

# Localization of estrogen receptors- $\alpha$ and - $\beta$ and androgen receptor in the human growth plate at different pubertal stages

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## Abstract

Sex steroids are required for a normal pubertal growth spurt and fusion of the human epiphyseal growth plate. However, the localization of sex steroid receptors in the human pubertal growth plate remains controversial. We have investigated the expression of estrogen receptor (ER)  $\alpha$ , ER $\beta$  and androgen receptor (AR) in biopsies of proximal tibial growth plates obtained during epiphyseal surgery in 16 boys and eight girls. All pubertal stages were represented (Tanner stages 1–5). ER $\alpha$ , ER $\beta$  and AR were visualized with immunohistochemistry and the number of receptor-positive cells was counted using an image analysis system. Percent receptor-positive chondrocytes were assessed in the resting, proliferative and hypertrophic zones and evaluated for sex differences and pubertal trends. Both ER $\alpha$ - and ER $\beta$ -positive cells were detected at a greater

frequency in the resting and proliferative zones than in the hypertrophic zone ( $64 \pm 2\%$ ,  $64 \pm 2\%$  compared with  $38 \pm 3\%$  for ER $\alpha$ , and  $63 \pm 3\%$ ,  $66 \pm 3\%$  compared with  $53 \pm 3\%$  for ER $\beta$ ), whereas AR was more abundant in the resting ( $65 \pm 3\%$ ) and hypertrophic zones ( $58 \pm 3\%$ ) than in the proliferative zone ( $41 \pm 3\%$ ). No sex difference in the patterns of expression was detected. For ER $\alpha$  and AR, the percentage of receptor-positive cells was similar at all Tanner pubertal stages, whereas ER $\beta$  showed a slight decrease in the proliferative zone during pubertal development ( $P < 0.05$ ). In summary, our findings suggest that ER $\alpha$ , ER $\beta$  and AR are expressed in the human growth plate throughout pubertal development, with no difference between the sexes.

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## Introduction

Longitudinal bone growth occurs at the growth plate, a thin cartilage structure found at the ends of long bones, by a process called endochondral ossification, in which cartilage is first formed and then remodeled into bone tissue. The growth plate consists of three histologically and functionally discrete layers: the *resting zone*, containing cells capable of differentiating into proliferative chondrocytes (Abad *et al.* 2002); the *proliferative zone*, where chondrocytes actively proliferate (Kember & Walker 1971) and line up in columns (Abad *et al.* 2002), thereby directing growth primarily in one dimension; and the *hypertrophic zone*, where the terminally differentiated chondrocytes calcify their matrix and attract the invading bone and endothelial cells, a crucial step in endochondral bone formation (Baron *et al.* 1994, Gerber *et al.* 1999).

During puberty, sex steroids from the developing gonads induce a pubertal growth spurt, which occurs during early puberty in girls and mid-puberty in boys. Sex steroids, specifically estrogen, also cause the cessation of growth and epiphyseal fusion (Goldzieher 1956).

The pivotal role of estrogen in epiphyseal fusion in both boys and girls was confirmed by the recognition of two rare genetic disorders: estrogen deficiency resulting from mutations in the aromatase gene (Morishima *et al.* 1995), and estrogen resistance resulting from a mutation in the estrogen receptor (ER)  $\alpha$  gene (Smith *et al.* 1994). In both these conditions, longitudinal bone growth continues well into adulthood. In contrast, patients with complete androgen insensitivity syndrome have a nearly normal pubertal growth spurt (the amplitude of the spurt is intermediate between male and female) and undergo epiphyseal fusion during pubertal development (Zachmann *et al.* 1986).

Androgens are thus not necessary for the pubertal growth acceleration or for the cessation of linear growth that occurs in late puberty. However, *in vitro* (Carrascosa *et al.* 1990, Schwartz *et al.* 1994) and *in vivo* (Jansson *et al.* 1983, Ren *et al.* 1989) studies suggest that androgens do stimulate longitudinal bone growth, through a direct effect on the growth plate, and this is further supported by the observation that both dihydrotestosterone and oxandrolone, both non-aromatizable androgens, accelerate growth velocity without an increase in growth hormone secretion (Stanhope *et al.* 1988, Keenan *et al.* 1993, Nilsson *et al.* 1996).

