Regulators of growth plate maturation

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Genome wide screening in human growth plates at early and progressed stage puberty of a single patient suggests a role of Elk1, Stat5b and RunX2 in growth plate maturation.

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Abstract

In late puberty, estrogen is responsible for the deceleration of growth by stimulating growth plate maturation. The mechanism of action is largely unknown. We obtained pubertal growth plate specimens of the same girl at Tanner stage B2 and B3, which allowed us to address this issue in more detail. Histological analysis showed that progression of puberty coincided with characteristic morphological changes associated with growth plate maturation, such as decreases in total growth plate height ($p=0.002$), height of the individual zones ($p<0.001$) and an increase in intercolumnar space ($p<0.001$). Microarray analysis identified 394 genes (72% upregulated, 28% downregulated) changing with progression of puberty. Overall changes in gene expression were small (average 1.38-fold upregulated and 1.36-fold downregulated genes). The 394 genes mapped to 13 significantly changing pathways ($p<0.05$) in majority belonging to extracellular matrix, cell cycle and cell death, all related to growth plate maturation. We next scanned the upstream promoter regions of the 394 genes for the presence of evolutionarily conserved binding sites for transcription factors implemented in growth plate maturation such as Estrogen Receptor, Androgen Receptor, Elk1, Stat5b, CREBP and Runx2. High quality motif sites for Runx2 (87 genes), Elk1 (43 genes) and Stat5b (31 genes), but not estrogen receptor, were evolutionarily conserved, indicating their functional relevance across primates.
In conclusion, our data suggest a role for Runx2, Elk1 and Stat5b in growth plate maturation and provides suggestive evidence that the effect of estrogen on growth plate maturation is not mediated by activating genomic estrogen signalling in growth plate chondrocytes.

Introduction

Longitudinal growth occurs at the epiphyseal growth plate, a thin layer of cartilage entrapped between epiphyseal and metaphyseal bone at the distal ends of the long bones. In the normal growth plate, immature cells are located towards the epiphysis, called the resting zone, with mature chondrocytes in the proliferating zone, which hypertrophy in the hypertrophic zone adjacent to this (1). At the beginning of puberty longitudinal growth rate first increases, but with progression of puberty, growth rate is decelerating due to growth plate maturation, and at the end of puberty the growth plate eventually disappears due to epiphyseal fusion. The molecular mechanisms underlying these distinct phases of growth plate activity during puberty are largely unknown but a role for estrogen has been suggested (2;3).
Endocrinological observations suggest that at the beginning of puberty relatively low levels of estrogen initiate the growth spurt. With progression of puberty, estrogen levels further increase which drives growth plate maturation and finally growth plate fusion. The most compelling evidence for a role of estrogen is provided by clinical observations in a patient with an inactivating mutation in the estrogen receptor alpha and in patients with a mutation in the aromatase gene resulting in lack of estrogen. These patients did not experience a growth spurt, and lack growth plate maturation and fusion (4;5). Furthermore, from clinical observations it is known that high levels of estrogen inhibit longitudinal bone growth (6).
The mechanism by which estrogens exert these effects on growth plate activity is not fully understood. It has been postulated that estrogen accelerates the senescent decline of the growth plate (7). Senescence is a term for the structural and functional changes over time in the growth plate, such as a gradual decline in the overall growth plate height, proliferative zone height, hypertrophic zone height, size of hypertrophic chondrocytes, proliferation rate and column

density (7). It is believed that the growth plate fuses when senescence reaches a critical point in the growth plate. Recent evidence indicates that senescence might occur because stem-like cells in the resting zone have a finite proliferative capacity, which is exhausted gradually. This process is accelerated by estrogen (8;9).

Estrogen induces cell responses by activating the so-called genomic signaling pathway involving the nuclear estrogen receptor alpha (ERa) and beta (ERb) or of a non-genomic signaling pathway involving membrane bound receptors like GPR30 resulting in activation of adenylyl cyclase and MAPKs (10-13). ERa, ERb and GPR30 are all expressed in human growth plate chondrocytes (14;15). Their expression is not limited to the stem-like cells of the resting zone, which are the main target cells of estrogen action based on the senescence hypothesis, but is more broadly distributed in the growth plate. It is still largely unknown whether the pubertal phenomena in relation to growth rate are caused by direct effects of estrogen on chondrocytes or by indirect effects via, for example, activation of the Growth Hormone/IGF-I axis.

During puberty both sex steroids, growth hormone (GH) and IGF-1 levels increase (16). It is well known that GH and IGF-1 can increase growth velocity as well as accelerate bone maturation measured by a decrease in growth plate height in children (17;18). Also receptors for GH and IGF-1 are present on human chondrocytes (19), indicating that both hormones can have direct effects on the growth plate. Stimulation of the GH-receptor activates an intracellular signal transduction cascade eventually converging to the transcription factor Stat5b (20). Likewise, IGF-1 signalling results in the activation of signalling routes involving for example the transcription factor Elk1 (21). The exact contributions of these hormones in growth plate maturation and epiphyseal fusion still need to be clarified.

Alternatively, estrogen may regulate, either directly or indirectly, the expression of paracrine regulators of growth plate activity such as Parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (Ihh). These secreted growth factors coordinate endochondral ossification by regulating chondrocyte proliferation and differentiation as well as osteoblast differentiation (22;23). PTHrP signals, amongst others, via activation of the cyclic AMP response element binding protein (24). Both factors have been identified in the postnatal growth plate and have been postulated to play a role in growth plate fusion (25).

In the growth plate, the transcription factor Runx2 plays an important role in the regulation of chondrocyte hypertrophy and the associated changes in the extracellular matrix (26). The expression and activation of this transcription factor is in part regulated by PTHrP and Ihh (27).

Studies on the regulation of growth plate activity during puberty are hampered by the lack of easy accessible and representative animal models. For example, rodents do not fuse their growth plates at the end of sexual maturation and discrepancies exist between human and mouse models with respect of the role of ERa in growth plate regulation (28-30). In addition, human growth plate specimens are very difficult to obtain.

We were fortunate to obtain growth plate samples of a single patient at two different stages of puberty. The growth plate tissues are genetically identical and from the same anatomical location. In this study we have performed a morphological analysis of these growth plate specimens complemented with a detailed microarray and bioinformatic analysis and identified 394 differentially expressed genes which were representative for processes that occur during growth plate maturation. We subsequently searched the promoter regions of these genes for evidence of involvement of hormones and paracrine factors in their expression regulation during growth plate maturation. Assuming that the regulation of processes such as growth plate maturation is conserved across primates, we identified functional transcription factor binding sites as those motif sites with a better evolutionary conservation than sites occurring by chance, related to

phylogenetic footprinting (31). More specifically, we searched the promoter regions of genes that were differentially expressed in the two growth plate specimens for evidence of direct effects of estrogen, androgen, GH, IGF-I, PTHrP and Runx2 on their expression.

