Regulators of growth plate maturation

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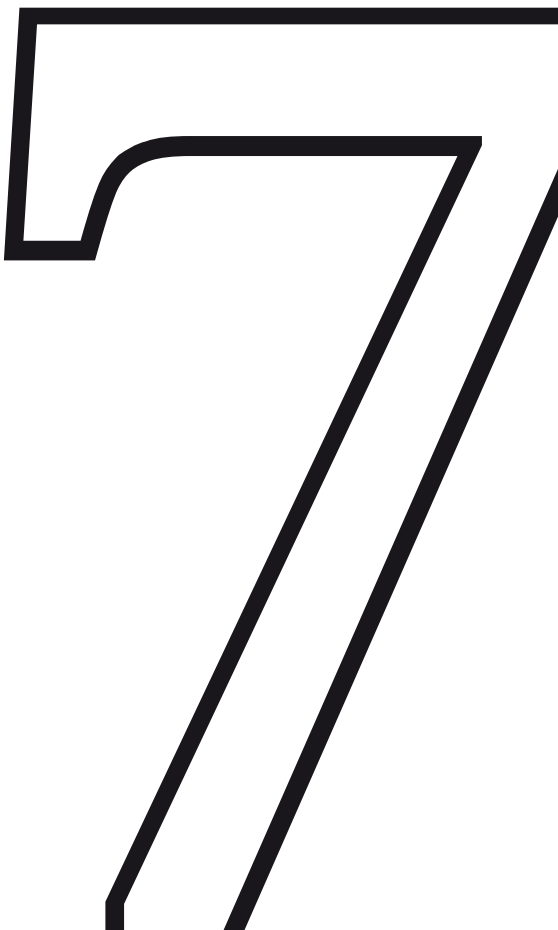

A cross-sectional microarray study of human epiphyseal growth plates during pubertal maturation.

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Abstract

In puberty growth velocity first accelerates, followed by a phase of deceleration and eventually cessation of growth with fusion of the epiphyses. Little is known about the exact mechanisms underlying the pubertal growth spurt, growth plate maturation and epiphyseal fusion. In this study, human growth plate specimens were collected during distinct phases of puberty. High quality RNA was isolated, amplified, labelled and subjected to Affymetrix microarray analysis (HG-U133 Plus 2). We compared growth plate tissue from the distal femur and proximal tibia and found no great difference between expression patterns of these two growth plates from different anatomical locations. Secondly, we performed a cross-sectional microarray study with female tibial growth plate specimens obtained at different stages of pubertal development (prepuberty, early puberty and more progressed puberty). Progression of puberty is associated with many, however mostly small, changes in gene expression that occurred most frequently late in puberty. Pathway analysis revealed 11 pathways, associated with the extracellular matrix homeostasis, hormonal pathways and programmed cell death, changing with maturation of the growth plate. Significance in this comparison was lost when the results were corrected for multiple testing, probably due to the small sample size. Power calculations revealed that at least 5 growth plates are needed in each group to detect a 1.5 fold difference. For the first time we show a developmental regulation of the gene expression pattern in the human epiphyseal growth plate with most alterations occurring during late puberty. However more future studies are needed to validate our findings.

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 (1). Epiphyseal chondrocytes are under tight control of a variety of hormones and growth factors acting directly or indirectly on the growth plate (2). At onset of puberty, the levels of various hormones, including sex steroids, increase in circulation. Sex steroids induce the external signs of puberty (secondary sex characteristics) and the accompanying rise in growth velocity, called the pubertal growth spurt. Typically, pubertal growth consists of a phase of acceleration, followed by a phase of deceleration, and the eventual cessation of growth with the fusion of the epiphyses. One of the important hormones regulating pubertal bone growth in both males and females is estrogen. Estrogen levels increase about 10-fold along with a 1.5 to 3-fold increase in GH and IGF-1 secretion in puberty (3;4).

