Expression of vascular endothelial growth factor in the growth plate is stimulated by estradiol and increases during pubertal development

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Abstract

Longitudinal bone growth is regulated in the growth plate. At the end of puberty, growth velocity diminishes and eventually ceases with the fusion of the growth plate through mechanisms that are not yet completely understood. Vascular endothelial growth factor (VEGF) has an important role in angiogenesis, but also in chondrocyte differentiation, chondrocyte survival, and the final stages of endochondral ossification. Estrogens have been shown to up-regulate VEGF expression in the uterus and bone of rats. In this study, we investigated the relation between estrogens and VEGF production in growth plate chondrocytes both *in vivo* and *in vitro*. The expression of VEGF protein was down-regulated upon ovariectomy and was restored upon

estradiol (E₂) supplementation in rat growth plates. In cultured rat chondrocyte cell line RCJ3.1C5.18, E₂ dose dependently stimulated 121 and 189 kDa isoforms of VEGF, but not the 164 kDa isoform. Finally, VEGF expression was observed at both protein and mRNA levels in human growth plate specimens. The protein level increased during pubertal development, supporting a link between estrogens and local VEGF production in the growth plate. We conclude that estrogens regulate VEGF expression in the epiphyseal growth plate, although the precise role of VEGF in estrogen-mediated growth plate fusion remains to be clarified.

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Introduction

Longitudinal growth occurs at the epiphyseal plate, a thin layer of cartilage entrapped between epiphyseal and metaphyseal bones, at the distal ends of the long bones (Kronenberg 2003). In the growth plate, immature cells lie toward the epiphysis, called the resting zone, with flat more mature chondrocytes in the proliferating zone and large chondrocytes in the hypertrophic zone adjacent to this. At the end of puberty, longitudinal growth ceases with total replacement of avascular cartilage by highly vascularized bone, eventually resulting in epiphyseal fusion. Estrogens are known to be important hormones in the regulation of growth plate maturation and epiphyseal fusion; they regulate and can accelerate the programmed senescence of the growth plate, leading to proliferative exhaustion of chondrocytes and epiphyseal fusion (Weise *et al.* 2001, Chagin & Savendahl 2007). At a low concentration, estrogens are known to increase growth velocity, an effect possibly mediated through the GH–insulin-like growth factor 1 (IGF1) axis, while at a

high concentration, estrogens inhibit growth and promote epiphyseal fusion in the long bones (Ross *et al.* 1986, Metzger & Kerrigan 1994, Klein *et al.* 1996).

A critical step in endochondral ossification is when blood vessels enter from the primary spongiosum and osteoblasts invade from the bone marrow to lay down trabecular bone (Kember 1993, Hunziker 1994). Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis, but it has also been shown to modulate chondrocyte differentiation and survival, osteoblast differentiation, and osteoclast recruitment (Zelzer *et al.* 2004, Zelzer & Olsen 2005, Dai & Rabie 2007). VEGF is expressed by growth plate chondrocytes and osteoblasts in different species including humans (Gerber *et al.* 1999, Carlevaro *et al.* 2000, Garcia-Ramirez *et al.* 2000, Haeusler *et al.* 2005). Human VEGF is present in six different proteins, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and VEGF-F, where VEGF-A has been shown to be expressed in the growth plate and also believed to be most important in the regulation of longitudinal bone growth. VEGF-A has six alternatively spliced isoforms: VEGF-121,

VEGF-145, VEGF-165, VEGF-183, VEGF-189, and VEGF-206 (Robinson & Stringer 2001). The receptors involved in VEGF-A signaling are VEGFR-1 (also known as fms-like tyrosine kinase receptor 1, FLT1) and VEGFR-2 (also known as kinase insert domain-containing receptor, KDR), with almost all responses being mediated through the second receptor, which has also been detected at the chondro-osseous junction in the mouse growth plate (Gerber *et al.* 1999, Ferrara *et al.* 2003, Dai & Rabie 2007).

Inactivating VEGF in mice and monkeys resulted in impaired trabecular bone formation and expansion of the hypertrophic zone, indicating inhibition of cartilage resorption (Gerber *et al.* 1999, Ryan *et al.* 1999). In addition, *Vegfa* conditional knockout mice driven by a *Col2a1* promoter showed delayed invasion of blood vessels into the primary ossification center and delayed removal of terminal hypertrophic chondrocytes together with massive cell death in chondrocytes throughout the growth plate, demonstrating the importance of VEGFA in chondrocyte survival (Zelzer *et al.* 2004).