Material and Methods

The study was approved by the local medical ethical committee and informed consent was obtained. Two epiphyseal growth plate samples, from the left and right proximal femur were obtained from the same girl with a 1 year interval. In this period the girl progressed from early (Tanner B2) to a progressed stage of puberty (Tanner B3). The patient suffered from cerebral palsy and underwent resection of her femur head twice because of painful luxations. She did not use any long-term medication. Both epiphyseal samples were longitudinally cut with a bone saw and pieces were covered by Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands), directly frozen in liquid isopentane and stored at –80ºC or fixed in 10% formalin, decalcified with EDTA and embedded in paraffin.

Histological analysis

Paraffin embedded samples were cut into longitudinal 5 mm thick sections using a Reichert Jung 2055 microtome (Leica, Rijswijk, The Netherlands). The sections were mounted on glass slides and stained with Haematoxylin. Total height was measured at three points parallel to the chondrocyte columns, height of each zone was measured at 10 different places for each zone and results were averaged. The space between columns in the proliferative and hypertrophic zone was measured at 20 different places.

RNA isolation

Bone was removed from both epiphyseal growth plate 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. (32) except that the protocol was started by homogenizing the sections in 1 ml guanidine thiocyanate solution. RNA extraction was followed by purification with a RNeasy kit according to the manufacturers protocol (Qiagen) and quality and integrity of each sample were checked with the Agilent 2100 Bioanalyzer.

Microarray

RNA was tested by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent) and high quality was confirmed. 100 ng of total RNA was then amplified and labeled using the GeneChip Two-Cycle cDNA Synthesis Kit (Affimetrix) and the MEGAscript T7 Kit (Ambion). The labeled cRNA was further used for the hybridization to Affymetrix Human Genome U133 PLUS 2.0 Array Genechips and hybridized according to Affymetrix manufacturer's protocol. RNA was extracted from two different sections of each growth plate. A Custom CDF Version 11 with Entrez based gene definitions was used to annotate the arrays (33). The Raw fluorescence intensity values were normalized applying quantile normalization using a commercial software package SAS JMP7 Genomics, version 3.1, from SAS (SAS Institute, Cary, NC, USA). Gene annotation was obtained through the Affymetrix NetAffx website (http://www.affymetrix.com/analysis/index.affx). The quality control, normalisation and statistical modelling were performed by array group correlation,

mixed model normalisation and mixed model analysis respectively. For the presence/absence analysis for a single-array, GeneChip® Operating Software version 1.4 (GCOS) from Affymetrix was used. Analysis of differential gene expression was based on loglinear mixed model of perfect matches (34). A false discovery rate of a=0.05 with FDR-correction for multiple testing was used to make a selection of most differentially expressed genes. These affected genes were further investigated to identify pathways that are likely to be affected by differential expression. Pathways were generated either from the KEGG database (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.ad.jp/kegg/pathway.html) or from manual annotation. The selection of affected genes were also analysed with a genome wide analysis of gene sets defined by the Gene Ontology (GO) Consortium and classified as GO-terms (35). In this analysis, an enrichment of affected genes within a GO-term suggests that this GO-term is affected by maturation of the growth plates. Analyses were done with the Gene Ontology Tree Machine program (http://bioinfo.vanderbilt.edu/gotm). The raw and normalized data are deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession No. GSE-18338).

Reverse transcription- Polymerase Chain Reaction (RT-PCR)

RNA was reverse transcribed into cDNA using First Strand cDNA Synthesis kit for qPCR (Roche Diagnostics Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Expression of collagen 3A1 (COL3A), CDKN1B (p27Kip1), dolichyl-phosphate mannosyltransferase polypeptide 1 (DPM1), Thrombospondin 4 (THBS4), and ribosomal protein L15 (RPL15) mRNA was quantified by real-time PCR using the Bio-Rad iCycler with SYBR Green. QuantiTect Primer Assays for each of these genes were purchased from Qiagen (Qiagen Benelux B.V., Venlo, the Netherlands) and used according to the manufacturer's protocol. Threshold cycles 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, quality of total RNA and in the efficiency of the reverse transcription reaction. Delta Ct was calculated by extracting the threshold cycle for β_2-microglobulin from the threshold cycle for the gene of interest followed by calculation of the change in delta Ct with progression of puberty.

Transcription factor binding sites

Upstream regions of 5000nt were downloaded from the 394 genes that changed with progression of puberty. The promoter regions were scanned for six transcription factor binding motifs selected from Jaspar 3.0 (36) and Transfac 7.0 (http://www.gene-regulation.com). The motifs were (see supplemental table 1): estrogen receptor (Jaspar MA0112), androgen receptor (Jaspar MA0007), Elk-1 (Transfac M00025), CREB (Jaspar MA0018), Runx2 (Jaspar MA0002) and STAT5B (Transfac M00459). A selection was made of the fraction of the highest scoring positions as potential regulatory sites. Two types of randomization controls were included. Firstly, we scanned the 5,000nt upstream regions of 100 sets of 394 randomly chosen genes for the six motifs mentioned above (random genes). Secondly, we scanned the 5,000nt upstream regions of the 394 differentially expressed genes for 100 versions of the six motifs with randomized columns (random motifs).

Because we expected that meaningful binding sites may be distinguished from spurious high scoring hits by their evolutionary conservation, we assessed the conservation of each of the binding sites across nine primate genomes. For this purpose the phastCons (37) primates conservation track was downloaded from the UCSC Genome Browser download page (38) and the average conservation score for all positions aligned with the motif were calculated.

Results

Quantitative Histology

Histology of the samples showed a clear decrease in overall height of the growth plate at the more progressed stage of puberty (figure 1). This was confirmed by quantitative measurements showing a significant decrease in the average height of the growth plate, and a significant decrease in the height of the resting, proliferative and hypertrophic zone at Tanner stage 3. The mean space between columns was increased in the more matured growth plate. These data are summarized in table 1.

Gene expression microarray analysis

RNA of both growth plate samples was amplified, labelled and subjected to Affymetrix microarray analysis (HG-U133 Plus 2) in duplicate. The technical and biological reproducibility was good, with correlations above 0.97. The raw and normalized data are deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE-XXXX). Presence and Absence analysis for each probe set was employed by using the GeneChip® Operating Software version 1.4 (GCOS) from Affymetrix. On average 5043 genes were present; with progression of puberty the number of genes present in the growth plate increased slightly (5069 vs 5016) (table 2). The microarray data was validated by quantitative PCR for 5 randomly chosen genes. Similar trends in gene expression (up- or downregulation) were found in qPCR and microarray analysis for all genes (Figure 2). THSB4 showed a more pronounced increase in expression in the microarray results compared to the qPCR results.