In puberty, humans gain on average 27.5-31 centimeters in height, accounting for approximately 17% of adult height (5). The dramatic effects on growth velocity during distinct phases of puberty are a reflection of the activity of the growth plate which consequently undergoes substantial changes over time. Little is known about the exact mechanisms underlying the pubertal growth spurt, growth plate maturation and epiphyseal fusion. Most previous studies studying growth plate regulation have used animal models, which poorly represent characteristic growth patterns of human during puberty.

In chapter 6 we investigated gene expression levels during pubertal growth plate maturation within one patient. This revealed a multitude of changes in gene expression. In addition, 13 pathways changed with maturation. Several of these pathways were related to the extracellular matrix. An increase in matrix / cell ratio is a typical characteristic of a maturing growth plate. These analyses were, however, based on one single patient. In order to validate these findings in

a larger population we have started collecting human growth plate specimens. Human growth plate specimens become occasionally available during surgical procedures for diverse medical indications. These medical indications are founded in various diseases, some related to growth others. These specimens were processed for histology and for RNA extraction. In this study, we report preliminary results of the microarray analysis of human growth plate specimens collected during distinct phases of puberty.

Material and Methods

Patients and tissue preparation

Human proximal tibia growth plate tissues were collected from patients at different pubertal stages, undergoing surgery for different medical indications (Table 1). The study protocol was approved by the Local Medical Ethics Committees of The Leiden University Center, Leiden, the Netherlands and The Karolinska University Hospital, Stockholm, Sweden. Informed consent was obtained from all patients and their parents. Epiphyseal samples were embedded in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and frozen in liquid isopentane or immediately fixed in 10% formaldehyde for 24hrs, decalcified in 10% EDTA and embedded in paraffin. RNA was extracted and checked for quality and integrity as previously described in chapter 6.

Table 1

sample	diagnosis	sex	location in fumer	age (yr:m)	Tanner	parafin/RNA	origin	code
1	elective abortion	f	tibia	fetal		RNA	Leiden	fetal
2	constitutional tall stature	f	distal	11:06	B1	paraffin	Stockholm	pt2
3	leg length difference	f	distal	9:06	B1	paraffin	Stockholm	pt22
4	Osteosarcoma	f	distal	8	B1	RNA	Leiden	GP1234
5	Upper limb amputation of the leg, tibia	f	proximal	9	B1	RNA	Leiden	GP_t_8
6	Upper limb amputation of the leg, femur	f	distal	9	B1	RNA	Leiden	GP_f_6
7	Chondroid osteosarcoma, Rothmund Thompson S	f	distal	12	B1	RNA	Leiden	GP1142
8	leg length difference	f	distal	9:06	B1-2	paraffin	Stockholm	pt23
9	hip luxations, femur head resection	f	proximal	12:05	B2	paraffin/RNA	Leiden	GS 3
10	leg length difference	f	distal	12:08	B2	paraffin	Stockholm	pt19
11	constitutional tall stature	f	distal	13:07	B2	paraffin	Stockholm	pt25
12	constitutional tall stature	f	distal	12:01	B2	paraffin	Stockholm	pt30
13	Tall stature	f	distal	10:06	B2	RNA	Stockholm	GP 12h
14	leg length difference	f	distal	14:02	B2-3	paraffin	Stockholm	pt17
15	Tall stature	f	distal	11:05	B2-3	RNA	Stockholm	GP 10h
16	hip luxations, femur head resection	f	proximal	13:05	B3	paraffin/RNA	Leiden	GS 7
17	leg length difference	f	distal	13:08	B3	paraffin	Stockholm	pt15
18	Tall stature	f	distal	12	B3	RNA	Stockholm	GP 22h
19	cerebral palsy, femur head resection	f	proximal	15:01	B4	paraffin	Leiden	GS 8
20	leg length difference	f	distal	13:03	B4	paraffin	Stockholm	pt8
21	femur head resection	f	proximal	14:00	B4	RNA	Leiden	5233
22	Tall stature	f	distal	15:07	B4	RNA	Stockholm	GP 21h
23	cerebral palsy, femur head resection	f	proximal	17:00	B5	paraffin	Leiden	GS 9
24	leg length difference	f	distal	1:00	B5	paraffin	Stockholm	pt31
25	CHD, femur head resection	m	proximal	13:01	G1	RNA	Leiden	GS 13
26	Marfan	m	distal	12:03	G1	paraffin	Stockholm	pt 5
27	cerebral palsy, AIS, femur head resection	m	proximal	10	G1/B1	RNA	Leiden	GP6
28	cerebral palsy, femur head resection	m	proximal	16:10	G2	RNA	Leiden	GP25
29	leg length difference	m	distal	12:06	G3	paraffin	Stockholm	pt 24
30	leg length difference	m	distal	15:04	G3-4	paraffin	Stockholm	pt 26
31	leg length difference	m	distal	14:08	G4	paraffin	Stockholm	pt 18
32	47XYY	m	distal	14	G4-5	paraffin	Stockholm	pt 27