In vitro VEGF expression can be up-regulated by factors known to be important in the regulation of longitudinal bone growth such as fibroblast growth factor, transforming growth factor β , and IGF1 (Garcia-Ramirez *et al.* 2000). Studies on rats showed that in uterus and bone tissue, VEGF expression is up-regulated by estrogens (Hyder *et al.* 1996, Mekraldi *et al.* 2003). In humans, bone growth and estrogen levels increase in parallel during earlier phases of puberty, while at the end of puberty, growth ceases with a total replacement of cartilage by bone resulting in the fusion of the growth plate.

We hypothesized that estrogens have the capacity to stimulate local VEGF production in growth plate chondrocytes, and that this could be a possible mechanism involved in the process of growth plate maturation and fusion in humans. To address this, we performed *in vivo* studies in ovariectomized rats supplemented with estradiol (E_2) and also *in vitro* studies in cultured rat chondrocytes exposed to E_2 , and assessed chondrocyte-specific expression of VEGF. In addition, we measured VEGF expression in growth plate specimens obtained from humans in different pubertal stages.

Materials and Methods

Animals and study protocol

Female Sprague–Dawley rats were purchased from Scanbur BK AB (Sollentuna, Sweden). The animals were housed in a temperature- and humidity-controlled room under a 0600 h light:0600 h darkness cycle, and allowed a soy-free diet containing 0.7% of calcium and 0.5% of phosphorus (R70; Lactamin AB, Kimstad, Sweden) and tap water *ad libitum*. All procedures were approved by the Ethics Committee at Göteborg University, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were randomly divided into three

groups: sham operation + vehicle treatment (sham, $n=12$), OVX + vehicle treatment (OVX, $n=10$), and OVX + E_2 treatment (E_2 , $n=11$). At 12 weeks of age (body weight, 251 ± 2 g), the rats were either sham-operated or OVX under isoflurane anesthesia (Baxter Medical AB, Kista, Sweden), and small silastic implants were placed subcutaneously in the cervical region. The silastic implants were prepared as described previously, releasing $2.5 \mu\text{g/day}$ of E_2 (Vandenput *et al.* 2002). Vehicle-treated animals received an empty implant. E_2 was obtained from Sigma Chemical. After 6 weeks of treatment, the animals were killed by excision of the heart under isoflurane anesthesia, and the right proximal tibia was fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. Uterus size, correlating with estrogen levels, was smaller in the ovariectomized rats and slightly larger in the estrogen-supplemented animals compared with the sham-operated animals, indicating supra-physiological levels of estrogens in the estrogen-treated group. Study details were described previously (Tivesten *et al.* 2004, 2006).

Patients and tissue preparation

Human proximal and distal femur growth plate tissues were collected from 12 girls at different pubertal stages who were undergoing surgery for different medical indications (Table 1). One fetal sample was collected from a female donor of 23 weeks of gestational age. The study protocol was approved by the local medical ethics committees of the Leiden University Center, Leiden, The Netherlands, and by the Karolinska University Hospital, Stockholm, Sweden. Informed consent was obtained from all patients and their parents. Epiphyseal samples were either directly frozen in liquid isopentane and stored at -80°C or fixed in 10% formalin, decalcified, and embedded in paraffin. All tissue samples were processed in the same way.

Immunohistochemistry

All tissues were cut into $5\text{-}\mu\text{m}$ sections and mounted on histological glass slides (Starfrost, Knittel Glaser, Braunschweig, Germany), dried at 37°C overnight, and heated at 60°C for 1 h before immunohistochemical treatment. Immunohistochemistry was performed as described previously (Nilsson *et al.* 2003), with the modification that antigen retrieval was achieved by incubating with 0.1% trypsin (Invitrogen) for 10 min at 37°C . Anti-VEGF antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and was used in a 1:200 dilution for rat tissues and 1:50 dilution for human tissues. Secondary anti-rabbit biotinylated antibody (Jackson ImmunoResearch Lab, West Grove, PA, USA) was used in a 1:1000 dilution, followed by incubation with avidin-biotin Vectastain ABC reagent according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Digital images were collected employing a Nikon Eclipse E800 microscope equipped with an Olympus DP70 digital camera.

