

Genome-wide screening in human growth plates during puberty in one patient suggests a role for RUNX2 in epiphyseal maturation

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Abstract

In late puberty, estrogen decelerates bone growth by stimulating growth plate maturation. In this study, we analyzed the mechanism of estrogen action using two pubertal growth plate specimens of one girl at Tanner stage B2 and Tanner stage B3. Histological analysis showed that progression of puberty coincided with characteristic morphological changes: a decrease 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 of the specimens identified 394 genes (72% upregulated and 28% downregulated) that changed with the 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 ($P<0.05$) associated with growth plate maturation (e.g. extracellular matrix, cell cycle, and cell death). We next scanned the upstream promoter regions of the 394 genes for the presence of evolutionarily conserved binding sites for transcription factors implicated in growth plate maturation such as estrogen receptor (ER), androgen receptor, ELK1, STAT5B, cyclic AMP response element (CREB), and RUNX2. High-quality motif sites for RUNX2 (87 genes), ELK1 (43 genes), and STAT5B (31 genes), but not ER, were evolutionarily conserved, indicating their functional relevance across primates. Moreover, we show that some of these sites are direct target genes of these transcription factors as shown by ChIP assays.

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Introduction

Longitudinal growth occurs at the epiphyseal growth plate, a thin layer of cartilage between epiphyseal and metaphyseal bone at the distal ends of the long bones. In the normal growth plate, immature cells are located toward the epiphysis, called the resting zone, with mature chondrocytes in the proliferating zone, which dramatically increase in size in the hypertrophic zone adjacent to this (Kronenberg 2003). At the beginning of puberty, the longitudinal growth rate first increases, but with progression of puberty, the growth rate decelerates due to growth plate maturation, and eventually, at the end of puberty, the growth plate disappears due to epiphyseal fusion. The molecular mechanisms underlying these distinct phases are largely unknown, but a role for estrogen has been suggested (MacGillivray *et al.* 1998, Chagin & Savendahl 2007a).

Endocrinological observations suggest that the growth spurt is initiated at the beginning of puberty by relatively low levels of estrogen. The increase in estrogen levels that occur with the progression of puberty drives growth plate maturation and finally growth plate fusion. The most compelling evidence for the role of estrogen is provided by clinical observations in a patient with an inactivating mutation in the estrogen receptor α (ER α) and in patients with a mutation in the aromatase gene resulting in lack of estrogen. These patients did not experience a growth spurt and lacked growth plate maturation and fusion (Smith *et al.* 1994, Morishima *et al.* 1995). Furthermore, clinical observations have shown that high levels of estrogen inhibit longitudinal bone growth (Turner *et al.* 1994).

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 (Weise *et al.* 2001). 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 (Weise *et al.* 2001). It is believed that the growth plate fuses when senescence reaches a critical point. Recent evidence indicates that senescence might occur because stem-like cells in the resting zone have a finite proliferative capacity, which is gradually exhausted. This process is accelerated by estrogen (Gafni *et al.* 2001, Schrier *et al.* 2006).

Estrogen induces cell responses by activating the so-called genomic signaling pathway involving the nuclear ER α and ER β (Greene *et al.* 1986). Moreover, estrogen also works through a non-genomic signaling pathway involving membrane-bound receptors like GPR30, resulting in the activation of adenylyl cyclases and MAPKs (Filardo *et al.* 2002). In addition, Teplyuk *et al.* (2008) describe a direct role for Runx2 in G-protein-coupled signaling for controlling growth of osteoblast progenitor cells. ER α , ER β , and GPR30 are all expressed in human growth plate chondrocytes (Nilsson *et al.* 2003, Chagin & Savendahl 2007b). 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 GH/insulin-like growth factor 1 (IGF1) axis.

During puberty, the levels of both sex steroids, GH and IGF1, increase (Perry *et al.* 2008). It is well known that GH and IGF1 can increase growth velocity and accelerate bone maturation measured by a decrease in growth plate height in children (de Zegher *et al.* 1997, Kamp *et al.* 2002). Moreover, human chondrocytes contain receptors for GH and IGF1 (Werther *et al.* 1993), 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 (Rosenfeld & Hwa 2009). Likewise, IGF1 signaling results in the activation of signaling routes involving, for example, the transcription factor ELK1 (Bruning *et al.* 2000). The exact contributions of these hormones in growth plate maturation and epiphyseal fusion remain to be elucidated.