Patients information. Samples #4 and 5 are derived from the same patient. Samples #8 and 15 are derived from the same patient

Microarray analyses

100 ng of total RNA was amplified and labeled using the GeneChip Two-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA) and the MEGAscript T7 Kit (Ambion, Austin, TX). The labeled cRNA was further used for the hybridization to Affymetrix Human Genome U133 PLUS 2.0 Array Genechips and hybridized according to Affymetrix manufacturers protocol. In a first approach we compared growth plate tissue from the distal femur and proximal tibia to investigate the influence of location on gene expression profiles. In this analysis samples #4 and #5 were used.

In a second approach we performed a cross-sectional microarray study with growth plate specimens obtained at different stages of pubertal development. To minimize the variability we only included growth plate specimens from the female sex derived from the proximal tibia. The samples were divided in 3 different groups: 1. prepubertal (pt #3 and #4); 2. early puberty (defined as Tanner stage B2 and B2-3, pt #12 and #14); 3. more progressed stage puberty (defined as Tanner stage B3 and B4 pt #17 and #20). A genewise linear model was fitted to the six arrays using the R package Limma to test differences between the three stages of puberty. Because of the limited power due to the small sample size, no adjustment was made for multiple testing, but attention was focused on the patterns in the uncorrected P values. Differentially expressed genes were further investigated as previously described with a global test in order to identify pathways that are likely to be affected by growth plate maturation (6).

Thirdly, a power calculation was performed to see how many growth plate specimens are needed to ensure that with 80% probability a 1.5 fold change can be detected at 5% level of significance after correction for multiple testing.

Besides the growth plate samples used in the experiments described in the above table 1 also contains additional growth plate samples not just yet, but available for future experiments.

Results

Proximal vs distal femoral growth plate expression

The expression profiles of distal and proximal femoral growth plate were largely overlapping. Most genes showed a small non-significant change (<1.4 fold). Only eight genes were showing a more than 2-fold difference. Figure 1 shows a volcano plot of all genes. From this we conclude that growth plates from different anatomical locations are similar in gene expression pattern. Subsequently, they can be used together in one comparison studying other parameters like pubertal maturation.

Pubertal maturation

Because of the small sample size (n=2 in each group) attention was focused on the patterns in the uncorrected P values. This revealed 605 genes changing from prepuberty to early puberty, while 1275 genes changed from early to late puberty and as many as 2362 genes changed from prepuberty to late puberty. Many genes were changing in more than one comparison (Figure 2). Significance was lost when results were corrected for multiple testing. The changes in expression level were mostly in a small range. The most affected upregulated gene (matrilin-1) showed a 32 fold change in expression from prepuberty to late puberty. The most affected downregulated gene showed a 11 fold change in expression level from prepuberty to late puberty (TCDD-inducible poly(ADP-ribose) polymera). The percentage of probes, out of the genes changing with maturation, with ≥2 fold change are shown in table 2. Results indicate that most alterations occurred during late puberty with 48.6% of genes increasing in expression level and 51.4% of genes decreasing in expression level.

Power calculation

Although the expression of many genes tended to change with progression of puberty, in this limited analysis none of these changes reached significance after correction for multiple testing. This was due to the small sample size, the biological variability between the specimens and the unexpectedly low levels of fold change. Based on this first analysis, we performed a power calculation to determine the number of growth plate specimens needed to ensure that with 80% probability a 1.5 fold change at 5% level of significance after correction for multiple testing can be detected.