Table 1 Patient information and human tissues. The diagnosis of each patient, location of growth plate, age, in years, Tanner pubertal breast stage (B1–B5), and experiment in which growth plate sample was used (IHC and/or qPCR) are given in the table

Patient	Diagnosis	Bone	Age (years)	Puberty	Experiment
1	Leg length difference	Distal femur	9	B1–B2	IHC
2	Hip luxations, femur head resection	Proximal femur	12	B2	qPCR, IHC
3	Leg length difference	Distal femur	12	B2	IHC
4	Leg length difference	Distal femur	14	B2–B3	IHC
5	Hip luxations, femur head resection	Proximal femur	13	B3	qPCR, IHC
6	Cerebral palsy, femur head resection	Proximal femur	15	B4	IHC
7	Osteosarcoma in tibia	Distal femur	8	B1	qPCR
8	Upper limb amputation of the leg	Distal femur	9	B1	qPCR
9	Tall stature	Distal femur	10	B2	qPCR
10	Tall stature	Distal femur	12	B3	qPCR
11	Tall stature	Distal femur	15	B4	qPCR
12	Hip luxations, femur head resection	Proximal femur	14	B4	qPCR
13	Fetal, selective abortion	Distal femur	23 weeks	Fetal	qPCR

IHC, immunohistochemistry.

Image analysis

Images of the central two-thirds of the rat growth plates were captured in three visual fields. All pictures were taken at 200× magnification with a 2040×1536 resolution, and were further analyzed in Image Pro Plus 5.0 software (Silver Spring, MD, USA). Pictures were converted into grayscale-8 mode, and were inverted in order to obtain correct optical density (OD) values in immunopositive areas. An automatic bright object counting was performed to identify the number of immunopositive objects above the defined thresholds. Threshold level for cell size was defined as objects with an area over 12 and 20 μm^2 in the proliferative and hypertrophic zones respectively. The total OD of the immunopositive objects was calculated automatically, a function referred as density sum in the Image Pro Plus software. The analyzed areas were measured in mm^2 , and results are expressed as the number of positive cells/ mm^2 , protein expression (OD arbitrary unit)/ mm^2 , and protein expression (OD arbitrary unit)/per cell. Data are presented as mean $\pm$ S.E.M.

RNA isolation

Bone was removed from all epiphyseal samples, and 40- μm thick sections were cut with a cryostat. Every fifth section was followed by a 5- μm thick section, which was studied with hematoxylin staining to ensure lack of bone contamination. Total RNA isolation was performed with an optimized method for RNA extraction from cartilage as described by Heinrichs *et al.* (1994), except that the protocol was started by homogenizing the sections in 1 ml guanidine thiocyanate solution. RNA samples from bladder and prostate tissue were obtained from Gentaur molecular products (Brussel, Belgium). RNA extraction was followed

by purification using an RNeasy kit according to the manufacturer's protocol (Qiagen), and the quality and integrity of each sample were checked with the Agilent 2100 Bioanalyzer.

Real-time reverse transcription-PCR

RNA was reverse transcribed into cDNA using a First Strand cDNA Synthesis kit for quantitative PCR (qPCR; Roche Diagnostics GmbH) according to the manufacturer's instructions. Expression of *VEGF-A* and *VEGFR-2 (KDR)* mRNA was quantified by real-time PCR using the Bio-Rad iCycler with SYBR Green. QuantiTect Primer Assays were purchased from Qiagen Benelux BV, and were used according to the manufacturer's protocol. Threshold cycles (C_t) were estimated and averaged for the triplicates. Relative amounts of mRNA were normalized to β_2 -microglobulin expression in the same sample to account for variability in the initial concentration and quality of total RNA and in the efficiency of the reverse transcription reaction.

Western blotting

Nontransformed clonal rat chondrogenic cells RCJ3.1C5.18 (C5.18 cells) were differentiated for 10 days (Lunstrum *et al.* 1999), and were subsequently treated for 24 h with a dose range of E_2 . Cells were lysated, and the protein concentration was measured by the Bradford protein assay (Bio-Rad Laboratories AB). Proteins were separated on acrylamide gels (Bio-Rad Laboratories), and transferred to polyvinylidene fluoride membrane. Three different isoforms of VEGFA were detected employing anti-VEGF rabbit Ab (1:1500; sc-152, Santa Cruz Biotechnology Inc). Secondary goat anti-rabbit antibody was peroxidase labeled and used in a 1:10 000 dilution (Santa Cruz Biotechnology Inc). The resulting bands

were confirmed by comparing the size of the protein in the cell extract with that of the known molecular weight markers. The antigen–antibody complexes were then detected by chemiluminescence. After the films had been developed, blots were stained with Coomassie blue to ensure equal loading of total protein. Density measurements were normalized per density of Coomassie blue staining (Chrysis *et al.* 2005). Each experiment was repeated at least three times.