Several *in vitro* and *in situ* studies have suggested that the sex steroid responsiveness of growth plate cartilage is dependent on the sex (Carrascosa *et al.* 1990, Schwartz *et al.* 1991, 1994, Nasatzky *et al.* 1993, van der Eerden *et al.* 2002b) or the age, or both (Corvol *et al.* 1987, Blanchard *et al.* 1991, Pinus *et al.* 1993) of the individual. However, few studies have addressed this issue in human growth plate cartilage.

To gain insight into the mechanisms by which sex steroids cause the pubertal growth pattern, we examined the patterns of expression of ER $\alpha$ , ER $\beta$  and androgen receptor (AR) in tibial growth plates from girls and boys at different stages of puberty.

## Materials and Methods

### Patients and tissue preparation

Biopsies from human tibial growth plates were collected from 16 boys and eight girls, at different pubertal stages (Table 1), who were undergoing epiphyseal surgery to arrest longitudinal bone growth. Surgery was performed because of either leg-length inequality or expected extremely tall stature (Table 1). The local ethics committees at Karolinska Hospital, Stockholm, Sweden and at Turku University Hospital, Turku, Finland, gave approval for the study, informed assent was obtained from all the patients, and informed consent was obtained from a parent. All biopsies were of the same size and processed in the same way. The specimens were immediately fixed in 4% formaldehyde for 16 h (pH 7.4), decalcified (10% EDTA, 2 days), dehydrated, and then embedded in paraffin. The tissues were cut into 5  $\mu$ m sections and mounted on histological glass slides (Superfrost/+, Menzel-Gloeser, Braunschweig, Germany), dried at 37 °C overnight and heated at 60 °C for 1 h. For bone age assessment, radiographs of the hand and wrist were obtained within 6 months of the date of surgery. The bone age was assessed according to the method of Gruelich & Pyle (1959) by two experienced readers blinded to the identity of the patient. The resulting two bone ages were averaged.

### Immunohistochemistry

For immunohistochemistry, slides were treated with xylene to remove paraffin and rehydrated in graded alcohol

baths. Antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0) at 94–97 °C for 20 min. After one wash in Tris-buffered saline (TBS; 0.05 M Tris, 0.85% saline, pH 7.4), endogenous peroxidase activity was reduced by incubation in 3.0% hydrogen peroxide in TBS for 5 min at room temperature. To decrease non-specific binding, slides were incubated with 1.0% BSA (fraction V powder, Sigma, St Louis, MO, USA) in TBS at room temperature for 1 h. Primary antibodies were diluted in TBS with 0.1% BSA and sections were incubated overnight at +4 °C. The same concentrations of primary antibodies were used to stain prostate (positive control) and growth plate tissue.

Control sections were incubated with preadsorbed antibodies, non-specific rabbit IgG or non-specific mouse IgG1, as described below. Sections were washed five times in TBS and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA) or goat anti-mouse IgG1 (1:200; Santa Cruz Biotechnology, CA, USA) for 45 min at room temperature. They were then washed five times in TBS, incubated with avidin-conjugated peroxidase (Vector Laboratories) for 30 min, and the resulting peroxidase activity was detected using a DAB-kit (Vector Laboratories). Growth plate sections were counterstained with methyl-green, whereas sections of ovary and liver were counterstained with hematoxylin.

### Antibodies

**Anti-ER $\alpha$**  For ER $\alpha$  localization, we used a monoclonal mouse antibody (ER-6F11; Novocastra Lab. Ltd, Newcastle, UK). ER-6F11, raised in mouse against the complete human ER $\alpha$  protein, was diluted to 2  $\mu$ g/ml before use. In all experiments, control sections incubated with non-immune mouse IgG1 were included. As an additional negative control, ER-6F11 (2.0  $\mu$ g/ml) was incubated with recombinant ER $\alpha$  protein (2.0  $\mu$ g/ml; catalog no. P2187, Panvera LLC, Madison, WI, USA) for 2 h at room temperature, before it was applied on the tissue. The antibody recognizes an epitope located within the first 184 amino acids of the human ER $\alpha$  (Novocastra Lab. Ltd, personal communication) and is therefore unlikely to recognize any isoform that is truncated from the N-terminal (Denger *et al.* 2001).