In this analysis the total number of genes has to be considered, given that including many genes which are not involved in the regulation of growth during puberty makes it more difficult to flag the genes that do play a role. The variance of the expression levels between the samples also has to be considered. If a given gene naturally varies very much between individuals, it will consequently be hard to detect a signal. To get an idea of the amount of variability we might expect, we computed the variance for each gene in our data. We found that the mean variance was around 0.2, but variances above 0.8 did also occur.

In Figure 3 we plotted the required sample size (in each group) as a function of the variance. The red line is based on testing 200 genes, the black line on testing 1000 genes. From this can be concluded that about 5 growth plates in each group are needed to detect a fold change of 1.5 when the variance is 0.2.

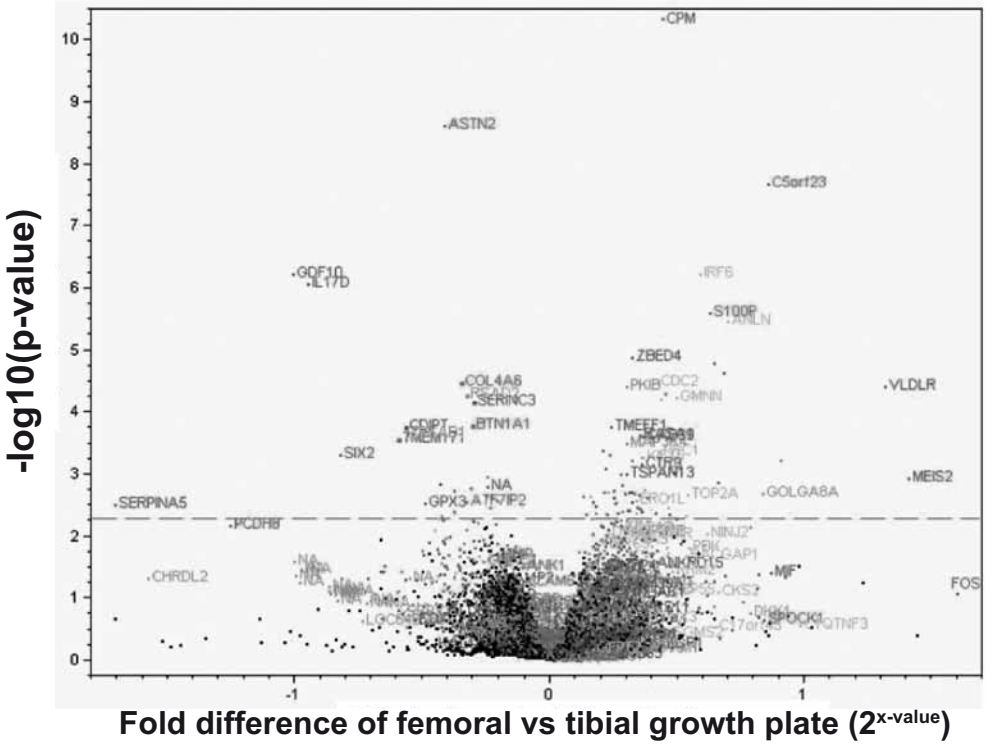

Figure 1. Proximal vs distal femoral growth plate expression.

In this figure the gene expression levels of the tibial growth plate versus the femoral growth plate is plotted. On the x-axis is written the fold-difference which can be calculated by $2^{x\text{-value}}$. On the y-axis the –log10(p-value) which indicates the level of significance. The higher these value the more significant and values above the dashed line are significant without correction for multiple testing. Expression profiles were largely overlapping. Most genes showed a small non-significant change (x<0.5= fold change< 1.4 fold). Only eight genes were showing a more than 2-fold difference.