Statistical analysis

The human sections were blinded, and a relative staining intensity was scored (score 0–3) for the proliferative and hypertrophic zones of each growth plate. Scores were displayed in a scatter plot, and a linear regression analysis was performed to calculate significance. The same was done for the qPCR results.

For the rat data, six growth plate sections for every animal were analyzed by the image analysis protocol described above, and means were calculated in terms of VEGF-positive cells/ μm^2

Figure 1 VEGF immunohistochemistry staining. VEGF protein expression was detected in the rat growth plate (panel A, 100 $\times$ magnification) and the human pubertal growth plate (panel B, 100 $\times$ magnification). Panels Ai and Bi show 5 $\times$ enlargements of the proliferative zone in panels A and B. Panels Aii and Bii show 5 $\times$ enlargements of the hypertrophic zone in panels A and B. Pre-incubation of the primary antibody with recombinant VEGF abolished the staining in both proliferative and hypertrophic chondrocytes (panel C, 400 $\times$). Panel D shows a negative control for the human growth plates. Bars indicate 100 μm .

Figure 2 Quantification of VEGF staining in the proliferative and hypertrophic zones. VEGF staining was quantified with a computerized method for the proliferative zone. Panel A shows a significant decrease in the amount of positive cells/mm² growth plate after ovariectomy, which was completely restored by 17 β -estradiol. Staining intensity per cell (OD arbitrary unit/cell) showed no difference between all groups (panel B). The hypertrophic zone showed the same trend as the proliferative zone; however, there were no statistically significant differences (panel C). Staining intensity per cell (OD arbitrary unit/cell) showed a significant decrease after ovariectomy in the hypertrophic zone, but there was no restoration with estrogen supplementation (panel D). Data are presented as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

and VEGF expression (OD arbitrary unit)/per cell for each animal. Significance was calculated by one-way ANOVA followed by Fisher's protected least significant difference test.

Results

VEGF protein expression in the rat growth plate

Sham-operated rats were used as an internal control which confirmed abundant VEGF expression in the growth plates, in both the proliferative and hypertrophic zones (Fig. 1A). Pre-incubation of the primary antibody with recombinant VEGF abolished the staining in both proliferative and hypertrophic chondrocytes (Fig. 1C). Staining was analyzed by a computerized method for the proliferative and hypertrophic zones (see Materials and Methods section).

To reveal any possible regulation of VEGF expression by estrogens, we analyzed the number of VEGF-expressing chondrocytes in rats upon ovariectomy and E₂ supplementation. Ovariectomy resulted in a significant decrease in the number of VEGF-positive cells in the proliferative zone

Figure 3 VEGFA isoform western blot analysis in chondrocyte culture. Panel A illustrates that 17 β -estradiol directly stimulated the expression of isoforms VEGF-189, VEGF-164, and VEGF-121. The isoforms VEGF-121 and VEGF-189 showed a very strong response, and in contrast, VEGF-164 showed just a marginal response. In panel B, the expression levels are calculated as a percentage of the VEGF expression in the nontreated control.

(1173 $\pm$ 93 vs 1556 $\pm$ 100 cells/mm² in sham-operated animals; $P < 0.01$), an effect that was completely restored by E₂ replacement (1713 $\pm$ 81 vs 1173 $\pm$ 93 cells/mm² in vehicle alone; $P < 0.001$; Fig. 2A). A similar trend was observed in hypertrophic chondrocytes, albeit not statistically significant (Fig. 2C). The level of VEGF per cell (expressed as OD arbitrary units/cell) did not differ significantly between the groups in the proliferative zone (Fig. 2B). However, in the hypertrophic zone, ovariectomy resulted in a significant decrease in the level of VEGF per cell (35 747 $\pm$ 1989 vs 43 240 $\pm$ 1900 in sham-operated animals; $P < 0.01$), an effect which was not restored by E₂ supplementation (36 601 $\pm$ 1615 vs 35 747 $\pm$ 1989 in vehicle alone; $P = 0.74$; Fig. 2D).

VEGF-A isoform expression in cultured chondrocytes

In order to distinguish between direct and systemic effects of estrogens on VEGF expression, we performed experiments in the rat chondrogenic cell line RCJ3.1C5.18 (C5.18 cells), which can be differentiated into hypertrophic chondrocytes (Lunstrum *et al.* 1999). The cells were differentiated for 10 days and were then treated with E₂ for 24 h. E₂ dose dependently stimulated the expression of VEGF-121 and VEGF-189, while VEGF-164 expression was not affected (Fig. 3). VEGF-189 was suppressed by low E₂ concentrations, and it was increased by high concentrations.