Analysis with a loglinear mixed model of perfect matches and a false discovery rate of a=0.05 and a Bonferroni-correction for multiple testing revealed 460 affymetrix probe IDs changing in expression, of which 330 were upregulated and 130 were downregulated. Using BioMart 0.7 (39) these probes were mapped to 394 genes changing with maturation of the growth plate (see table 2 supplemental data). The overall changes in gene expression were small; on average 1.38-fold increase for upregulated and 1.36-fold decrease for down regulated genes. Cytokine-like 1 was the most upregulated gene showing a 6.48 fold increase in expression and the most affected downregulated gene, pannexin 3, showed a 2.02 fold decrease in expression level.

The 394 differentially expressed genes were further investigated with Fisher's exact tests using SAS and the KEGG database. 111 of the 394 genes could be mapped to 13 enriched pathways ($p<0.05$) (Table 3). Several of the differentially expressed genes were present in more than one of the above pathways. These pathways were mostly related to the extracellular matrix, cell communication and metabolism. We studied these genes independently for their up or down regulation (see table 3 supplemental data). Most genes, 89 out of 111, were upregulated in the growth plate with progression of puberty. In addition, differentially expressed genes were further investigated with the Gene Ontology Tree Machine. This revealed 49 different Gene Ontology terms (GO terms) relatively enriched ($p<0.01$). Enriched GO terms were related to the extracellular matrix, cell cycle, cell growth and ligase activity (see figure 1 supplemental data).

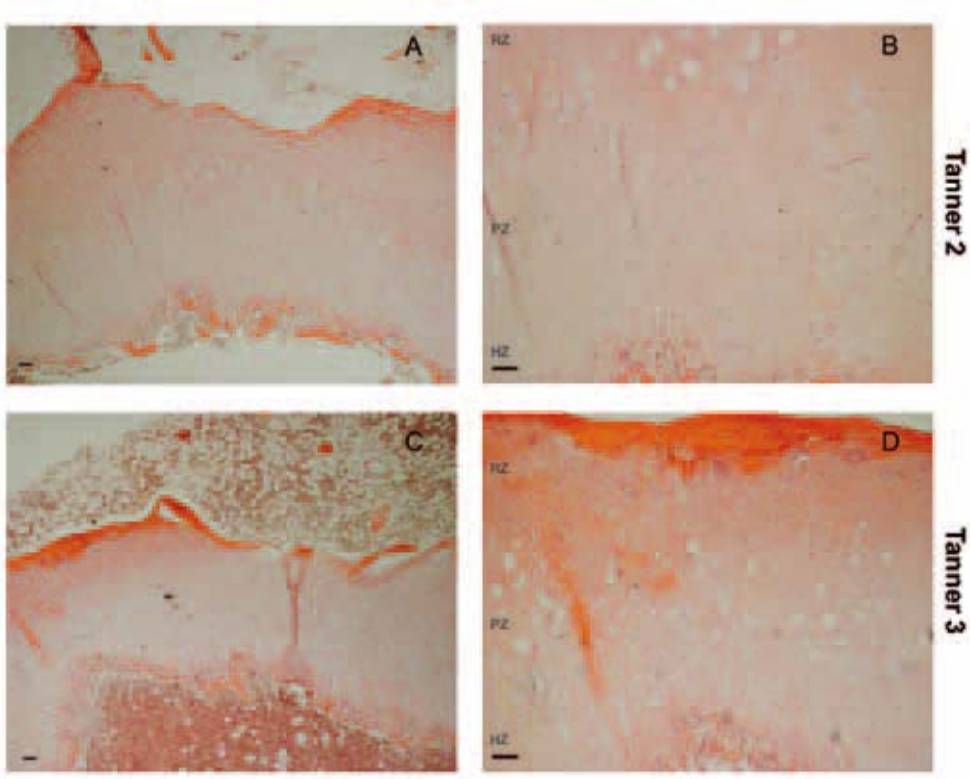

Figure 1: Histology of growth plate Tanner 2 and Tanner 3.

Panel A and B; pictures of growth plate of patient in Tanner stage 2 in respectively 40x and 100x magnification. Panel C and D; pictures of growth plate of patient in Tanner stage 3 in respectively 40x and 100x magnification. The more mature growth plate (Tanner stage 3) shows a decrease in total growth plate height, a decrease in height of each separate zone and an increase in the mean space between columns. RZ means resting zone, PZ means proliferative zone and HZ means hypertrophic zone. Bars indicate 200μm.

Table 1: Quantitative Histology growth plate Tanner stage 2 and 3.

	Tanner stage 2	**Tanner stage 3**	**P-value**
Total height (mm)	0.16 ± 0.01	0.097 ± 0.012	0.002
Height resting zone (mm)	0.073 ± 0.003	0.037 ± 0.009	<0.001
Height proliferative zone (mm)	0.047 ± 0.003	0.033 mm ± 0.004	<0.001
Height hypertrophic zone (mm)	0.024 ± 0.003	0.016 ± 0.003	<0.001
Intercolumn space (mm)	$4.87*10\text{-}4 \pm 0.34*10\text{-}^{4}$	$7.52*10\text{-}4 \pm 0.45*10\text{-}^{4}$	<0.001

Table showing measurements of total height, height of each individual zone and intercolumn space of the growth plate in Tanner stage 2 and the more progressed growth plate in Tanner stage 3.

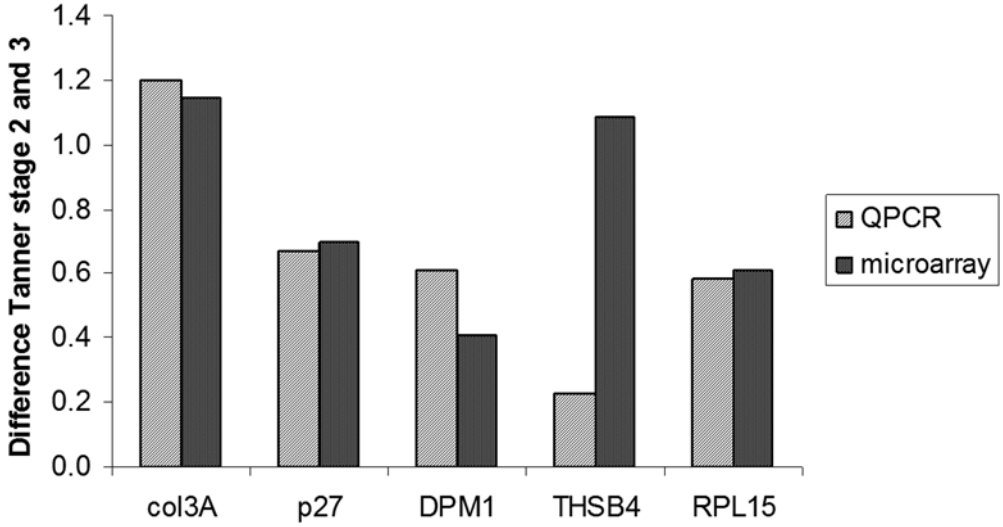

Figure 2: RT-PCR validation of microarray data.