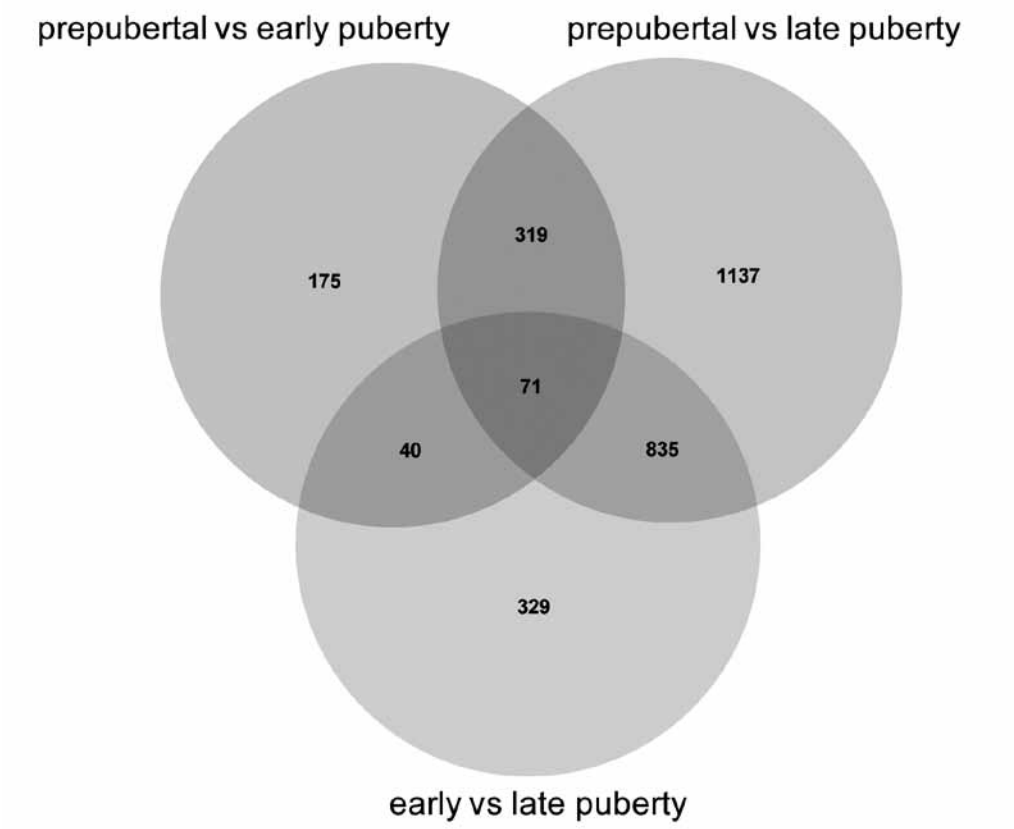

Figure 2: Venn diagram of significantly different genes in each group.
This figure shows the number of genes significantly different in each of the 3 comparisons between the different groups. It also shows the number of genes significant in more than one comparison.

Table 2

	Percentage of probes ≥2 fold change
prepuberty to early puberty	0.4%
early puberty to late puberty	2.0%
prepuberty to late puberty	3.1%

Percentage of probes with a ≥2 fold change between the different groups of pubertal maturation.
A pathway-based analysis with a global test for all genes revealed 11 pathways changing significantly from early to late puberty (table 3). Affected pathways associated with the extracellular matrix homeostasis, hormonal pathways and programmed cell death. Significance was lost when the results were corrected for multiple testing.