VEGF protein and mRNA expressions in human growth plates

Growth plate biopsies were obtained from girls at different stages of pubertal development. To verify how VEGF is distributed in the human pubertal growth plate, we analyzed VEGF expression levels in these rare tissue samples. Of the 13 collected growth plates, we analyzed six human growth plates for VEGF protein expression and nine for VEGF mRNA expression (Table 1). In all these human growth plates, VEGF protein was detected in both proliferative and hypertrophic

zone chondrocytes (Fig. 1B). When the relative staining intensity was scored (score 0–3), a significant increase in VEGF expression with progression of puberty was found in both the proliferative ($P = 0.022$) and hypertrophic zones ($P = 0.017$; Fig. 4 panels A and B). Negative controls showed no staining (data not shown).

Studies of mRNA levels with qPCR confirmed VEGF expression in pubertal as well as fetal human growth plates, albeit expression of VEGF mRNA in the prepubertal growth plate was ~ 200 -fold lower compared with the expression in prostate and bladder tissues (positive controls). The C_t for VEGF expression were subtracted from the $\beta 2$ -microglobulin C_t in order to calculate the ΔC_t . Average values for the different groups were calculated and compared with the prepubertal growth plate. The fetal growth plate showed a 4.2-fold higher expression of VEGF compared with the prepubertal growth plate. The pubertal growth plate samples ($n = 6$) showed on average a 1.6-fold higher expression of VEGF mRNA compared with the prepubertal growth plate ($n = 2$), but in contrast to VEGF protein expression, we did not find a significant correlation between VEGF mRNA levels assessed by qPCR and the stage of pubertal development ($P = 0.183$, $R = 0.238$). The VEGF receptor,

Figure 4 Scatter plot of VEGF expression in the human growth plate. Relative intensity of VEGF protein expression was scored for each growth plate and plotted in relation to the pubertal stage for the proliferative zone (panel A) and the hypertrophic zone (panel B). R^2 values were respectively 0.766 ($P = 0.022$) for the proliferative zone and 0.794 ($P = 0.017$) for the hypertrophic zone.

VEGFR2, was also expressed at mRNA level in all our growth plate samples that were analyzed. Similar to VEGF, the average *VEGFR2* mRNA level was 1.6-fold higher in pubertal girls ($n=6$) compared with the prepubertal girls ($n=2$), but there was no statistically significant correlation with pubertal progression ($P=0.585$, $R=0.045$) likely due to a high variation between samples and low number of patients.

Discussion

Our *in vivo* and *in vitro* data demonstrate that E_2 directly stimulates the expression of VEGF in rat growth plate chondrocytes. Furthermore, we confirmed that VEGF is expressed in the human pubertal growth plate and that the VEGF protein level increases with pubertal progression, supporting a link between estrogens and local VEGF production in the growth plate.

VEGF was previously detected mostly in hypertrophic chondrocytes of human growth plate samples by immunohistochemistry (Haeusler *et al.* 2005). We observed VEGF expression not only in the hypertrophic zone, but also in the proliferative zone, which is in line with the observations of Horner *et al.* who studied VEGF protein expression in neonatal human growth plate cartilage (Horner *et al.* 1999). *VEGF* mRNA was also detected in the proliferative zone of murine growth plate cartilage (Cramer *et al.* 2004). VEGF expression in proliferating chondrocytes and the significant change in expression with alternating estrogen levels were observed in both human and rat growth plates in our study. This slight divergence in results could be due to technical issues attributed to immunohistochemistry such as antigen retrieval or type of antibody.

We confirmed our immunohistochemistry data by qPCR analysis. mRNA expression of VEGF and the *VEGFR-2* was, to our knowledge for the first time, detected in adolescent and pubertal human growth plates. *VEGF* mRNA was previously detected in the fetal growth plate by others (Garcia-Ramirez *et al.* 2000, Petersen *et al.* 2002). Expression of *VEGFR-2* was detected earlier at the chondro-osseous junction in mice (Gerber *et al.* 1999), in epiphyseal cartilage of pigs (Kim *et al.* 2009), in the avian growth plate (Rath *et al.* 2007), and in hypertrophic chondrocytes of the fetal growth plate (Petersen *et al.* 2002). In contrast to protein levels, mRNA did not reveal a significant increase in VEGF expression with progression of puberty. This discrepancy in results could be due to a difference between mRNA and protein expression, a difference in tissue preparation for RNA extraction when the surrounding bone was removed or alternatively due to a change in morphological organization of the growth plate during progression of puberty (e.g. a decreased hypertrophic layer).