Correlation between RT-PCR and microarray results for (A) collagen 3A1 (COL3A), (B) Thrombospondin 4 (THBS4), (C) CDKN1B (p27Kip1), (D) ribosomal protein L15 (RPL15), (E) dolichyl-phosphate mannosyltransferase polypeptide 1 (DPM1). Results are expressed as changes with progression of puberty (value Tanner B3- Tanner B4) for both the RT-PCR (delta Ct = $Ct_{\text{gene of interest}}$ - $Ct_{\beta 2\text{-microglobulin}}$) and microarray results (least square means). Similar trends in gene expression (up- or downregulation) were found in qPCR and microarray analysis for all genes, however THSB4 showed a more pronounced increase in expression in the microarray results compared to the qPCR results.

Table 2: Number of expressed and non-expressed genes.

	Absent	**Present**	**Unknown**
Growth plate Tanner 2	10255	5016	5555
Growth plate Tanner 3	10118	5069	5639

Table showing the number of genes absent or present in each of the growth plate. In the column defined as unknown is the number of genes not consistent in the present/absent analysis.

Table 3: Pathways significantly changing with progression of puberty.

	pathway	genes found	total genes pathway	%	p.
1	Proteasome	9	23	39	***
2	Cholera_Infection	10	30	33	***
3	Oxidative_phosphorylation	20	89	22	***
4	N_Glycan_biosynthesis	9	27	33	**
5	ATP_synthesis	9	28	32	**
6	Adherens_junction	14	60	23	**
7	Aminosugars_metabolism	6	17	35	**
8	Regulation_of_autophagy	6	17	35	**
9	Ribosome	9	35	26	**
10	ECM_receptor_interaction	14	67	21	**
11	Cell_cycle	15	84	18	*
12	Cell_Communication	13	74	18	*
13	Ubiquitin_mediated_proteolysis	7	32	22	*

***= p < 0.05, **= p < 0.01, ***= p < 0.001**

Table showing the 13 significant pathways associated with pubertal maturation of the growth plate.

Table 4: Top 0.001% genes with a transcription factor binding site for 6 motifs; Estrogen receptor, Elk-1, STAT5B, RunX2, Androgen receptor and CREB.

Motif	no. genes	% of 394 genes	p-value	average conservation score	% genes up	% genes down
Estrogen receptor	49	13	0,25	0,19	73	27
Elk-1	43	9	<0,01	0,33	70	30
STAT5B	31	8	0,04	0,25	81	19
RunX2	87	22	<0,01	0,23	76	24
Androgen receptor	46	12	0,07	0,22	80	20
CREB	44	11	0,16	0,20	75	25

Number and percentage of genes plus the average conservation score containing an transcription factor binding site for each of the 6 motifs. Results are presented for the top 0.0001% of sites and 0.001% of sites. For each motif is the percentage given of genes going up and down in expression.

Transcription factor binding sites

We next scanned the promoter regions of the 394 differentially expressed genes for the presence of conserved transcription factor binding sites. We limited our search to transcription factor binding sites which are activated by hormones and paracrine factors that have previously been implicated in growth plate maturation: Estrogen response elements (EREs) and androgen response elements (ARE) for activity of sex-steroids, Stat5b for GH (20), Elk-1 for IGF-I (21), Cyclic AMP response element (CREB) for PTHrP (24) and Runx2 for growth plate hypertrophy (40). We limited our analysis to the top 0.001% of the highest scoring motifs and determined the evolutionary conservation score of these sites. We found 215 genes with one or more transcription factor binding motif using the cut off of 0.001% of the top scoring motifs. The motifs and genes are listed in table 4 of the supplemental data. As a control, a similar analysis was performed using 100 sets of 394 randomly chosen genes. In addition, the promoter regions of the 394 genes were screened with randomized motifs for each transcription factor binding site and their evolutionary conservation score was also determined. These randomizations were used to calculate the statistical confidence score (p-value). The data are summarized in table 4.

We found 87 genes with a transcription factor binding site for RUNX2, 76% of genes going up and 24% going down in expression. The average evolutionary conservation score of the motif was significantly higher ($p<0.01$) compared to the findings in randomly chosen genes. Likewise, evolutionary conservation of the ELK-1 (49 genes) and STAT5B (31 genes) binding sites in the panel of 394 genes associated with growth plate maturation was significantly higher than random. We subsequently repeated the statistical analysis of the conservation score by including the top 0.01, top 0.1, top 1 and top 10% of the highest scoring sites in the analysis. By including up to 10% of the highest scoring sites of ELK-1 and STAT5B, the evolutionary conservation score was still significantly higher than for the controls. Significance for RUNX2 was lost by increasing the number of motif sites from the top 0.001 to the top 0.01 % (data not shown).

In marked contrast, the average evolutionary conservation scores of EREs (49 genes), AREs (46 genes) and CREB (44 genes) in the set of 394 genes were not significantly higher than in the randomly chosen controls.

In summary, the highest scoring motif sites for RUNX2, Elk-1 and STAT5B were also the most conserved across primates, suggesting that the presence of these motifs may play a functional role in the regulation of expression of the genes related to growth plate maturation. Conversely, high scoring ER, AR and CREBP motif sites were not better conserved than those in random gene sets, suggesting that their presence is coincidental.

Discussion

In the present study we compared gene expression levels in two epiphyseal growth plate samples obtained from one girl at early and mid puberty (Tanner stage 2 and 3) with a 1 year interval. Maturation of the epiphyseal growth plate in mid puberty is associated with a multitude of changes in morphology and expression levels of genes associated with the extracellular matrix, cell death, cell communication and metabolism. In the panel of 394 genes changing with growth plate maturation we found evidence, based on the evolutionary conservation of the highest scoring transcription factor binding sites, for regulation of expression by the transcription factors RUNX2, ELK-1 and STAT5B.