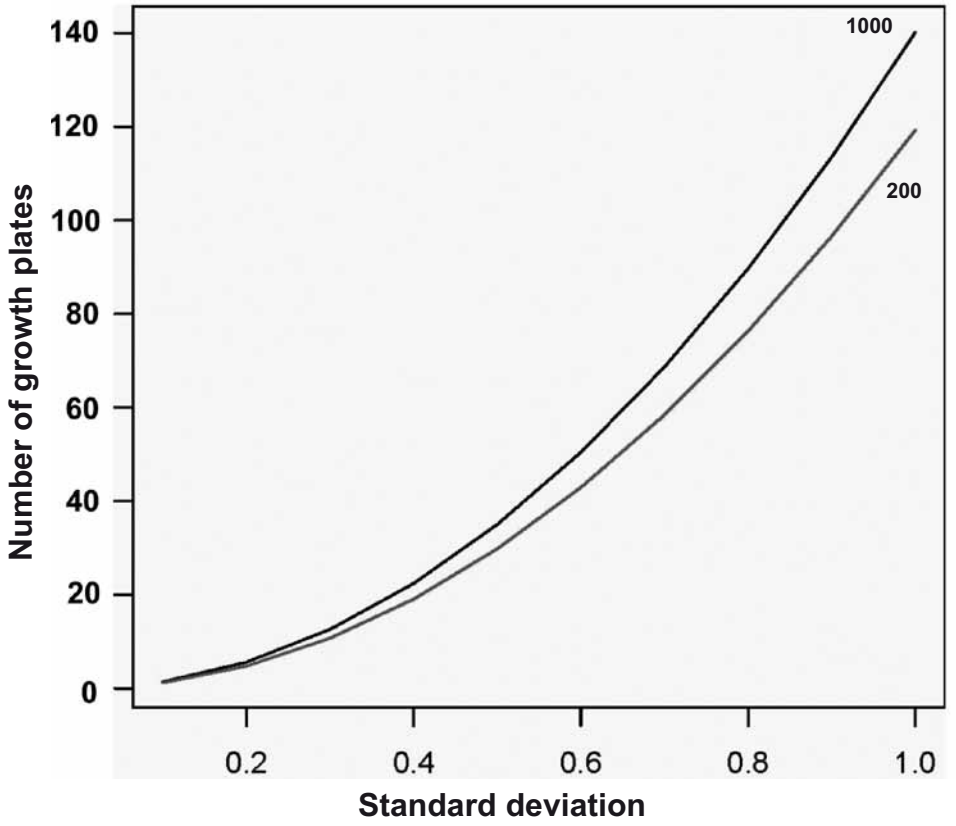

Figure 3: Required number of growth plates for different standard deviation scores.
One line is based on a selection of 200 genes and the other one for a selection of 1000 genes.

Tabel 3

	prepubertal. vs.early puberty	prepubertal.vs.late puberty	early.vs.late puberty
Regulation of autophagy	0.518	0.046	0.005
N-Glycan biosynthesis	0.879	0.403	0.006
RNA polymerase	0.767	0.195	0.016
Peptidoglycan biosynthesis	0.266	0.053	0.020
Lysine degradation	0.631	0.151	0.026
Biosynthesis of steroids	0.802	0.262	0.026
Terpenoid biosynthesis	0.749	0.358	0.028
Apoptosis	0.658	0.106	0.034
Complement + coagulation cascades	0.677	0.199	0.042
Fc epsilon RI signaling pathway	0.749	0.314	0.047
GnRH signaling pathway	0.715	0.168	0.049

Affected pathways and p-values for each comparison.

Discussion

A limited microarray analyses of 6 human growth plate samples at prepuberty, early puberty and advanced puberty revealed a large number of changes in gene expression with progression of puberty. Changes in expression levels were on average small and most alterations occurred during late stage of puberty. Furthermore, the variations in gene expression in between growth plate samples isolated from different patients at an identical stage of pubertal development were relatively large. Given the high impact of genetic composition on growth and final height this is not surprising.

A number of pathways changed with progression of puberty. Affected pathways were associated with extracellular matrix homeostasis, hormonal pathways and programmed cell death which have all been implicated in growth plate maturation. None of the identified changes reached significance when the statistical analyses were corrected for multiple testing. This was due to small sample size, large variation between specimens at the same developmental stage and overall small changes in gene expression levels.

The overall small change in gene expression levels in growth plate chondrocytes with progression of puberty was unexpected, since puberty is associated with dramatic changes in growth velocity which is mediated by the activity of growth plate chondrocytes. This is exemplified by the pubertal growth spurt, which accounts for approximately 17% of final height (3-5). In addition, puberty is related to a large increase in expression of hormones like sex steroids, Growth Hormone and IGF-I. These hormones have been postulated to regulate the changes in growth plate activity in part by exerting direct effects on growth plate chondrocytes. Of these hormones, estrogen demonstrates the most dramatic changes particularly in girls who were subject of the present study and is postulated to be the driving factor of the growth spurt, growth plate maturation and fusion. This might indicate that gene expression in the human growth plate is not as sensitive to circulating estrogen levels as was expected.

