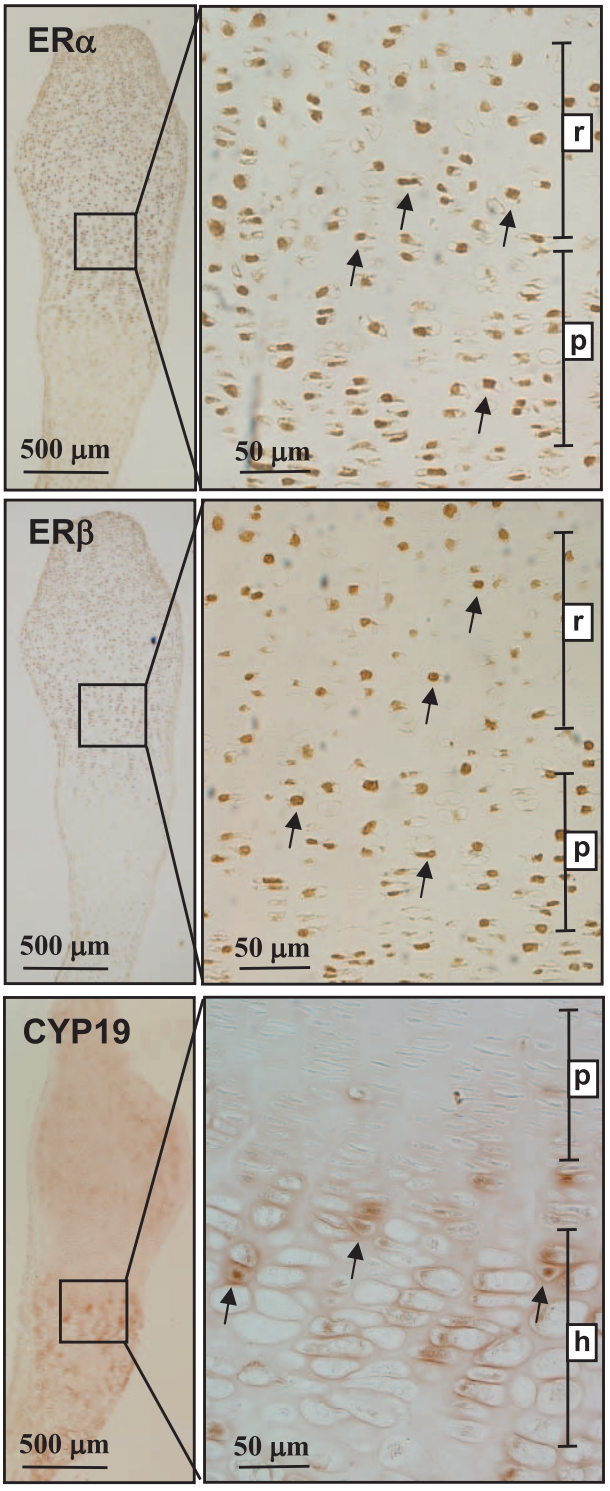


Figure 4 Expression of ERα, ERβ and aromatase P450 in fetal rat metatarsal bones. The bones were cultured for 7 days, then fixed and paraffin embedded. The expression patterns of ERα (upper panels) and ERβ (middle panels) and aromatase P450 (CYP19; lower panels) were analyzed by immunohistochemistry in 5 μM-thick sections (see Material and Methods). Immuno-positive cells for both ERα and ERβ were commonly detected in resting (r) and proliferative (p) chondrocytes, while immuno-positive cells for aromatase P450 were detected in hypertrophic (h) chondrocytes (arrows show a few typical cells).