**Anti-ER $\beta$**  To localize ER $\beta$ , a polyclonal rabbit anti-rat ER $\beta$  (06–629; Upstate Biotechnology, Lake Placid, NY, USA), raised against a synthetic peptide corresponding to amino acids 99–116 of human ER $\beta$  (Genbank AF051427) was used. The optimal working concentration was found to be 2.5  $\mu$ g/ml. Control sections incubated with non-immune rabbit IgG (2.5  $\mu$ g/ml) were included in all experiments. In addition, 06–629 was incubated with a 10-fold molar excess of immunizing peptide (629P; Upstate Biotechnology) for 2 h at room temperature. The

**Table 1** Patients, physical findings and expression of ER $\alpha$ , ER $\beta$  and AR in the resting, proliferative and hypertrophic zones

Patient no.	Sex	Tanner pubertal stage	Age (yr:month)	Bone age (yr:month)	Diagnosis	ER $\alpha$ (%pos. cells)			ER $\beta$ (%pos. cells)			AR (%pos. cells)		
						RZ	PZ	HZ	RZ	PZ	HZ	RZ	PZ	HZ
1	F	B3/Ph4	13:8	12:4	Leg length difference	58	83	55	54	55	73	60	47	42
2	F	B2-3/Ph2	14:2	13:4	Leg length difference	61	66	55	50	74	61	61	47	55
3	F	B2/Ph2	12:8	11:2	Leg length difference	51	72	33	71	68	71	74	45	67
4	F	B2/Ph1	12:7	12:4	Leg length difference	58	76	50	53	68	45	41	15	75
5	F	B2/Ph4	13:7	13:0	Constitutional tall stature	61	60	31	64	49	42	56	42	32
6	F	B4/Ph4	13:3	13:6	Leg length difference	72	49	28	52	56	35	58	23	71
7	F	B1/Ph1	9:6	11:0	Leg length difference	75	67	59	56	73	68	88	54	65
8	F	B2/Ph2	12:6	12:0	Constitutional tall stature	50	67	15	59	58	51	61	32	69
9	M	G1/Ph1	12:3	12:4	Marfan syndrome	50	60	42	52	74	50	64	32	69
10	M	G4/Ph4	13:5	15:4	Leg length difference	54	76	31	68	58	51	75	27	63
11	M	G4-5/Ph4	14:0	14:8	Klinefelter's syndrome	44	57	40	68	40	64	68	40	64
12	M	G3-4/Ph3-4	15:4	13:6	Leg length difference	90	75	33	78	73	44	72	23	75
13	M		16:2	14:6	Leg length difference	54	55	73	88	75	71	54	64	70
14	M	G4/Ph4	14:3	14:7	Leg length difference	61	76	29	38	66	59	63	59	24
15	M	G4-5/Ph4	14:1	12:4	Leg length difference	71	56	38	63	56	56	71	56	38
16	M	G4/Ph4	13:8	13:6	Leg length difference	64	76	28	28	54	44	43	24	53
17	M	G3/Ph3	14:3	13:10	Leg length difference	69	40	42	72	72	49	80	51	67
18	M	G3/Ph3	14:7	15:3	Leg length difference	59	62	39	89	80	42	95	77	44
19	M	G4/Ph4	16:0	14:6	Leg length difference	64	67	35	62	69	62	64	29	76
20	M	G4/Ph4	14:7	14:0	Leg length difference	61	49	28	64	52	42	64	45	29
21	M	G3/Ph3	12:3	14:0	Leg length difference	63	49	31	63	53	46	61	31	67
22	M	G3/Ph3	14:10	13:0	Constitutional tall stature	65	70	29	89	81	47	77	15	77
23	M	G4/Ph3	16:4	14:3	Constitutional tall stature	90	81	33	55	85	33	62	39	54
24	M	G1/Ph1	12:7	12:6	Constitutional tall stature	81	47	31	70	88	65	50	63	58

yr:month, years:months; %pos. cells, percentage of positive cells; RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone.

antibody is likely to detect all isoforms of ER $\beta$  that have an intact A/B domain, for example hER $\beta$ 1–3 (reviewed in Lewandowski *et al.* 2002).