Alternatively, estrogen may directly or indirectly regulate 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 (van der Eerden *et al.* 2000, Karp *et al.* 2000). PTHrP signals exert their influence by activating the cyclic AMP response element (CREB) binding protein (Mak *et al.* 2008). Both factors have been identified in the postnatal growth plate and have been postulated to play a role in growth plate fusion (Kindblom *et al.* 2002).

The transcription factor Runx2 plays an important role in the growth plate by regulating chondrocyte hypertrophy and the associated changes in the extracellular matrix (ECM; Yoshida & Komori 2005). The expression and activation of this transcription factor is in part regulated by PTHrP and Ihh (Yoshida *et al.* 2004).

Studies on the regulation of growth plate activity during puberty are hampered by the lack of easily accessible and representative animal models. For example, in rodents, growth plates are not fused at the end of sexual maturation and discrepancies exist between human and mouse models with respect to the role of ER α in growth plate regulation (Smith *et al.* 1994, Vidal *et al.* 1999, Nilsson *et al.* 2003). 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, some of which are 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 (Tagle *et al.* 1988, Kouwenhoven *et al.* 2010). In this study, 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, IGF1, PTHrP, and RUNX2 on their expression. Next, we compared the predicted transcription factor binding with transcription factor binding data from CHIP assays and RNA interference experiments to verify the interaction of transcription factors with the regulatory region of these target genes.

Materials and Methods

This study was approved by the local medical ethical committee and informed consent was obtained. Two epiphyseal growth plate samples, from the left and the right proximal femur, were obtained from the same girl with a 1-year interval. In this period, the girl progressed from early (Tanner stage B2) at age 12.5 years to a progressed stage of puberty (Tanner stage B3) at age 13.5 years. 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 the epiphyseal samples were longitudinally cut with a bone saw and the pieces were

covered by Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), directly frozen in liquid isopentane, and stored at -80°C or fixed in 10% formalin, decalcified with EDTA and embedded in paraffin.

Histological analysis

Paraffin-embedded samples were cut into longitudinal $5\ \mu\text{m}$ thick sections using a Reichert Jung 2055 microtome (Leica, Rijswijk, The Netherlands). The sections were mounted on glass slides and stained with hematoxylin. Total height was measured at three points parallel to the chondrocyte columns, height of each zone was measured at ten 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 $40\ \mu\text{m}$ thick sections were cut with a cryostat. Every fifth section was followed by a $5\ \mu\text{m}$ 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 extraction was followed by purification with a RNeasy kit according to the manufacturer's 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 Technologies, Santa Clara, CA, USA) and high quality was confirmed. Total RNA (100 ng) was then amplified and labeled using the GeneChip Two-Cycle cDNA Synthesis Kit (Affymetrix, High Wycombe, UK) and the MEGAscript T7 Kit (Ambion/Applied Biosystem, Carlsbad, CA, USA). 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 (Sandberg & Larsson 2007). 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 web site (<http://www.affymetrix.com/analysis/index.affx>). The quality control, normalizations, and statistical modeling were performed by array group correlation, mixed model normalization, and mixed model analysis respectively. For the presence/absence analysis for a single

array, GeneChip Operating Software version 1.4 (GCOS; Affymetrix) was used. Analysis of differential gene expression was based on loglinear mixed model of perfect matches (Chu *et al.* 2002). A false discovery rate of $\alpha=0.05$ 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 Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.ad.jp/kegg/pathway.html>) or from the manual annotation. The selection of affected genes were also analyzed with a genome-wide analysis of gene sets defined by the Gene Ontology (GO) Consortium and classified as GO terms (Beissbarth 2006). 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-PCR

RNA was reverse transcribed into cDNA using First Strand cDNA Synthesis kit for qPCR (Roche Diagnostics GmbH) according to the manufacturer's instructions. Expression of collagen 3A1 (COL3A), CDKN1B (p27Kip1), dolichylphosphate 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.) 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. ΔC_t 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 ΔC_t 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 well-described transcription factor binding motifs selected from Jaspar 3.0 (Sandelin *et al.* 2004) and Transfac 7.0 (<http://www.gene-regulation.com>). The motifs were (Supplementary Table 1, see section on supplementary data given at the end of this article) ER (Jaspar MA0112; Welboren *et al.* 2009), androgen receptor (Jaspar MA0007; Roche *et al.* 1992), ELK1 (Transfac M00025; Treisman *et al.* 1992), CREB (Jaspar MA0018;