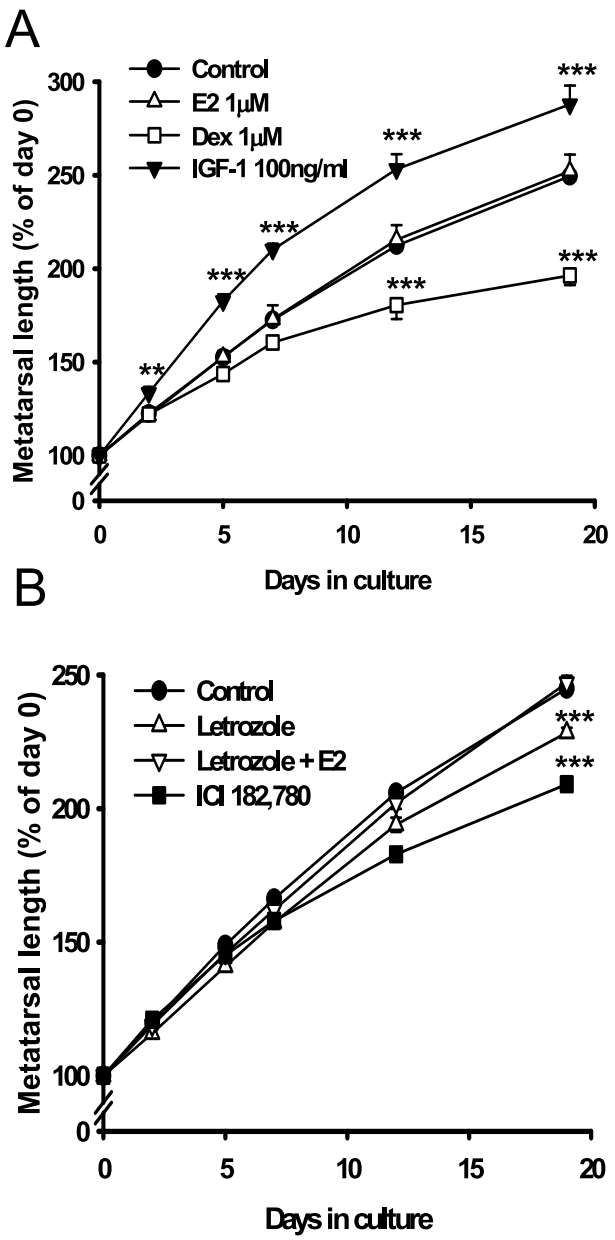


Figure 5 Effect of locally produced estrogen on longitudinal growth. Fetal rat metatarsal bones were cultured for 19 days in the presence of E2 (1 μM), dexamethasone (1 μM), or IGF-I (100 ng/ml) (A), or in the presence of letrozole (1 μM), letrozole plus estrogen (10 nM) or ICI 182,780 (10 μM) (B). At days 0, 2, 5, 7, 12 and 19, the lengths of the metatarsal bones were measured (see Material and Methods). Data represent the mean ± S.E.M. for at least 3 independent experiments. $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ vs control.

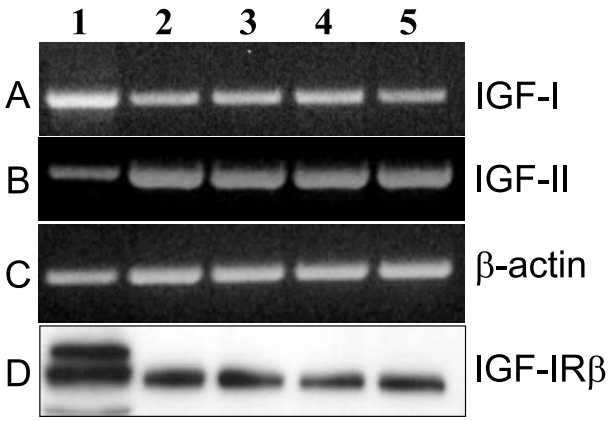


Figure 6 Effect of estrogen on IGF-I, IGF-II and IGF-IRβ expression in HCS-2/8 cells. HCS-2/8 cells were cultured for 48 h in 1% CTS in the presence of vehicle (0·1% ethanol, lane 2), E2 (10 nM, lane 3), letrozole (10 μM, lane 4) or ICI 182,780 (10 μM, lane 5). Human testis was used as a positive control (lane 1). Expression of IGF-I (A), IGF-II (B) and β-actin (C) was analyzed by RT-PCR and expression of IGF-IRβ (D) was analyzed by Western immunoblot as described in the Material and Methods.