Estrogen accelerates the senescent decline (7). Senescence is a term for the structural and functional changes over time in the growth plate. Stem-like cells in the resting zone have a finite proliferative capacity, which is gradually exhausted resulting eventually in epiphyseal fusion (8;9). Based on this hypothesis, cells that are most sensitive to estrogen are growth plate stem cells. These cells are located in the resting zone of the growth plate adjacent to the epiphyseal bone and form a minority in the growth plate. By removing the bone from the cartilage by micro dissection, it is likely that many of the growth plate stem cells are removed as well. Likewise, to prevent contamination of the RNA prepartions with cells of the metaphyseal bone, large parts of the terminal hypertrophic cell layer will be sacrificed as well. Our growth plate RNA samples are therefore enriched in proliferating and early hypertrophic cells. If stem cells are indeed most responsive to estrogen by changes in gene expression it is likely that such an effect will be missed in our experiment. Micro dissection with a laser for example, which allows a more precise selection of cell types for RNA isolation than manual micro dissection, might be considered as an alternative approach. Our findings do not support nor contradict the senescence hypothesis. A suggested biological marker for growth plate senescence is loss of DNA methylation and this cannot be detected by gene expression microarray analysis (10).

Although the analysis of the microarray data did not reveal findings that could withstand correction for multiple testing, it was remarkable that genes changing over time could be mapped to pathways like ‘regulation of autophagy’ and ‘apoptosis’. Chondrocyte death is presumed to be a characteristic of growth plate maturation and fusion. In a previous study we studied apoptosis in a collection of growth plate tissues together with a unique growth plate specimen in the process

of epiphyseal fusion (11). In this study we were not able to detect any signs of autophagy and/or classical apoptosis with pubertal maturation morphologically nor at the protein level. We did observe early signs of necrosis and signs of hypoxia. The results from this microarray study are indicative for more cell death markers in late stages of puberty. Results do not identify the exact mechanism since both pathways contain many genes, and also overlapping genes, suggestive for some sort of programmed cell death which might be a combination of autophagy, apoptosis and perhaps a third kind of cell death like necrosis.

The exact mechanism through which chondrocytes disappear during epiphyseal fusion in humans still needs to be clarified. By increasing the power of the analysis by including new growth plate specimens, microarray analysis may shed more light on the process of chondrocyte death during the advanced stages of puberty. Alterations in genes associated with hormonal pathways are in line with a rat study, reporting an increase in sex steroid synthesis in sexually maturing rats (12).

A number of growth plates in our collection were derived from pathological conditions. We believe that it is a safe assumption that the underlying mechanism of epiphyseal maturation and fusion is the same for all growth plates regardless of the underlying disease of each patient since eventually longitudinal growth stops in all patients by the end of puberty and all patients undergo growth plate maturation as a function of puberty.

Power calculations showed that 5 growth plates in each group are needed to detect a fold change of 1.5 with an overall variance of 0.2. Unfortunately, we did not have that many growth plates derived from female tibias. Differences in genetic background were omitted in the longitudinal analysis of the 2 growth plate samples from one patient as described in chapter 6. Comparing data from this longitudinal analysis with the present cross-sectional study did not show a perfect match in respect to individual genes. However, results of pathway analysis are more in agreement with the longitudinal study. Both analyses showed changes in pathways associated with the extracellular matrix and programmed cell death. In addition, the longitudinal study confirmed extracellular matrix changes on protein level.

Our microarray results are promising and contain a great amount of data; however more samples are needed to validate our findings. With a larger collection, results could serve as a database and clarify multiple research questions involving the growth plate. In addition, we now have a large collection of paraffin embedded tissues on which studies can be performed on protein expression.

In conclusion, for the first time we show a developmental regulation of the gene expression pattern in the human epiphyseal growth plate. Most alterations occurred during late puberty suggesting that the affected pathways may be involved in the regulation of human epiphyseal growth plate maturation and fusion. However, more future studies are needed to validate our findings.

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