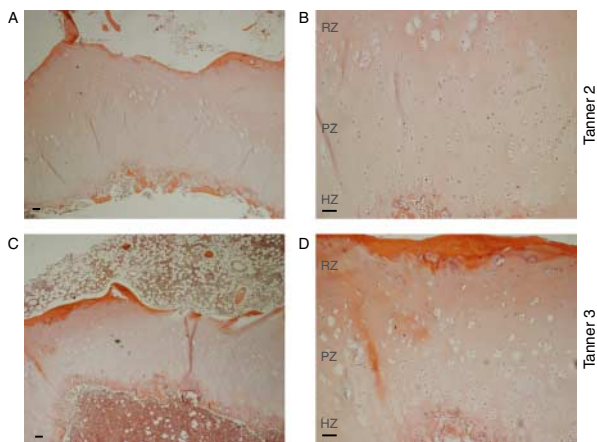


Figure 1 Histology of growth plate Tanner stage B2 and Tanner stage B3. Panels A and B: pictures of growth plate of patient in Tanner stage B2 in 40 \times and 100 \times magnification respectively. Panels C and D: pictures of growth plate of patient in Tanner stage B3 in 40 \times and 100 \times respectively magnification. The more mature growth plate (Tanner stage B3) 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, resting zone; PZ, proliferative zone; HZ, hypertrophic zone. Bars indicate 200 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-10-0219>

Portales-Casamar *et al.* 2007), Runx2 (Jaspar MA0002), and STAT5B (Transfac M00459; Soldaini *et al.* 2000). The highest scoring positions were selected as potential regulatory sites. Two types of randomization controls were included. First, we scanned the 5000nt upstream regions of 100 sets of 394 randomly chosen genes for the six motifs mentioned above (random genes). Secondly, we scanned the 5000nt 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 (Kouwenhoven *et al.* 2010), we assessed the conservation of each of the binding sites across nine primate genomes. For this purpose, the PhastCons (Siepel *et al.* 2005) primates conservation track was downloaded from the UCSC Genome Browser download page (Kuhn *et al.* 2009) and the average conservation score for all positions aligned with the motif were calculated.

Table 1 Quantitative histology growth plate Tanner stage B2 and B3. Table showing measurements of total height, height of each individual zone, and intercolumn space of the growth plate in Tanner stage B2 and the more progressed growth plate in Tanner stage B3. The relative distribution of the different zones is given in percentage of the total height of the growth plate

	Tanner stage B2	%	Tanner stage B3	%	P value
Total height (mm)	0.16 \pm 0.01		0.097 \pm 0.012		0.002
Height resting zone (mm)	0.073 \pm 0.003	46	0.037 \pm 0.009	38	<0.001
Height proliferative zone (mm)	0.047 \pm 0.003	29	0.033 \pm 0.004	34	<0.001
Height hypertrophic zone (mm)	0.024 \pm 0.003	15	0.016 \pm 0.003	16	<0.001
Intercolumn space (mm)	4.87 $\times 10^{-4}$ \pm 0.34 $\times 10^{-4}$		7.52 $\times 10^{-4}$ \pm 0.45 $\times 10^{-4}$		<0.001

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 (Fig. 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 mature growth plate. These data are summarized in Table 1.

Gene expression microarray analysis

RNA of both growth plate samples was amplified, labeled, 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-18338). The 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 the progression of puberty, the number of genes present in the growth plate slightly increased (5069 vs 5016) (Table 2).

The microarray data was validated by quantitative PCR (qPCR) for five randomly chosen genes. Similar trends in gene expression (up- or downregulation) were found in qPCR and microarray analysis for all genes (Fig. 2). THSB4 showed a more pronounced increase in expression in the microarray results compared with the qPCR results.

Analysis with a loglinear mixed model of perfect matches and a false discovery rate of $\alpha=0.05$ 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 (Haider *et al.* 2009), these probes were mapped to 394 genes changing with maturation of the growth plate (Supplementary Table 2, see section on supplementary data given at the end of this article). The overall changes in gene expression were small: on average, 1.38-fold increase for upregulated and 1.36-fold decrease for downregulated genes. Cytokine-like 1 was the

Table 2 Number of expressed and non-expressed genes. The table shows the number of genes absent or present in each of the growth plates. In the column defined as unknown is the number of genes not consistent in the present/absent analysis

	Absent	Present	Unknown
Growth plate Tanner B2	10255	5016	5555
Growth plate Tanner B3	10118	5069	5639

