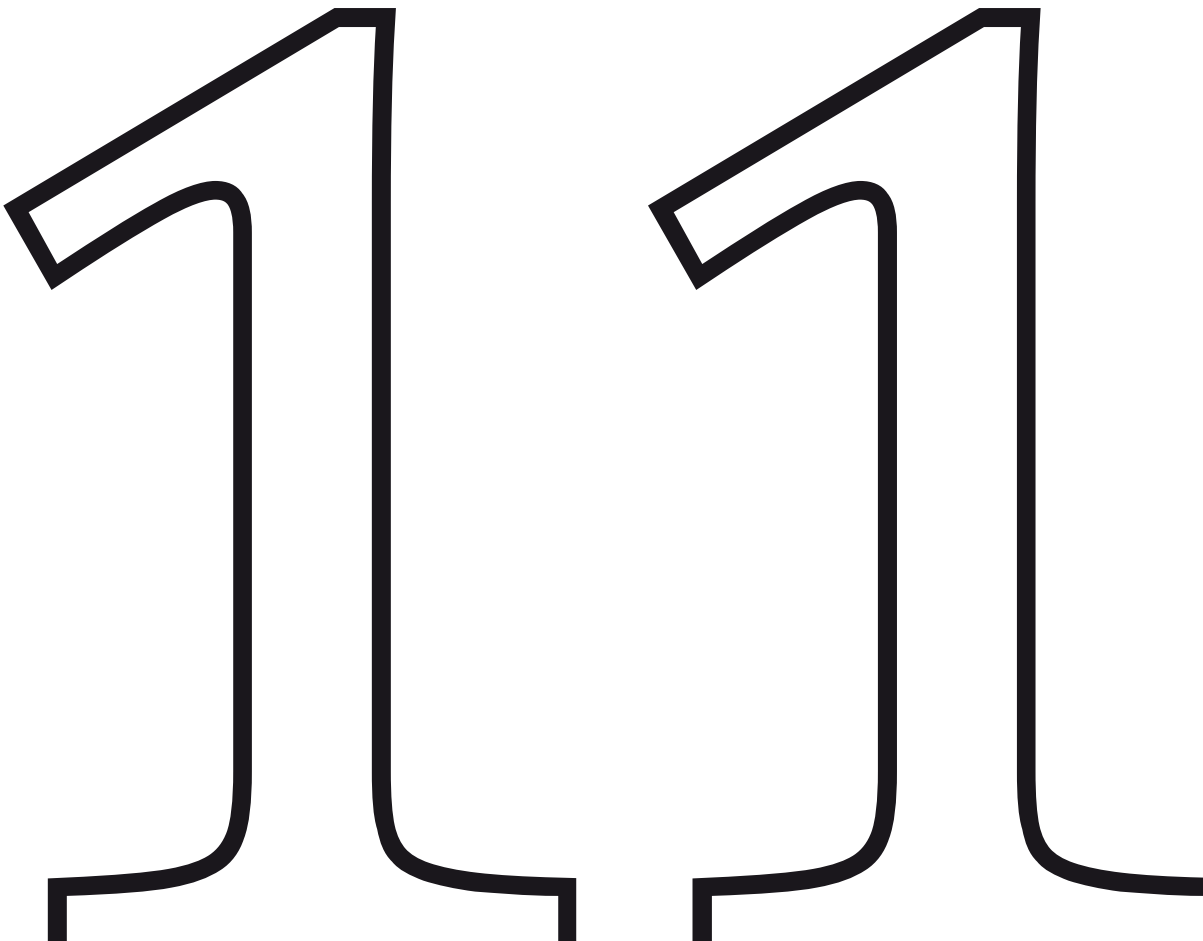
Regulators of growth plate maturation

Emons, J.A.M.



General discussion

General Discussion

Longitudinal bone growth occurs throughout childhood and is the key characteristic that distinguishes children from adults. During puberty longitudinal growth rate first increases, but finally as the growth plate further matures growth decreases and eventually ceases with epiphyseal fusion at the end of puberty. Mechanisms underlying growth plate maturation and epiphyseal fusion with termination of longitudinal growth are still largely unknown. With puberty estrogen levels increase and this is supposed to play an important role in growth plate maturation and epiphyseal fusion. In this thesis we investigated:

1. The route and effects of estrogens (17β-estradiol or/and SERMs) on growth plate structure and VEGF expression.

2. Maturation of the growth plate: regarding proliferation and the cell cycle, the delayed growth plate senescence theory, pubertal changes and epiphyseal fusion.