which blocks the production of estradiol or the pure non-selective anti-estrogen, ICI 182,780 (10 μM), which efficiently blocks signaling through ERα and ERβ (Fig. 5B). The growth retardation was significant already after 7 days of culture ($P<0{\cdot}01$ vs control for both letrozole and ICI). E2 restored letrozole-induced growth retardation to control levels (Fig. 5B). Detailed analysis of proliferation and apoptotic indices within metatarsal chondrocytes revealed that blocking of estrogen receptors with ICI 182,780 (10 μM) was associated with decreased chondrocyte proliferation compared with control bones (170 ± 13 versus 277 ± 38 cells per mm^2, respectively; $P<0{\cdot}05$) and increased apoptosis ($55{\cdot}2 \pm 4{\cdot}2$ versus $33{\cdot}9 \pm 1{\cdot}9$ TUNEL-positive cells per mm^2, respectively; $P<0{\cdot}01$).

Cross-talk between estrogens and IGF-I system

In order to study any cross-talk between the local estrogen and the IGF system, we analyzed the expression of IGF-I, IGF-II and the IGF-I receptor beta subunit (IGF-IRβ). As shown in Fig. 6, exogenous E2, letrozole and ICI 182,780 were all unable to affect the expression of IGF-I, IGF-II and IGF-IRβ in HCS-2/8 cells. Immunohistochemical localization of IGF-I, IGF-II and IGF-IRβ within fetal rat metatarsal bones revealed that IGF-I is mainly localized within the resting zone, IGF-II mainly in late proliferative chondrocytes and IGF-IRβ in prehypertrophic and early hypertrophic chondrocytes (Fig. 7, left column, upper, middle and lower panels, respectively; and data not shown). Pre-incubation of the IGF-I and IGF-II antibodies with IGF-I completely abrogated IGF-I staining but did not affect IGF-II positive cells (Fig. 7, right column, upper and middle panels, respectively). Pre-incubation of the IGF-IRβ antibody with the corresponding peptide abrogated IGF-IRβ staining (Fig. 7, right column, lower panel). After culturing fetal rat metatarsal bones in the presence of ICI 182,780, we found a relative decrease of the intensity of the IGF-I, IGF-II