This is the first report to demonstrate a link between estrogens and VEGF expression in the epiphyseal growth plate. *In vivo* treatment of rats with E_2 increased the number of

growth plate chondrocytes expressing VEGF. In line with this, in the hypertrophic zone of ovariectomized animals, the number of VEGF-positive cells/mm² growth plate was decreased. In addition, *in vitro* data in a rat chondrocytic cell line showed a dose-dependent stimulatory effect of E_2 on the expression of VEGF-121 isoform. VEGF-189 was suppressed by low E_2 concentrations and was stimulated by higher concentrations. This might counterbalance the slight concomitant increase of VEGF-121, thereby protecting the growth plate when exposed to low concentrations of E_2 . Our growth plate findings are in line with previous reported effects of estrogens on VEGF expression in bone, uterus, and breast cancer tissues (Mekraldi *et al.* 2003, Kazi *et al.* 2005, Garvin *et al.* 2006).

Systemic estrogen levels increase with puberty eventually resulting in epiphyseal fusion by the end of puberty (Juul 2001), presumably due to acceleration of growth plate senescence through proliferative exhaustion of chondrocytes (Weise *et al.* 2001). From our results obtained in rats, we hypothesized that estrogens not only accelerate senescence of the growth plate, but also stimulate chondrocytes to secrete VEGF, which might contribute to the process of epiphyseal fusion. VEGF protein was detected in human pubertal growth plates, and indeed, the expression level significantly increased during pubertal progression. This observation in humans supports our findings in rats, and strengthens our hypothesis that estrogens stimulate VEGF expression in the growth plate.

A 200-fold lower *VEGF* mRNA level in the growth plate was observed compared with the prostate or bladder tissues. We believe that the observed stimulation of VEGF expression by estrogens in avascular growth plate chondrocytes can substantially affect the growth plate. Indeed, VEGF has an important role in chondrocyte differentiation, chondrocyte survival, and endochondral ossification (Zelzer *et al.* 2004, Zelzer & Olsen 2005, Dai & Rabie 2007). In several studies, inhibition of VEGF showed dramatic effects on the growth plate, such as expansion of the hypertrophic zone and delayed removal of terminal hypertrophic chondrocytes (Gerber *et al.* 1999, Zelzer *et al.* 2004). Conversely, one might speculate that an increase in expression leads to a smaller hypertrophic zone, a more rapid removal of terminal hypertrophic chondrocytes, and eventually epiphyseal fusion. To our knowledge, reports on an increase in VEGF expression have not been published before.

Estrogen levels were higher in the estrogen-supplemented rats compared with the sham-operated rats. Uterus size, correlating with estrogen levels, was small in ovariectomized rats compared with the sham-operated animals and was slightly larger in the estrogen-supplemented animals, indicating supra-physiological levels of estrogens in the estrogen-treated group (Tivesten *et al.* 2004, 2006). Serum levels of E_2 were not measured in patients from whom growth plate tissue samples were collected. However, serum levels of E_2 are well known to positively correlate with the stage of pubertal development (Norjavaara *et al.* 1996).

The collection of human samples is small and originates from patients having a variability of disorders. However, human growth plate samples are extremely difficult to obtain. We believe that even though patients suffered from diverse disorders, the underlying mechanism of epiphyseal maturation and fusion will be the same for all growth plates. Eventually, longitudinal growth stops in all patients, with only few exceptions, at the end of puberty. The human data are in line with both *in vivo* and *in vitro* rat data, thereby strengthening our conclusion that estrogens stimulate VEGF expression in the growth plate. Estrogen levels were not analyzed in these patients, and our assumption of different levels of estrogen exposure is based on the fact that tissue samples were obtained from girls in different pubertal stages.

In summary, we demonstrated that VEGF protein expression in the growth plate is elevated by estrogens *in vivo* in ovariectomized rats and *in vitro* in a rat chondrocytic cell lines. Our findings are supported by human expression studies in girls in different pubertal stages. From this, we conclude that estrogens stimulate VEGF expression in the growth plate, although the exact role of VEGF in estrogen-mediated growth plate fusion remains to be clarified.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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