3. A relatively new human growth plate model using mesenchymal stem cells.

Actions of estrogens and selective estrogen receptor modulators

Estrogens play a key role in longitudinal bone growth through growth plate maturation, epiphyseal fusion and augmentation of accrual of bone. In the human lack of estrogen action leads to continuation of growth and subsequently tall stature by absence of growth plate fusion (1;2). Conversely high levels of estrogen inhibit longitudinal bone growth and can clinically be used to prevent tall stature, but treatment is associated with side-effects (3). Therefore it is important to investigate the actions of estrogens in more detail.
In the sexually immature rat we observed an increase in body weight gain, tibia length and width of the growth plate with an increased proliferating zone after removal of estrogens by ovariectomy. Suppletion of estrogen showed the reverse effects in line with the clinical observations in humans. The effects of estrogen have been suggested to be mediated mainly through the nuclear receptors ERα and ERβ, which are both expressed in growth plate chondrocytes of several species, including the human (4-7). We detected these estrogen receptors in the rat predominantly in late proliferating and early hypertrophic chondrocytes and observed a decrease in ERα and ERβ staining with estrogen suppletion in chapter 2.
Estrogen can exert its effects through a genomic or non-genomic pathway, however the contribution of these routes to longitudinal growth has not been clarified up to now. Desoxyestrone administration, a synthetic compound that exclusively acts through the non-genomic pathway, resulted in a decrease in longitudinal growth although to a smaller extent than 17β-estradiol. This indicates that estrogenic effects on longitudinal growth are regulated both through genomic and nongenomic pathways with genomic signalling prevailing.
Estrogen acts on a number of diverse tissues resulting in potential side-effects of estrogen treatment (3). Selective estrogen receptor modulators (SERMs) were developed to circumvent these side effects (8). 2-methoxyestradiol was suggested to be tissue-selective in the growth plate of female rats (9). We administered 2-methoxyestradiol to sexually immature rats and indeed found a decreased thickness of the proliferative zone in female rats and a decrease in the thickness of the total growth plate and of the hypertrophic zone in the male rat, but no general

effect on longitudinal growth was observed. Sibonga et al did find an effect on longitudinal bone growth when 2-methoxyestradiol was administered orally to older (10 weeks) and more mature rats (10). Therefore we conclude that 2-methoxyestradiol indeed has a growth plate selective estrogenic effect. However, more studies in animal and human models are needed to optimize the effects on longitudinal bone growth and to assess potential side-effects before clinical use can be considered. Recent studies have assigned 2-methoxyestradiol as a promising anticancer agent in the treatment of cancer, since it induces apoptosis in cancer cells (11;12). Nowadays more extensively investigated SERMs in relation to skeletal growth are raloxifene and tamoxifen. Both compounds act as estrogen agonists and impair longitudinal bone growth (13-15).
Estrogens can have a direct effect, but also an indirect effect on growth by stimulating the GH-IGF-I axis resulting in an increase of IGF-I levels (16;17). In our estrogen treated rats we were unable to measure circulating IGF-1 levels, but we did investigate local IGF-1 expression. We did not observe an effect on IGF-1 expression in the growth plate.

VEGF expression in the growth plate

Estrogen and its receptors interact with various growth factors in order to control longitudinal growth (16-19). One of these growth factors is Vascular Endothelial Growth Factor (VEGF). It was previously shown that estrogen up-regulated VEGF expression in uterus and bone tissue (20;21). In chapter 3 we demonstrate that VEGF expression in chondrocytes was elevated by estrogen treatment *in vivo* and *in vitro*. Removal of estrogens by ovariectomy resulted in a decrease in VEGF expression. Moreover, pubertal human growth plate maturation resulted in an increase in VEGF expression. From these observations we concluded that estrogens regulate VEGF expression in the growth plate.
Vascular Endothelial Growth Factor (VEGF) is important in the final event of endochondral ossification, as it has a function in chondrocyte differentiation and chondrocyte survival (22-24). Inhibition of VEGF resulted in dramatic effects on longitudinal growth and the growth plate in previous studies (22;25). At the end of puberty longitudinal growth ceases with total replacement of avascular cartilage by highly vascularized bone eventually resulting in epiphyseal fusion. VEGF could play an important role in this process. Our results are in line with this hypothesis, since estrogen and pubertal maturation both upregulated VEGF expression in growth plate chondrocytes suggesting a potential role for VEGF in estrogen-induced growth plate fusion. However, this suggested role needs to be confirmed in future experiments.