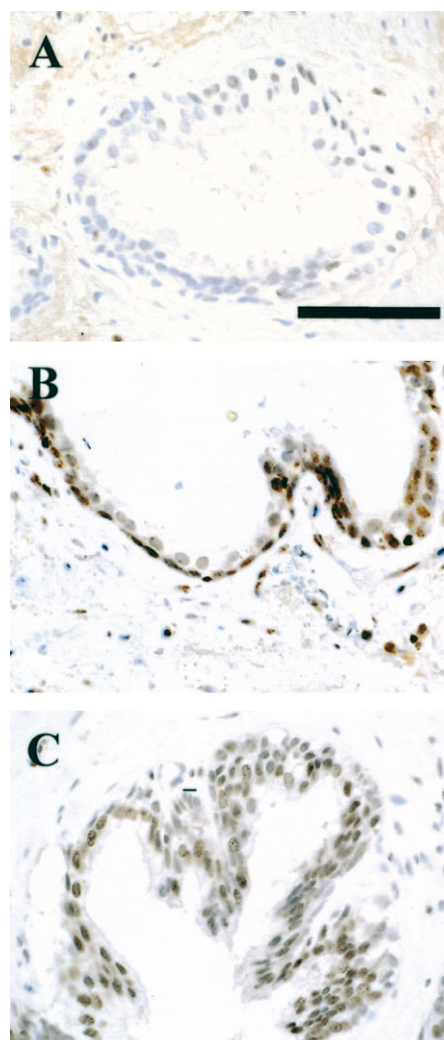
**Anti-AR** AR was localized using a polyclonal rabbit anti-human AR (N-20; catalog no. sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA), raised against a synthetic peptide mapping at the amino terminus of the human AR. The optimal working concentration was found to be 0.5  $\mu$ g/ml. Control sections incubated with non-immune rabbit IgG (0.5  $\mu$ g/ml) were included in all experiments. For further confirmation of the specificity, the anti-AR antibody (N-20) was incubated with a 10-fold molar excess of the blocking peptide (catalog no. sc-816P, Santa Cruz Biotechnology) for 2 h at room temperature.

#### Image analysis

Digital images of stained samples were transferred from a DMR-X microscope (Leica, Wetzlar, Germany) into a computerized image-analysis system (Quantimet 550IW; Leica, Cambridge, UK). To calculate the percentage of positive cells, three visual fields were assessed for positively stained cells and for the total number of cells. Methyl-green stains the nuclei to a faint green color; only those cells in which a visible nucleus could be detected were counted. The epiphyseal bone, the metaphyseal bone and areas where the tissue sections had detached from the slides were excluded. The positively stained cells in the resting, proliferative and hypertrophic zones were detected by the program and counted manually by one observer unaware of the pubertal stage of the patient. Chondrocytes distal to the epiphyseal bone and proximal to the proliferative columns were regarded as resting chondrocytes. Proliferative cells were defined as flat chondrocytes (cell height <10  $\mu$ m) arranged in columns, whereas the hypertrophic chondrocytes were defined by a cell height of 10  $\mu$ m or more. Results are given as the percentage of cells in the region that was stained. The sensitivity and specificity of the imaging system has been previously evaluated (Bjork *et al.* 1997).

#### Statistics

For each growth plate zone, the results from each antibody were evaluated independently. The overall effects of sex and growth plate zone on the percent positive cells in each growth plate zone were evaluated by two-way ANOVA. For evaluation of developmental trends, regression analysis was applied with pubertal stage and bone age as the independent variables and percent positive cells as the dependent variable. Patient no. 13 was excluded because of lack of pubertal staging.