Histological experiments and measurements showed a clear decrease in total growth plate height with maturation. This is in line with the observations in rabbits, where growth plate height gradually declines with age and even more rapidly under the influence of estrogen (7). In humans it is known and widely used for assessing skeletal maturation that radiographically the epiphyseal width varies in different stages and declines in its progress toward maturity. In the more mature growth plate, columns were more widely spaced with more intervening extracellular matrix. These changes are described as senescence of the growth plate and confirm earlier results in rabbits and rats (7;41). Histological observations and measurements were in line with the microarray results, showing significant changes in the extracellular matrix compartment with maturation of the growth plate.The ECM receptor interaction pathway changed significantly with 14 out of 67 genes affected in this pathway. Associated with the extracellular matrix are the aminosugars metabolism pathway and the N-Glycan biosynthesis pathway, both changing significantly with maturation. The ECM is composed of a variety of macromolecules like proteoglycans and polysaccharides (glycosaminoglycans) that are secreted locally and assembled into an organized network (42;43). Most genes in these three pathways are upregulated with maturation suggesting an increase in pathway activity and extracellular matrix production. In addition to the pathway and morphology data, the GO term analyses also showed many enriched GO categories that are involved and associated to the extracellular matrix, which strengthens our findings. Blanchard et al demonstrated previously that estrogens and testosterone stimulate proteoglycan synthesis *in vitro* in male and female human epiphyseal chondrocytes, consistent with our results (44). Besides extracellular matrix pathways, also cell death pathways were enriched in the differentially expressed gene sets, e.g. proapoptotic and anti-apoptotic genes, but also genes involved in the regulation of autophagy. Apoptosis and autophagy are closely related and there is an overlap in signaling proteins (45;46). Previously, we found no signs of classical apoptosis in the human growth plate with pubertal maturation and epiphyseal fusion (47). The results of this study are in line with this and suggestive for a non-classical and perhaps intermediate mechanism of different types of cell death.

The overall change in gene expression levels in growth plate chondrocytes with progression of puberty was unexpectedly small, particularly since puberty is associated with dramatic changes in growth velocity and hormone levels like sex steroids, Growth Hormone and IGF-I (48-50). Our microarray data is in line with the histological changes observed with growth plate maturation providing support that the differentially expressed gene set is representative for the changes that occur during growth plate maturation. We hypothesized that analysis of the promoter regions of these genes may provide clues for transcription factors and signaling pathways that are involved in growth plate maturation. More specifically the promoter regions were analyzed for the presence

of evolutionarily conserved binding sites for Estrogen and Androgen Receptors, ELK-1 for IGF-I, STAT5b for GH, CREB for PTHrP and RUNX2 for growth plate hypertrophy.

Despite strong clinical and experimental evidence for the role of sex steroids and in particular estrogen in growth plate maturation, the potential EREs and also AREs in the promoter regions of the 394 genes were not conserved in other primate species. Although these motif sites may still be functional in human, the fact that they are uniquely human makes this less likely since sequences conserved along species are more likely to have functional roles (37). Thus, estrogen may not have a direct genomic effect in pubertal growth plate maturation. This contrasts with findings of Windahl et al., who previously detected an ERE-mediated response in the hypertrophic zone of mice (51). This discrepancy might be explained by a species difference, as illustrated before by the divergent phenotypes of the ERα knockout mice and man with respect to growth plate regulation. Our data does not exclude a role for non-genomic estrogen signalling in growth plate maturation nor for an indirect effect of estrogen. Likewise, no enrichment was found for CREB binding sites which are activated by intracellular cAMP levels via for example PTHrP.

Interestingly, the high scoring ELK-1, STAT5b and RUNX2 motif sites were conserved across primates. ELK-1 and STAT5b are activated by, amongst others, IGF-I and GH for which receptors are present in growth plate chondrocytes. In animal models local effects of GH and IGF-1 on growth plate chondrocytes have been established (52;53). Besides the increase in levels of estrogen, also the levels of GH and IGF-I increase significantly with the progression of puberty. In addition, it is well known that GH-treatment accelerates growth as well as growth plate maturation, either directly or indirectly via IGF-I. Our conservation analysis of the transcription factor binding motifs in the promoters of differentially expressed genes supports a direct role for GH and IGF-I in growth plate maturation, resulting in activation of STAT5b and ELK-1 mediated gene transcription, respectively. The effect of estrogen on the activity of the GH/IGF-I axis is well appreciated, demonstrated by increasing GH levels in patients with oral estrogen treatment (54;55). This may suggest that effects of estrogen on growth plate maturation might be mediated, at least in part, by GH and/or IGF-I.

Runx2 plays an important role in chondrocyte maturation and is involved in the production of bone matrix proteins (56). Our results are in line with this hypothesis, since we found many genes changing with maturation of the growth plate in puberty that contained evolutionarily conserved transcription factor binding site for Runx2. Previous studies have shown that Runx2 can mediate actions of estrogen in an osteoblastic cell line and that selective estrogen receptor modulators like tamoxifene and raloxifene can increase Runx2 promotor activity in an osteosarcoma cell line (57;58). This provides an additional mechanism by which estrogen can indirectly influence growth plate maturation.

While the changes in growth plate morphology are in line with the senescence hypothesis, our data do not allow testing the proposed effect of estrogen on the depletion of stem-like cells in the growth plate with progression of puberty.

The major limitation of our study is the small sample number. However these growth plate samples are unique and enable a longitudinal analysis within one patient, therefore excluding genetic confounders. Adult height is determined for 80-90% by genetic factors (58;59). Including additional patients would therefore result in increasing variability, which would complicate all subsequent analysis. To the best of our knowledge, no other microarray studies have been performed on human growth plate tissues. The observed changes in gene expression and subsequent pathway analysis were fully in line with morphological changes that were characteristic for growth plate maturation in animal studies. In addition, microarray data were confirmed by qPCR. This strengthens our

confidence that the set of 394 genes is representative for changes in growth plate maturation and that our findings are biologically relevant. However, additional studies have to be done in a larger number of samples and with more pubertal stages to confirm our findings.
In conclusion, maturation of the epiphyseal growth plate in mid-puberty is associated with morphological changes in line with the senescence theory. This was corroborated by a multitude of changes in gene expression. Thirteen pathways were affected with maturation, several related to the extracellular matrix, the cell cycle, and programmed cell death. Evolutionary conservation of binding sites provides evidence for a direct role for GH, IGF-I and RUNX2 in growth plate maturation. We did not find support for direct genomic effects of estrogen, suggesting that the well appreciated role of estrogen in growth plate maturation might perhaps be indirect by modulating GH, IGF-I and RUNX2 activity.