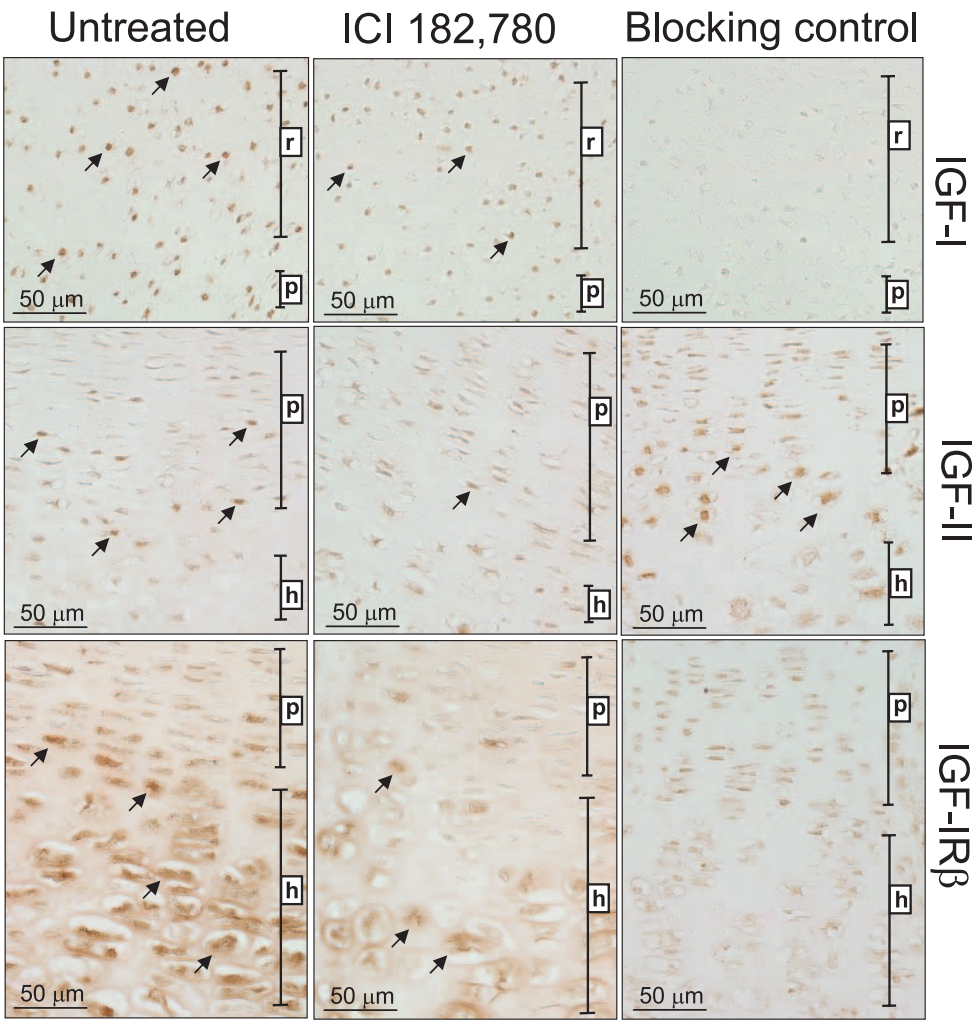


Figure 7 Effect of estrogen on IGF-I, IGF-II and IGF-IRβ expression in fetal rat metatarsal bones. Fetal rat metatarsal bones were cultured for 7 days in presence of vehicle (0·1% ethanol, left column) or ICI 182,780 (10 μM, middle column). Expression patterns of IGF-I (upper panels), IGF-II (middle panels), and IGF-IRβ (lower panels) were analyzed by immunohistochemistry in 5 μM-thick sections (see Material and Methods). Blocking experiments (right column) were performed by pre-incubating of anti-IGF-I or anti-IGF-II antibodies with IGF-I and anti-IGF-IRβ antibodies with corresponding peptide as indicated in the Material and Methods (arrows show a few typical cells).

and IGF-IRβ immunostaining (Fig. 7, middle column, upper, middle and lower panels, respectively).

Discussion

We here show that estrogen, locally produced by growth plate chondrocytes, promotes longitudinal growth of fetal rat metatarsal bones. In addition, we demonstrate that endogenously produced estrogen has the capacity to promote chondrocyte proliferation and protect from programmed cell death.

The possibility of intracrine actions of estrogen within the epiphyseal growth plate has been well discussed (Van Der Eerden *et al.* 2003) and supported by several recent reports showing that chondrocytes are able to synthesize estrogen *in vitro* and *in vivo* (Oz *et al.* 2001, Sylvia *et al.* 2002, Van Der Eerden *et al.* 2002). P450 aromatase activity has been demonstrated in human (Oz *et al.* 2001) and rat (Van Der Eerden *et al.* 2002) growth plate cartilage. It should be emphasized that aromatase expression was mainly detected in adolescent humans and pubertal animals but very weak activity and expression of aromatase was detected before sexual maturation

(Van Der Eerden *et al.* 2002). Interestingly, our observation of P450 aromatase expression in hypertrophic chondrocytes of fetal rat metatarsal bones is in line with several *in vivo* studies in different species (Oz *et al.* 2001, Van Der Eerden *et al.* 2002, Eshet *et al.* 2004). Several key enzymes needed for estrogen synthesis, including STS and 17β-HSD, has been demonstrated in rat growth plate cartilage (Van Der Eerden *et al.* 2002). Moreover, rat costal chondrocytes have been shown to synthesize estrogen (Sylvia *et al.* 2002). Our finding that HCS-2/8 chondrocytes endogenously produce estrogen is in line with those observations. Considering the poor vascularization of the growth plate, it can be assumed that the intracellular concentrations of the ligand are mainly dependent on its local production. Our finding that the amounts of estrogen produced by proliferative and hypertrophic chondrocytes in cell cultures are different, opens the possibility that estrogen synthesis is also regulated within the growth plate. This observation together with the previously described hormone- (Sylvia *et al.* 2002) and age-dependent (Van Der Eerden *et al.* 2002) regulation of estrogen synthesis within chondrocytes suggests physiological importance of intracrine estrogen action.