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. Of the 394 genes, 111 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 ECM, cell communication, and metabolism. We studied these genes independently for their up- or downregulation (Supplementary Table 3, see section on supplementary data given at the end of this article). 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 GO terms relatively enriched ($P < 0.01$). Enriched GO terms were related to the ECM, cell cycle, cell growth, and ligase activity (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Transcription factor binding sites

We next scanned the promoter regions up to 5000nt upstream of the translation start site of the 394 differentially expressed genes for the presence of evolutionarily conserved transcription factor binding sites. We limited our search to transcription factor binding sites that are activated by hormones and paracrine factors that have previously been implicated in growth plate maturation: estrogen response elements (EREs) and androgen response element (ARE) for activity of sex steroids, STAT5B for GH (Rosenfeld & Hwa 2009), ELK1 for IGF1 (Bruning *et al.* 2000), CREB for PTHrP (Mak *et al.* 2008), and Runx2 for growth plate hypertrophy (Solomon *et al.* 2008). We limited our analysis to the top 0.001% of the highest scoring motif sites in all the promoter regions, determined the evolutionary conservation score of these sites, and found 215 genes with one or more transcription factor binding motifs. The motifs and genes are listed in Supplementary Table 1. As a control, a similar analysis was performed using 100 sets of 394 randomly chosen genes and 100 randomized transcription factor binding motifs. These randomizations were used to calculate 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 in expression and 24% going down in expression. The average evolutionary conservation score of the motif was significantly higher ($P < 0.01$) compared with the findings in randomly chosen genes. Likewise, evolutionary conservation of the ELK1 (49 genes) and STAT5B (31 genes) binding sites in the panel of 394 genes associated with growth plate maturation was significantly higher than random. 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 randomized controls.

To verify that these motifs can actually be bound by the corresponding transcription factors, we searched the literature for known interactions with the corresponding genes. For each of the transcription factors, we found an overlap of genes that were shown to be bound in their promoter region by one or more of the transcription factors. For example, ChIP analysis of ELK1 binding in HeLa and Jurkat cells shows binding in nine of the genes found in our list of putative ELK1-regulated genes (Boros *et al.* 2009), and over 50 matches are found in genes that are shown to be bound by Runx2 in either ChIP-on-chip assays or responsive to modulations in Runx2 levels by siRNA knockdown experiments in both human and mouse cells (Young *et al.* 2007a,b, van der Deen *et al.* 2008, Teplyuk *et al.* 2008, 2009a). In summary, for ELK1, the identified transcription factor binding sites were confirmed in 21% of cases, RUNX2 57%, CREB 23%, ER 16%, and AR 20% (Supplementary Table 4, see section on supplementary data given at the end of this article).

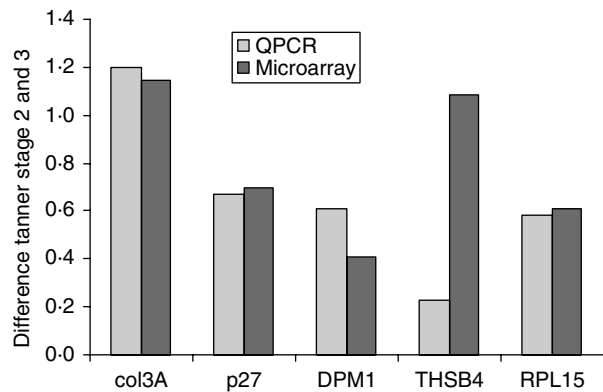


Figure 2 RT-PCR validation of microarray data. Correlation between RT-PCR and microarray results for (A) collagen 3A1 (COL3A), (B) thrombospondin 4 (THBS4), (C) CDKN1B (p27Kip1), (D) ribosomal protein L15 (RPL15), and (E) dolichyl-phosphate mannosyltransferase polypeptide 1 (DPM1). Results are expressed as changes with progression of puberty (value Tanner stage B3 - Tanner stage B2) for both the RT-PCR ($\Delta C_t = C_{t_{\text{gene of interest}}} - C_{t_{\beta_2\text{-microglobulin}}}$) and the 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 with the qPCR results.