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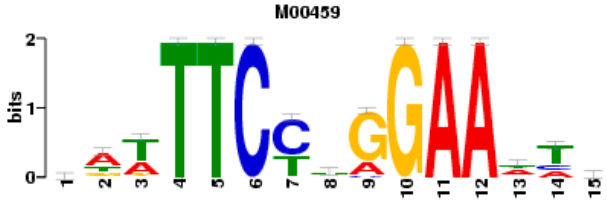
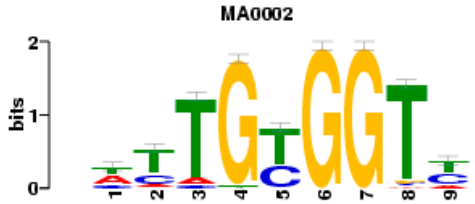
Supplemental table 1

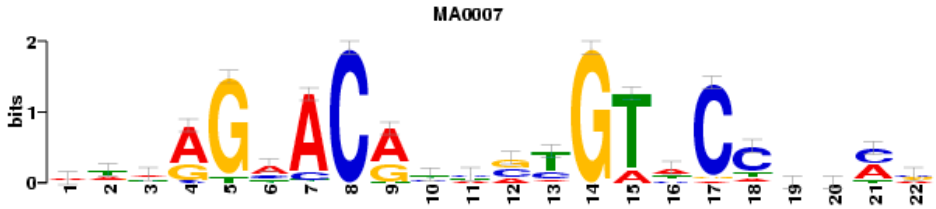
Stat5B



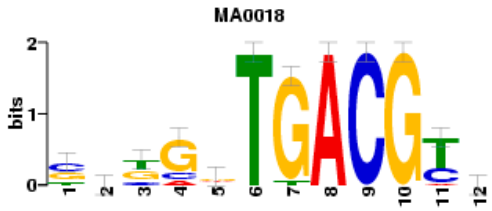
RunX2



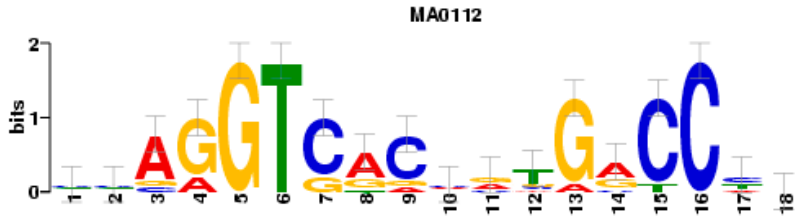
Androgeen Receptor



CREB



Estrogen Receptor



Elk-1

Supplemental table 2

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AC015922.3	ENSG00000220036	CFL2	ENSG00000165410	FAM96A	ENSG00000166797
AC022868.10-1	ENSG00000215034	CHCHD4	ENSG00000163528	FAU	ENSG00000149806
AC091047.10-3	ENSG00000218599	CHMP2B	ENSG00000083937	FBLN7	ENSG00000144152
ACADM	ENSG00000117054	CHMP4A	ENSG00000100931	FBXO28	ENSG00000143756
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ACPL2	ENSG00000155893	CIRBP	ENSG00000099622	FECH	ENSG00000066926
ACTL6A	ENSG00000136518	CLC	ENSG00000105205	FGD2	ENSG00000146192
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ADD3	ENSG00000148700	CLEC11A	ENSG00000105472	FKBP7	ENSG00000079150
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ANAPC5	ENSG00000089053	COL6A3	ENSG00000163359	FUBP1	ENSG00000162613
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ANXA7	ENSG00000138279	COX4NB	ENSG00000131148	FZD6	ENSG00000164930
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C5orf43	ENSG00000188725	EIF5	ENSG00000100664	KCTD13	ENSG00000174943
C6orf49	ENSG00000124593	ELK3	ENSG00000111145	KDELR2	ENSG00000136240
C7orf60	ENSG00000164603	EPAS1	ENSG00000116016	KIAA1370	ENSG00000047346
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CALD1	ENSG00000122786	ETS1	ENSG00000134954	KIAA1432	ENSG00000107036
CAPS2	ENSG00000180881	ETV5	ENSG00000171656	KIAA1524	ENSG00000163507
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Gene symbol	Ensemble ID
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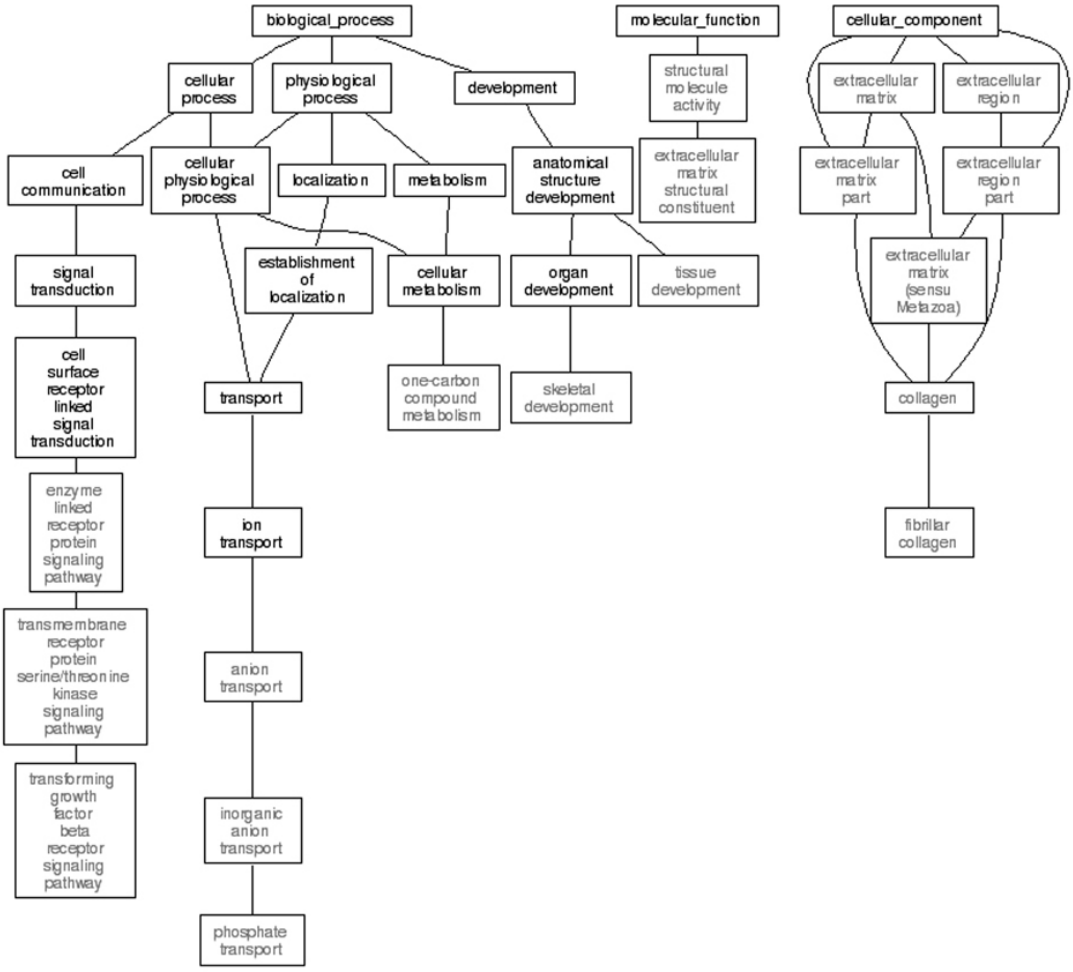
Supplemental figure 1