To understand the physiological role of locally produced estrogen within the growth plate, we blocked local estrogen synthesis by an aromatase inhibitor, letrozole, and estrogen receptors by the pure non-selective estrogen antagonist ICI 182,780. These experiments clearly show that endogenously produced estrogen stimulates chondrocyte proliferation and also longitudinal growth of fetal rat metatarsal bones. These data are in line with the recent observation that letrozole inhibits longitudinal growth velocity in boys (Wickman *et al.* 2001). Additionally, it is very well known that estrogen is able to stimulate longitudinal growth in humans (reviewed by Juul (2001). The mechanism behind this phenomenon is not clear, although low-dose estrogen treatment increases serum GH and IGF-I, which may contribute to the pubertal growth spurt (Juul 2001). Patients with growth hormone insensitivity do have a detectable pubertal growth spurt (Laron *et al.* 1980, 1993), which suggests that sex steroids also have a direct growth stimulatory effect which is independent of the GH/IGF-axis. In rodents, in contrast to humans, estrogen has a well known inhibitory effect on longitudinal growth. Treatment of mice with letrozole increases tibia length and epiphyseal growth plate height (Eshet *et al.* 2004). However, the effect was accompanied by an 8-fold increase in serum GH and a 6-fold increase in serum testosterone, which most likely reflects the systemic actions of the letrozole treatment. Indeed, GH is a well known stimulator of longitudinal bone growth. Interestingly, we recently showed that androgens can counteract the suppressive effect of estrogen on longitudinal growth (Tivesten *et al.* 2004). Altogether, these data suggest that letrozole stimulates longitudinal growth through systemic actions.

A direct proliferative effect of E2 on chondrocytes *in vitro* is well documented in literature (Somjen *et al.* 1989, 1991, Maor *et al.* 1999). However, estrogen has also been reported to inhibit chondrocyte proliferation (Nasatzky *et al.* 1993, Schwartz *et al.* 1997). Such a difference can be explained by the dose-dependent actions of estrogen. Indeed, it has been shown that at different concentrations of the ligand, estrogen receptors can exert different, often opposite, effects (Hall & McDonnell 1999, Pettersson *et al.* 2000).

We here report the novel finding that endogenously produced estrogen has the capacity to protect chondrocytes from undergoing programmed cell death. This finding is important as apoptosis is known to play an important role in growth plate homeostasis (Chrysis *et al.* 2002). Although estrogen is well known to have anti-apoptotic actions in other tissues (Contreras *et al.* 2002, Rau *et al.* 2003), it is not clear how estrogen affects chondrocyte cell death. Here, we demonstrate that the withdrawal of estrogen induces apoptosis in a caspase-dependent manner. This observation suggests involvement of specific anti-apoptotic pathways in the protective function of estrogen. However, we can not exclude the possibility that ICI 182,780 can act not only as a pure estrogen receptor antagonist but also as a selective estrogen receptor modulator. Additional studies are needed to reveal the exact mechanisms of the anti-apoptotic actions of estrogens in chondrocytes.

Despite the wide distribution of estrogen receptors, we did not find any effect of estrogen when added to cultures of HCS-2/8 chondrocytes or fetal rat metatarsal bones. One possible explanation could be that endogenously produced estrogen constantly activates estrogen receptors and thereby prevents further actions of exogenous estrogen. Local chondrocyte production of estrogens may vary between different experimental models which could explain the previously conflicting reports of varying estrogen effects on chondrocyte proliferation. If local estrogen synthesis is low due to specific experimental circumstances externally added estrogen will be capable to stimulate chondrocyte proliferation. Local estrogen production may be affected by factors such as age, sex and concomitant hormonal treatment or culture conditions.

In summary, we demonstrate a role of estrogen, locally produced by chondrocytes, in the normal regulation of growth plate cartilage. This endogenously produced estrogen maintains chondrocyte proliferation and protects cells from spontaneous apoptosis and thereby facilitates longitudinal growth. Our findings support the proposed intracrine actions of estrogen in growth plate cartilage.

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