Table 3 Pathways significantly changing with progression of puberty. The 13 significant pathways associated with pubertal maturation of the growth plate are shown

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	*

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Finally, we addressed the question whether these transcription factors co-operate in the regulation of growth plate maturation. Therefore, we checked whether there was any overlap in the motifs found in the regulatory region of the target genes. We assumed that the probability of functional regulation is higher in genes that contain multiple conserved binding motifs, as found for some of the target genes of the ELK1 family member Ets1 and the androgen receptor (Massie *et al.* 2007). Indeed, we found that four out of the 43 genes with an ELK1 binding motif also contain an ARE motif. We found that 29.6% of the genes contain motifs for two transcription factors and 3.5% of the genes contain three or more motifs. We have visualized the co-regulation of the transcription factors in Supplementary Figure 2, see section on supplementary data given at the end of this article.

In summary, the highest scoring motif sites for RUNX2, ELK1, 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 in the promoter regions investigated is coincidental.

Discussion

In this study, we compared gene expression levels in two unique human 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 ECM, 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.

A limitation of our study is the small sample number. However, these growth plate samples are unique and enable a longitudinal analysis within one patient, thereby excluding genetic confounders. Adult height is determined for 80–90% by genetic factors (Tou *et al.* 2001, Perola *et al.* 2007). Inclusion of additional patients would, therefore, result in increased 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 are 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.

The patient in this study suffered from cerebral palsy, which to our knowledge does not interfere with the molecular mechanisms of growth plate maturation and fusion. These patients have normal longitudinal bone growth, normal

Table 4 Genes with a transcription factor binding site (TFBS) for each of the six motifs. Number and percentage of genes plus the average conservation score containing a TFBS for each of the six motifs. Results are presented for the top 0.0001% of sites and top 0.001% of sites. For each motif the percentage of genes going up and down in expression is given. The last column shows the number of genes that are found in ChIP or knock-down assays for the corresponding transcription factors

Motifs	No. of genes	% of 394 genes	P value	Average conservation score	% genes up	% genes down	No. of TFBS confirmed
Estrogen receptor	49	13	0.25	0.19	73	27	8
Elk-1	43	9	<0.01	0.33	70	30	9
STAT5B	31	8	0.04	0.25	81	19	1
RUNX2	87	22	<0.01	0.23	76	24	50
Androgen receptor	46	12	0.07	0.22	80	20	9
CREB	44	11	0.16	0.20	75	25	10

puberty, and at the end of puberty longitudinal growth also stops with fusion of the growth plate as seen in healthy individuals. Therefore, we safely assume that the underlying mechanism of epiphyseal maturation and fusion is the same for the growth plates of healthy individuals and our patient.

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 (Weise *et al.* 2001). 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 ECM. These changes are described as senescence of the growth plate and confirm earlier results in rabbits and rats (Kember 1973, Weise *et al.* 2001). These changes in the ECM compartment with maturation of the growth plate were in line with the microarray results, demonstrating a significant change in expression of genes linked to ECM pathways. Histology shows that the absolute height of the growth plate changes during maturation, but not the relative height of the proliferative and hypertrophic zone (Table 1). This ratio is at least in part determined by the Ihh PTHrP feedback loop (Tryfonidou *et al.* 2010). Consequently, one would not expect differences in these pathways, and indeed, this is what we found (Supplementary Table 5, see section on supplementary data given at the end of this article).

