

**Title: MOLECULAR REGULATION OF THE GROWTH PLATE: Genome-wide expression analysis of human growth plate chondrocytes**

**Research groups:**

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**Background of the project**

It has become increasingly clear that frequently used *in vivo* and *in vitro* animal models are insufficient to obtain a complete understanding of the processes that take place in the human epiphyseal growth plate, the main organ responsible for longitudinal growth. Therefore, studies using human growth plate specimens and human models for chondrocyte differentiation and maturation are needed. In the past years, each of the participating research groups have invested in the collection of human growth plate material and in the development of *in vitro* models for chondrocyte differentiation based on culturing of human Mesenchymal Stem Cells (MSCs), primary chondrocytes and organ cultures of human growth plate slices. Our complementary expertise, common interest in growth plate regulation and recent technological developments have formed the basis for the ESPE-research unit application. The main objectives of the research proposal were obtaining a genome wide overview of gene expression patterns in the human growth plate which can be used to unravel various/multiple hypothesis regarding growth plate regulation and pubertal maturation.

**Summary of obtained results in 2010**

Within our growth plate collection we had the unique opportunity to obtain two epiphyseal samples of one female patient at different pubertal stages (Tanner stage B2 and Tanner stage B3). Microarray analyses of these two samples were used to receive a first insight in the molecular processes and interactions occurring during human growth plate maturation, coinciding with increasing serum estrogen concentrations.

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, 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 programmed 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, Androgen Receptor, ELK1, STAT5B, CREB 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. Moreover, we show that some of these sites are direct target genes of these transcription factors as shown by ChIP assays.

In conclusion, 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. Evolutionary conservation of binding sites provides evidence for a direct role for GH, IGF-I and RUNX2 in growth plate maturation. Results from this study were published in the *Journal of Endocrinology* (feb 2011).

In order to extend our microarray analyses in relation to pubertal growth plate maturation we collected additional human growth plate tissues. Growth plate tissues were obtained from patients who were undergoing surgery for a variety of disorders. Even though patients suffered from diverse disorders, we assume that the underlying mechanism of epiphyseal maturation and fusion is the same for all growth plates. Eventually longitudinal growth stops in all patients at the end of puberty. A cross-sectional microarray analysis was performed on 6 female tibial growth plates of various pubertal stages. Results suggested that many genes change from prepuberty to early puberty, however most alterations occurred during late puberty. Overall changes in gene expression were very small and only few genes changed more than 2 fold with progression of puberty. This shows that there is no large change in expression patterns with progression of puberty and that there rather might be an effect of multiple small factors. Affected pathways were associated with extracellular matrix homeostasis, hormonal pathways and programmed cell death. These findings in affected pathways are in agreement with the observed results in the previous study with the two growth plates of the single patient. However gene profiles were not fully overlapping and sets of other genes were at times in contrast as well.

In addition, we investigated a promising human model for the growth plate, human mesenchymal stem cells (hMSCs). hMSCs are multipotent and can differentiate into the chondrogenic lineage. The cartilage forming capacity of fetal hMSCs was studied in detail in order to explore the molecular processes and interactions taking place during early phases of chondrogenesis, chondrocyte proliferation and chondrocyte differentiation. To determine which type of hyaline cartilage is formed by differentiating hMSCs, we compared the gene expression profile with previously established gene expression fingerprints of human articular and epiphyseal growth plate cartilage. As differentiation towards chondrocytes proceeded, hMSCs gradually obtained a gene expression profile that was more overlapping with the fingerprint of epiphyseal growth plate cartilage than of articular cartilage. This study validates differentiating fetal bone marrow-derived hMSCs as an excellent model for the epiphyseal growth plate. This new human model now opens the opportunity to elucidate clinical conditions influencing chondrogenesis and cartilage homeostasis in more detail as well as the opportunity to develop strategies for new treatment options for cartilage disorders. Results from this study are summarized in a paper which has been submitted for publication.

The Stockholm group showed to be capable of culturing growth plate specimens *in vitro*. The chondrocytes were viable and had good cell morphology after 3 days of culture. Cultured growth plate samples were treated for subsequently 4hrs or 24hrs with estradiol followed by RNA extraction. Microarray analysis was performed on these samples. Changes in gene expression after estrogen treatment were very small and statistical significance was lost after correction for multiple testing. We have recently collected growth plate cartilage from 3 additional patients and plan to collect tissues from at least another 2 patients. We will then repeat the analysis to reveal if estradiol anyway may significantly affect the signaling pathways involved in the estrogenic response.

Two PhD students within the Leiden group finished their PhD thesis:.

- 1) Joyce AM Emons. Thesis: Regulators of growth plate maturation. Degree April 14<sup>th</sup> 2010. The growth plates obtained in our project were the origin of most studies in this thesis.
- 2) Sandy A. van Gool. Thesis: Regulation and modulation of growth. Insights from human and animal studies. Degree May 18<sup>th</sup>, 2011.

### **Future experiments**

A large data set of microarray results from the longitudinal growth plate study were sent to the Stockholm group together with extracted RNA and converted cDNA. This material can be used as a growth plate expression databank and will be used for validation of various experiments. At Twente University we have used this material for making a detailed comparison between the gene expression profiles of human growth plate cartilage and articular cartilage from the same patient. This has resulted in the identification of highly specific markers for articular cartilage that can be used to distinguish both types of hyaline cartilage. A manuscript reporting the results of this study is in preparation. The methodology developed to culture human growth plate tissue samples has now been applied to several research projects in Stockholm and 2 manuscripts are in preparation.