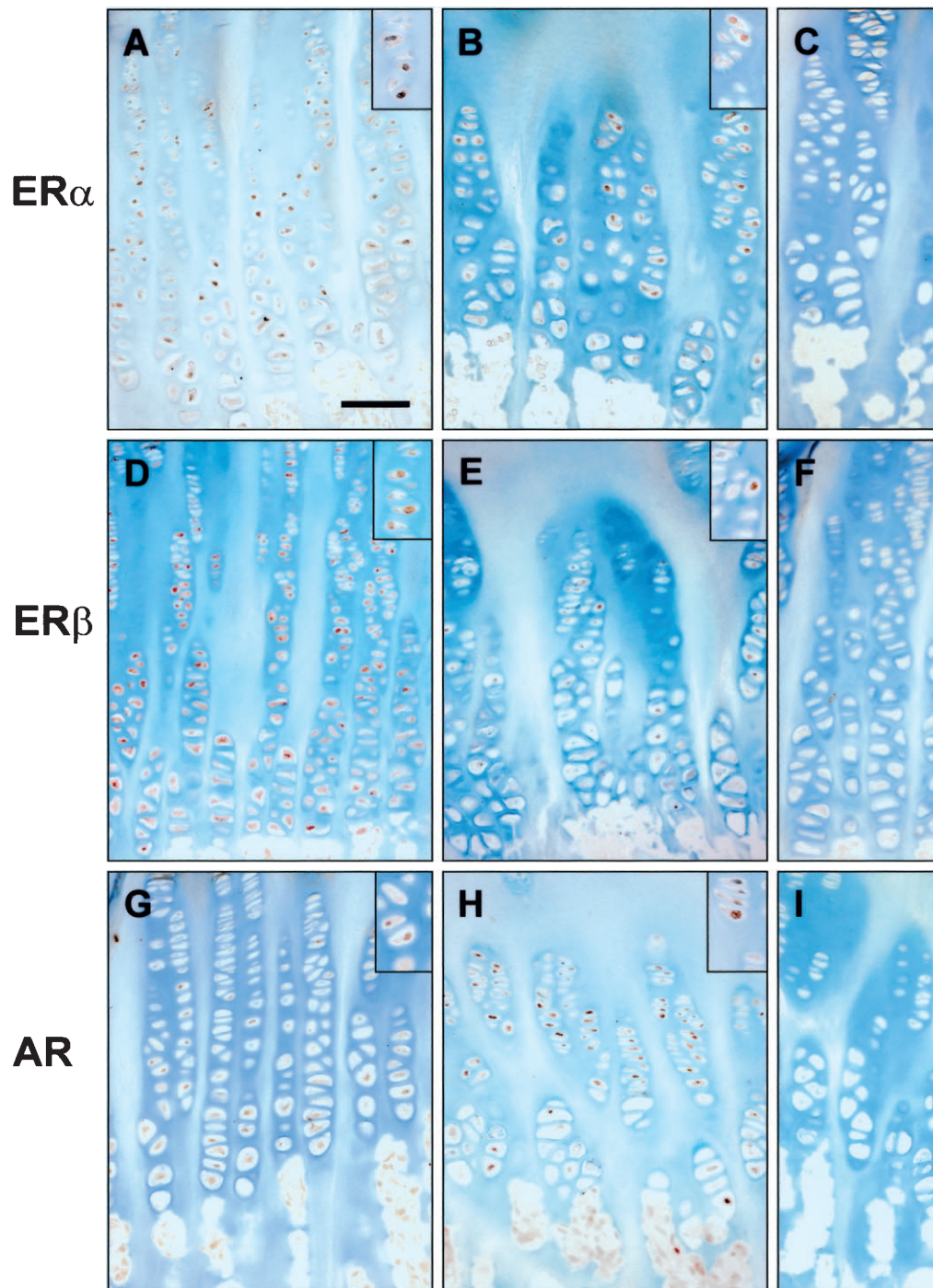


**Figure 1** Immunohistochemistry of (A) ER $\alpha$  (ER-6F11), (B) ER $\beta$  (06-629) and (C) AR (N-20) in human prostate. Positive immunoreactivity produces a brown coloration. Sections were counterstained with hematoxylin. The human prostate was mainly negative to ER $\alpha$  (A). ER $\beta$  was detected in basal and some epithelial cells (B), whereas AR was detected in most epithelial cells (C). Bar represents 100  $\mu$ m.