References

1. **Kronenberg HM** 2003 Developmental regulation of the growth plate. Nature 423:332-336

2. **Chagin AS, Savendahl L** 2007 Estrogens and growth: review. Pediatr Endocrinol Rev 4:329-334

3. **MacGillivray MH, Morishima A, Conte F, Grumbach M, Smith EP** 1998 Pediatric endocrinology update: an overview. The essential roles of estrogens in pubertal growth, epiphyseal fusion and bone turnover: lessons from mutations in the genes for aromatase and the estrogen receptor. Horm Res 49 Suppl 1:2-8

4. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

5. **Morishima A, Grumbach MM, Simpson ER, Fisher C, Qin K** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80:3689-3698

6. **Turner RT, Riggs BL, Spelsberg TC** 1994 Skeletal effects of estrogen. Endocr Rev 15:275-300

7. **Weise M, De Levi S, Barnes KM, Gafni RI, Abad V, Baron J** 2001 Effects of estrogen on growth plate senescence and epiphyseal fusion. Proc Natl Acad Sci U S A 98:6871-6876

8. **Gafni RI, Weise M, Robrecht DT, Meyers JL, Barnes KM, De Levi S, Baron J** 2001 Catch-up growth is associated with delayed senescence of the growth plate in rabbits. Pediatr Res 50:618-623

9. **Schrier L, Ferns SP, Barnes KM, Emons JA, Newman EI, Nilsson O, Baron J** 2006 Depletion of resting zone chondrocytes during growth plate senescence. J Endocrinol 189:27-36

10. **Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J** 1986 Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150-1154

11. **Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA** 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93:5925-5930

12. **Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER** 2005 A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625-1630

13. **Filardo EJ, Quinn JA, Frackelton AR, Jr., Bland KI** 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16:70-84

14. **Chagin AS, Savendahl L** 2007 GPR30 estrogen receptor expression in the growth plate declines as puberty progresses. J Clin Endocrinol Metab 92:4873-4877

15. **Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J, Martin RE, Savendahl L** 2003 Localization of estrogen receptors-alpha and -beta and androgen receptor in the human growth plate at different pubertal stages. J Endocrinol 177:319-326

16. **Perry RJ, Farquharson C, Ahmed SF** 2008 The role of sex steroids in controlling pubertal growth. Clin Endocrinol (Oxf) 68:4-15

17. **de ZF, Butenandt O, Chatelain P, bertsson-Wikland K, Jonsson B, Lofstrom A, Chaussain JL** 1997 Growth hormone treatment of short children born small for gestational age: reappraisal of the rate of bone maturation over 2 years and metanalysis of height gain over 4 years. Acta Paediatr Suppl 423:207-212

18. **Kamp GA, Waelkens JJ, de Muinck Keizer-Schrama SM, Delemarre-van de Waal HA, Verhoeven-Wind L, Zwinderman AH, Wit JM** 2002 High dose growth hormone treatment induces acceleration of skeletal maturation and an earlier onset of puberty in children with idiopathic short stature. Arch Dis Child 87:215-220

19. **Werther GA, Haynes K, Edmonson S, Oakes S, Buchanan CJ, Herington AC, Waters MJ** 1993 Identification of growth hormone receptors on human growth plate chondrocytes. Acta Paediatr Suppl 82 Suppl 391:50-53

20. **Rosenfeld RG, Hwa V** 2009 The growth hormone cascade and its role in mammalian growth. Horm Res 71 Suppl 2:36-40

21. **Bruning JC, Gillette JA, Zhao Y, Bjorbaeck C, Kotzka J, Knebel B, Avci H, Hanstein B, Lingohr P, Moller DE, Krone W, Kahn CR, Muller-Wieland D** 2000 Ribosomal subunit kinase-2 is required for growth factor-stimulated transcription of the c-Fos gene. Proc Natl Acad Sci U S A 97:2462-2467

22. **Karp SJ, Schipani E, St-Jacques B, Hunzelman J, Kronenberg H, McMahon AP** 2000 Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. Development 127:543-548

23. **van der Eerden BC, Karperien M, Gevers EF, Lowik CW, Wit JM** 2000 Expression of Indian hedgehog, parathyroid hormone-related protein, and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. J Bone Miner Res 15:1045-1055

24. **Mak KK, Bi Y, Wan C, Chuang PT, Clemens T, Young M, Yang Y** 2008 Hedgehog signaling in mature osteoblasts regulates bone formation and resorption by controlling PTHrP and RANKL expression. Dev Cell 14:674-688

25. **Kindblom JM, Nilsson O, Hurme T, Ohlsson C, Savendahl L** 2002 Expression and localization of Indian hedgehog (Ihh) and parathyroid hormone related protein (PTHrP) in the human growth plate during pubertal development. J Endocrinol 174:R1-R6

26. **Yoshida CA, Komori T** 2005 Role of Runx proteins in chondrogenesis. Crit Rev Eukaryot Gene Expr 15:243-254

27. **Yoshida CA, Yamamoto H, Fujita T, Furuichi T, Ito K, Inoue K, Yamana K, Zanma A, Takada K, Ito Y, Komori T** 2004 Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev 18:952-963

28. **Nilsson O, Chrysis D, Pajulo O, Boman A, Holst M, Rubinstein J, Martin RE, Savendahl L** 2003 Localization of estrogen receptors-alpha and -beta and androgen receptor in the human growth plate at different pubertal stages. J Endocrinol 177:319-326

29. **Vidal O, Lindberg M, Savendahl L, Lubahn DB, Ritzen EM, Gustafsson JA, Ohlsson C** 1999 Disproportional body growth in female estrogen receptor-alpha-inactivated mice. Biochem Biophys Res Commun 265:569-571

30. **Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331:1056-1061

31. **Tagle DA, Koop BF, Goodman M, Slightom JL, Hess DL, Jones RT** 1988 Embryonic epsilon and gamma globin genes of a prosimian primate (Galago crassicaudatus). Nucleotide and amino acid sequences, developmental regulation and phylogenetic footprints. J Mol Biol 203:439-455