The ECM receptor interaction pathway significantly changed with 14 out of 67 genes affected in this pathway. Both the pathways associated with the ECM, the aminosugars metabolism pathway and the *N*-glycan biosynthesis pathway, change 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 (Toole & Linsenmayer 1977, Toole *et al.* 1977). Most genes in these three pathways are upregulated with maturation implying an increase in pathway activity and ECM production. In addition to the pathway and morphology data, the GO term analyses also showed many enriched GO categories that are involved in and associated with the ECM, which strengthens our findings. Blanchard *et al.* (1991) demonstrated previously that estrogens and testosterone stimulate proteoglycan synthesis *in vitro* in male and female human epiphyseal chondrocytes, consistent with our results. Besides ECM pathways, not only 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 (Codogno & Meijer 2006, Heath-Engel *et al.* 2008). Previously, we found no signs of classical apoptosis in the human growth plate with pubertal maturation and epiphyseal fusion (Emons *et al.* 2009). The results of this study are in line with this and are 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, since puberty is associated with dramatic changes in growth velocity and hormone levels like sex steroids, GH and IGF1 (Juul *et al.* 1994, Abbassi 1998, Casazza *et al.* 2008). Our microarray data are 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 ER and androgen receptor, ELK-1 for IGF1, 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 less conserved in other primate species. Although motif sites that are conserved between species are more likely to have a functional role (Siepel *et al.* 2005, Kouwenhoven *et al.* 2010), the EREs we identified upstream of the 394 differentially regulated genes may still be functional in human. In the current analysis, we assessed only the 0.001% best matching motif sites in a promoter region of 5000nt upstream of the translation start site. We chose this rather conservative approach to minimize false positive predictions, which remains a challenge in bioinformatic transcription factor binding site prediction. Previous analyses have detected an ERE-mediated response in the hypertrophic zone of mice using reporter mice with a luciferase gene under the control of three EREs, enabling detection of *in vivo* activation of gene transcription (Windahl *et al.* 2007). It has been observed that most functional EREs and AREs are located far away from the transcription start site, and therefore these would not be found in our analysis (Carroll *et al.* 2006, Jariwala *et al.* 2007). However, 16% of the genes with conserved ERE motifs in the 5000nt upstream of the TSS were confirmed with ChIP analysis and 20% of the genes with conserved ARE motifs were confirmed by ChIP analysis (Table 4). So, although our stringency for the analysis is high, and ChIP data are only available for different tissues and cell types, we found that many of the putative interactions are indeed confirmed by ChIP analysis. This indicates that our method can be used to predict possible regulation of gene expression by these transcription factors in our human growth plates and provides clues as to which upstream signaling pathways are involved in the regulation of growth plate maturation. Our data do not exclude a role for non-genomic estrogen signaling 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.

In marked contrast, the high scoring ELK-1, STAT5B, and RUNX2 motif sites were conserved across primates. ELK-1 and STAT5B are activated by, amongst others, IGF1 and GH for which receptors are present in growth plate chondrocytes. In animal models, local effects of GH and IGF1 on growth plate chondrocytes have been established (Isgaard *et al.* 1986, Ohlsson *et al.* 1992). In addition to the increase in levels of estrogen, the levels of GH and IGF1 significantly increase with the progression of puberty. Moreover, it is well known that GH treatment accelerates growth as well as growth plate maturation, either directly or indirectly via IGF1. Our conservation analysis of the transcription factor binding motifs in the promoters of differentially expressed genes supports a direct role for GH and IGF1 in growth plate maturation, resulting in the activation of STAT5B- and ELK-1-mediated gene transcription respectively. The effect of estrogen on the activity of the GH/IGF1 axis is well appreciated, demonstrated by increasing GH levels in patients with oral estrogen treatment (Coutant *et al.* 2004, Veldhuis *et al.* 2008). This suggests that effects of estrogen on growth plate maturation might be mediated, at least in part, by GH and/or IGF1.

Runx2 plays an important role in the earliest stages of mouse chondrocyte maturation and hypertrophy, and Runx2 controls production of bone matrix proteins (Komori 2003, Lian *et al.* 2006). Our results are in line with this hypothesis, since we found many genes changing with maturation of the human 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 ER modulators like tamoxifen and raloxifene can increase RUNX2 promoter activity in an osteosarcoma cell line (Sasaki-Iwaoka *et al.* 1999, Tou *et al.* 2001). In addition, RUNX2 is a direct regulator of the non-genomic ER GPR30 (Teplyuk *et al.* 2008). These observations provide additional mechanisms by which estrogen can indirectly influence growth plate maturation. Several studies have examined Runx2-responsive genes in osteoprogenitors (Teplyuk *et al.* 2008, 2009a, 2009b), in mouse embryonic fibroblasts (MEFs; Wotton *et al.* 2008, Kilbey *et al.* 2010), and in osteosarcoma cells (Young *et al.* 2007a,b), as well as binding of Runx2 to promoters in genomic loci in osteosarcoma cells (van der Deen *et al.* 2008). Comparison of the corresponding gene lists reveals that at least ~60% (50 of 87) of all genes predicted to have Runx2 binding sites are regulated by Runx2, consistent with the biological role of Runx2 and other Runx proteins in the development and function of the growth plate.

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.

In conclusion, maturation of the epiphyseal growth plate in mid puberty is associated with morphological changes consistent with the senescence theory. This was corroborated by a multitude of changes in gene expression. In total, 13 pathways were affected with maturation, several related to the

ECM, the cell cycle, and the programmed cell death. 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, IGF1, and RUNX2 activity. Evolutionary conservation of binding sites provides evidence for a direct role for GH, IGF1, and RUNX2 in growth plate maturation.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-10-0219>.

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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