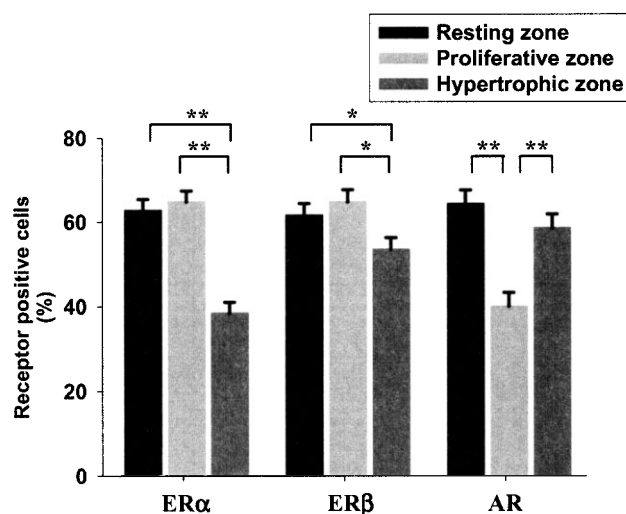
## Results

### *ER $\alpha$ , ER $\beta$ and AR immunoreactivity in prostate control tissue*

In the human prostate tissue, glandular epithelium was mainly negative to ER $\alpha$  (Fig. 1A), whereas ER $\beta$  was detected in basal and some secretory cells of the epithelium (Fig. 1B). Most stromal cells were negative to both ER $\alpha$  and ER $\beta$  (Fig. 1A and B). Nuclear immunoreactivity to AR was detected in some stromal and most epithelial cells (Fig. 1C).



**Figure 2** Immunohistochemical localization of ER $\alpha$ , ER $\beta$  and AR in human tibial growth plates at different pubertal stages. Sections of proximal tibial growth plates from one patient in early puberty (patient no. 7: A, C, D, F, and G) and two in late puberty (patient no. 11: B and E; patient no. 23: H and I) immunostained for ER $\alpha$  with the ER-6F11 antibody (A, B), for ER $\beta$  with the 06-629 antibody (D, E), and for AR with the N-20 antibody (G, H). Immunohistochemical staining produces a brown coloration. The sections were counterstained with methyl-green. Note that receptor-negative nuclei are stained in a light green color. When the primary antibodies, ER-6F11 (C), 06-629 (F) and N-20 (I) were preincubated with the corresponding antigen, no nuclear staining was detected. Original magnifications  $\times 20$ ;  $\times 40$  for insets. Bar represents 100  $\mu\text{m}$ .



**Figure 3** Levels of expression of ER $\alpha$ , ER $\beta$  and AR in the resting, proliferative and hypertrophic zones of the human tibial growth plate. Immunohistochemical localization of ER $\alpha$ , ER $\beta$  and AR was performed on sections of human growth plates. A computerized image-analysis system was used to detect immunostained cells. The percentage of positive cells (mean  $\pm$  S.E.M.) was assessed for each zone. Chondrocytes distal to the epiphyseal bone and proximal to the proliferative columns were regarded as resting chondrocytes. Proliferative cells were defined as flat chondrocytes (cell height  $<10\ \mu\text{m}$ ) arranged in columns, whereas the hypertrophic chondrocytes were defined by a cell height of  $10\ \mu\text{m}$  or more. \* $P<0.05$ , \*\* $P<0.001$ .