32. **Heinrichs C, Yanovski JA, Roth AH, Yu YM, Domene HM, Yano K, Cutler GB, Jr., Baron J** 1994 Dexamethasone increases growth hormone receptor messenger ribonucleic acid levels in liver and growth plate. Endocrinology 135:1113-1118

33. **Sandberg R, Larsson O** 2007 Improved precision and accuracy for microarrays using updated probe set definitions. BMC Bioinformatics 8:48

34. **Chu TM, Weir B, Wolfinger R** 2002 A systematic statistical linear modeling approach to oligonucleotide array experiments. Math Biosci 176:35-51

35. **Beissbarth T** 2006 Interpreting experimental results using gene ontologies. Methods Enzymol 411:340-352

36. **Sandelin A, Wasserman WW, Lenhard B** 2004 ConSite: web-based prediction of regulatory elements using cross-species comparison. Nucleic Acids Res 32:W249-W252

37. **Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, Clawson H, Spieth J, Hillier LW, Richards S, Weinstock GM, Wilson RK, Gibbs RA, Kent WJ, Miller W, Haussler D** 2005 Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res 15:1034-1050

38. **Kuhn RM, Karolchik D, Zweig AS, Wang T, Smith KE, Rosenbloom KR, Rhead B, Raney BJ, Pohl A, Pheasant M, Meyer L, Hsu F, Hinrichs AS, Harte RA, Giardine B, Fujita P, Diekhans M, Dreszer T, Clawson H, Barber GP, Haussler D, Kent WJ** 2009 The UCSC Genome Browser Database: update 2009. Nucleic Acids Res 37:D755-D761

39. **Haider S, Ballester B, Smedley D, Zhang J, Rice P, Kasprzyk A** 2009 BioMart Central Portal--unified access to biological data. Nucleic Acids Res 37:W23-W27

40. **Solomon LA, Berube NG, Beier F** 2008 Transcriptional regulators of chondrocyte hypertrophy. Birth Defects Res C Embryo Today 84:123-130

41. **Kember NF** 1973 Aspects of the maturation process in growth cartilage in the rat tibia. Clin Orthop Relat Res288-294

42. **Toole BP, Linsenmayer TF** 1977 Newer knowledge of skeletogenesis: macromolecular transitions in the extracellular matrix. Clin Orthop Relat Res258-278

43. **Toole BP, Okayama M, Orkin RW, Yoshimura M, Muto M, Kaji A** 1977 Developmental roles of hyaluronate and chondroitin sulfate proteoglycans. Soc Gen Physiol Ser 32:139-154

44. **Blanchard O, Tsagris L, Rappaport R, Duval-Beaupere G, Corvol M** 1991 Age-dependent responsiveness of rabbit and human cartilage cells to sex steroids in vitro. J Steroid Biochem Mol Biol 40:711-716

45. **Codogno P, Meijer AJ** 2006 Atg5: more than an autophagy factor. Nat Cell Biol 8:1045-1047

46. **Heath-Engel HM, Chang NC, Shore GC** 2008 The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. Oncogene 27:6419-6433

47. **Emons J, Chagin AS, Hultenby K, Zhivotovsky B, Wit JM, Karperien M, Savendahl L** 2009 Epiphyseal Fusion in the Human Growth Plate does not involve Classical Apoptosis. Pediatr Res

48. **Casazza K, Goran MI, Gower BA** 2008 Associations among insulin, estrogen, and fat mass gain over the pubertal transition in African-American and European-American girls. J Clin Endocrinol Metab 93:2610-2615

49. **Juul A, Bang P, Hertel NT, Main K, Dalgaard P, Jorgensen K, Muller J, Hall K, Skakkebaek NE** 1994 Serum insulin-like growth factor-I in 1030 healthy children, adolescents, and adults: relation to age, sex, stage of puberty, testicular size, and body mass index. J Clin Endocrinol Metab 78:744-752

50. **Abbassi V** 1998 Growth and normal puberty. Pediatrics 102:507-511

51. **Windahl SH, Lagerquist MK, Andersson N, Jochems C, Kallkopf A, Hakansson C, Inzunza J, Gustafsson JA, van der Saag PT, Carlsten H, Pettersson K, Ohlsson C** 2007 Identification of target cells for the genomic effects of estrogens in bone. Endocrinology 148:5688-5695

52. **Isgaard J, Nilsson A, Lindahl A, Jansson JO, Isaksson OGP** 1986 Effects of Local-Administration of Gh and Igf-1 on Longitudinal Bone-Growth in Rats. American Journal of Physiology 250:E367-E372

53. **Ohlsson C, Nilsson A, Isaksson O, Lindahl A** 1992 Growth hormone induces multiplication of the slowly cycling germinal cells of the rat tibial growth plate. Proc Natl Acad Sci U S A 89:9826-9830

54. **Coutant R, de Casson FB, Rouleau S, Douay O, Mathieu E, Gatelais F, Bouhours-Nouet N, Voinot C, Audran M, Limal JM** 2004 Divergent effect of endogenous and exogenous sex steroids on the insulin-like growth factor I response to growth hormone in short normal adolescents. J Clin Endocrinol Metab 89:6185-6192

55. **Veldhuis JD, Keenan DM, Bailey JN, Adeniji A, Miles JM, Paulo R, Cosma M, Soares-Welch C** 2008 Estradiol supplementation in postmenopausal women attenuates suppression of pulsatile growth hormone secretion by recombinant human insulin-like growth factor type I. J Clin Endocrinol Metab 93:4471-4478

56. **Komori T** 2003 Requisite roles of Runx2 and Cbfb in skeletal development. J Bone Miner Metab 21:193-197

57. **Sasaki-Iwaoka H, Maruyama K, Endoh H, Komori T, Kato S, Kawashima H** 1999 A trans-acting enhancer modulates estrogen-mediated transcription of reporter genes in osteoblasts. J Bone Miner Res 14:248-255

58. **Tou L, Quibria N, Alexander JM** 2001 Regulation of human cbfa1 gene transcription in osteoblasts by selective estrogen receptor modulators (SERMs). Mol Cell Endocrinol 183:71-79

59. **Perola M, Sammalisto S, Hiekkalinna T, Martin NG, Visscher PM, Montgomery GW, Benyamin B, Harris JR, Boomsma D, Willemsen G, Hottenga JJ, Christensen K, Kyvik KO, Sorensen TI, Pedersen NL, Magnusson PK, Spector TD, Widen E, Silventoinen K, Kaprio J, Palotie A, Peltonen L** 2007 Combined genome scans for body stature in 6,602 European twins: evidence for common Caucasian loci. PLoS Genet 3:e97