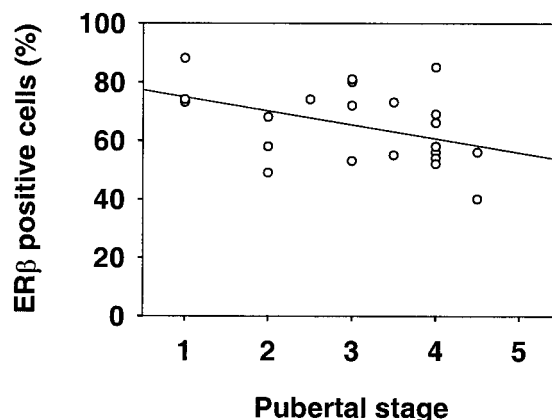
No nuclear staining could be detected when the primary antibodies were preadsorbed with their respective antigen, substituted for non-specific immunoglobulins, or excluded (not shown).

#### Expression of ER $\alpha$ , ER $\beta$ and AR in proximal tibial growth plates

ER $\alpha$ , ER $\beta$  and AR were detected in all zones of the growth plate, in all the specimens collected (Table 1). The immunoreactivity was predominantly localized to the nuclei (Fig. 2). The nuclear staining was abolished when the antibodies were preincubated with their respective antigen (Fig. 2C, F and I). Both ER $\alpha$ - and ER $\beta$ -positive cells were detected at a greater frequency in the resting and proliferative zones than in the hypertrophic zone (Fig. 3), whereas AR was more abundant in the resting and hypertrophic zones than in the proliferative zone (Fig. 3). No sex difference in the patterns of expression was detected. For ER $\alpha$  and AR, the percentage of receptor-positive cells was similar at all Tanner pubertal stages, whereas ER $\beta$  showed a slight decrease ( $P<0.05$ ) in the proliferative zone during pubertal development (Fig. 4).

#### Discussion

ER $\alpha$ , ER $\beta$  and AR immunoreactivity was detected in all zones of the growth plate in biopsies from all patients. This



**Figure 4** Correlation between the percentage of ER $\beta$ -positive cells in the proliferative zone and Tanner pubertal stages. A computerized image-analysis system was used to detect positively stained cells in sections of growth plate cartilage from boys and girls at different pubertal stages. The percentage of receptor-positive cells was calculated for each zone and analyzed for pubertal changes with linear regression analysis. Tanner pubertal stage was determined as breast (females) or genitalia (males) development. The percentage of ER $\beta$ -positive cells decreased with pubertal stage in the proliferative zone as indicated by the regression line ( $y=79.7-4.8 \times \text{pubertal stage}$ ;  $R=0.43$ ;  $P<0.05$ ).

finding suggests that sex steroid receptors are expressed in human growth plate cartilage throughout pubertal development. This is in agreement with previous studies localizing ER $\alpha$  (Kusec *et al.* 1998), ER $\beta$  (Nilsson *et al.* 1999) and AR (Abu *et al.* 1997) to the human growth plate during puberty. It also supports the hypothesis that sex steroids modulate linear bone growth through direct effects on growth plate chondrocytes (Jansson *et al.* 1983, Corvol *et al.* 1987, Ren *et al.* 1989, Blanchard *et al.* 1991).

ER $\alpha$ - and ER $\beta$ -positive cells were detected in a similar frequency in all zones of the growth plate. Thus, on the basis of our findings alone, the receptors could be assumed to have redundant roles in growth plate cartilage. However, lack of a functional ER $\alpha$  in humans (Smith *et al.* 1994) results in a phenotype similar to that of aromatase deficiency syndrome (Morishima *et al.* 1995), in which there is a complete lack of estrogen, suggesting that the two estrogen receptors are not redundant. In fact, studies on estrogen receptor-mediated transcription have suggested that ER $\beta$  acts as a negative regulator of ER $\alpha$ -mediated transcription at estrogen response elements (Hall & McDonnell 1999, Pettersson *et al.* 2000). It could thus be speculated that the observed decrease in ER $\beta$  during pubertal development could cause an enhanced ER $\alpha$ -mediated signaling in the growth plate during late puberty. Hypothetically, increased estrogen sensitivity in late puberty could contribute to the rapid decline in growth rate during this period.

The expression of ER $\alpha$  and ER $\beta$  in the resting and proliferative zones provides a possible explanation for the

effects of estrogen on chondrocyte proliferation and thus also on the rate of longitudinal bone growth. This effect may be mediated through estrogen receptors in the proliferative zone or, as suggested by Weise *et al.* (2001), indirectly through an effect on the replicative senescence of the resting chondrocytes.

We detected ER $\alpha$  and ER $\beta$  proteins in the hypertrophic zone in all growth plate samples from all our patients. The hypertrophic zone chondrocytes also produce Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) (van der Eerden *et al.* 2000, Kindblom *et al.* 2002), two polypeptides that have been identified as important mediators of a negative feedback-loop for the control of chondrocyte differentiation (Kronenberg *et al.* 1998). Histological studies of growth plates near fusion suggest that hypertrophic differentiation of all cells in the growth plate precedes epiphyseal fusion (Weise *et al.* 2001). It may therefore be speculated that estrogen induces epiphyseal fusion through a modulation of the Ihh-PTHrP feedback loop. This hypothesis is supported by the observation that expression of Ihh and PTHrP in the human growth plate decreases during pubertal development (Kindblom *et al.* 2002).

In the present study, immunoreactivity to ER $\beta$  was detected in all zones of the growth plate. It was thus detected in a wider distribution than we, and others, previously have reported in human growth plate cartilage (Nilsson *et al.* 1999, Egerbacher *et al.* 2002). This difference in results is attributable to the use of antigen retrieval, a technique that greatly enhances the sensitivity of immunoassays aimed at detecting nuclear receptors in formalin- and paraffin-embedded tissues (Taylor 1996). The increased sensitivity of the immunoassay also allowed us to decrease the primary antibody concentration, thereby minimizing the non-specific cytoplasmic staining frequently detected in the hypertrophic zone, especially when high concentrations of rabbit antibodies are used (unpublished data). Preadsorption of the primary antibodies with their respective immunizing peptide and control experiments in human prostate tissue confirmed that the immunohistochemical staining was specific for the respective ER $\alpha$ , ER $\beta$  and AR (Leav *et al.* 2001).

The expression of AR in the hypertrophic zone may indicate that androgens stimulate longitudinal bone growth by a modulation of the hypertrophic differentiation – that is, increased hypertrophic cell height (Breur *et al.* 1991). This is consistent with the findings of *in vitro* studies suggesting that androgens stimulate chondrocyte differentiation (Corvol *et al.* 1987, Blanchard *et al.* 1991, Schwartz *et al.* 1994). However, it has also been suggested that androgens stimulate growth plate chondrocyte proliferation (Carrascosa *et al.* 1990). Thus androgen-stimulated growth may be mediated through an increased proliferation or an increased size of the hypertrophic cells, or by a combination of these two effects. In addition, the observation that the growth-promoting effect of oxandrolone is

sustained after the cessation of treatment (Stanhope *et al.* 1988, Mallhotra *et al.* 1993) may suggest an androgenic effect on the resting zone chondrocytes.

Sex steroid receptor-positive cells were detected at a similar frequency in girls and boys. This is in agreement with the findings of most previous immunohistochemical studies in human (Abu *et al.* 1997, Kusec *et al.* 1998, Bord *et al.* 2001), rat (van der Eerden *et al.* 2002a, Nilsson *et al.* 2002) and rabbit (Kusec *et al.* 1998, Nilsson *et al.* 2002) growth plate cartilage. Thus the sex-specific effects of sex steroids suggested by the findings of some *in vitro* studies (Corvol *et al.* 1987, Blanchard *et al.* 1991, Schwartz *et al.* 1991, 1994), do not appear to be caused by sex-specific receptor expression. However, immunohistochemistry is a semi-quantitative method, and real differences could have been overlooked by this technique.

In summary, our immunohistochemical studies suggest that ER $\alpha$ , ER $\beta$  and AR proteins are expressed in all zones of the growth plate, in both boys and girls, throughout pubertal development. The patterns of expression may explain the effects of sex steroids on growth-plate cartilage, including the effects on proliferation, growth rate and epiphyseal fusion.

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