Growth plate senescence, proliferation and p27

One of the postulated hypotheses for epiphyseal fusion is that estrogen accelerates the senescent decline and that the growth plate fuses when senescence reaches a critical point (26). Senescence is a term for structural changes like a decrease in height of different zones in the growth plate, but also for the decline in chondrocyte proliferation (26;27). Senescence might influence proliferation and the cell cycle of chondrocytes by orderly inactivation and activation of cyclin-dependent kinases and kinase inhibitors like p27Kip1 (p27). p27-deficient mice have an increased size of long bones and weight compared to wild-type mice, suggestive for a functional role for p27 in the regulation of longitudinal growth (28-30). In chapter 4 we detected p27 mRNA in the growth plates of 5-wk-old mice by real-time PCR. The p27 mRNA levels in growth plate tissues were

approximately 2-fold lower than levels in the surrounding bone tissue. No significant difference was detected in p27 expression in the separate zones. Previous studies suggested a role for p27 in the differentiation of terminal growth plate chondrocytes. However, our results are in contrast with this since no elevated expression was seen in the hypertrophic zone (28;31;32). p27 ablation modestly increased chondrocyte proliferation in the growth plate and body length of 7 weeks old mice, however tibia length was not significantly greater than in controls. Presumably the increase in total length was a result of greater growth in the vertebrae and the increase in proliferation may have started shortly before sacrifice. The latter is supported by observations made by others who reported an increase in body size later in life, therefore most likely the increase in proliferation was too short to result in an increase in length of long bones (29;33). Another explanation for the modest effect of p27 in our study might be a difference in genetic background. Previous studies used a different background and have reported a more prominent growth phenotype (coisogenic 129S4 mice).

Treatment with glucocorticoids inhibited longitudinal growth in p27-deficient mice and controls to the same extent, indicating that p27 is not required for the negative effects of dexamethasone on longitudinal growth. Our findings in chapter 4 suggest that p27 negatively modulates growth plate chondrocyte proliferation, but it is not required for the conditional regulation of chondrocyte proliferation as induced by dexamethasone in the growth plate of rats.

In chapter 7 we used microarray techniques on human growth plate tissues and detected a significant change in the cell cycle pathway with progression of puberty. 15 out of 84 genes in this pathway were affected and among these was p27. p27 showed an up-regulation in expression with pubertal maturation of the growth plate. This indicates that the cell cycle activity and thereby proliferation decreased in line with the senescence hypothesis in the human pubertal growth plate and the reduced chondrocyte proliferation during growth plate maturation.

The delayed growth plate senescence hypothesis and catch-up growth

After a period of growth inhibition, the linear growth rate usually exceeds the normal range. This phenomenon is known as catch-up growth (34). Evidence from animal studies suggests that catch-up growth is due, at least in large part, to a delay in growth plate senescence. However, the relationship between catch-up growth and delayed growth plate senescence has only been studied in rabbits and rats (27;35). In chapter 5 we re-evaluated height and bone age measurements in patients with celiac disease before and on gluten-free diet. On gluten-free diet these patients experienced catch-up growth. We concluded that the pattern of catch-up growth in patients with celiac disease is consistent with the hypothesis of delayed growth plate senescence. We are the first showing human data in line with this hypothesis, however we cannot exclude that additional mechanisms like for example microRNAs and changes in gene expression may have contributed to the increase in height velocity as well.

Pubertal growth plate maturation

We had the unique opportunity to obtain two epiphyseal samples of one patient in different stages of puberty. 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 in chapter 6. Histological experiments and measurements showed a clear decrease in width of the total

growth plate, more widely spaced columns with less cells and consequently more extracellular matrix in the more mature growth plate. This confirmed earlier observations in rabbits and rats (36;37). Our microarray results were in line with our morphological data obtained with histology, showing many genes and pathways related to the extracellular matrix significantly affected with maturation. Progression of puberty did affect many genes in the cell cycle pathway.

In addition, we have performed a detailed bioinformatic analysis on these two growth plate specimens. More specifically, we searched the promoter regions of genes that are differentially expressed in the two growth plate specimens with pubertal maturation for evidence of direct effects of estrogen, androgen, GH, IGF-I and PTHrP on their expression and analyzed these genes for their evolutionary conservation across 9 primates. Sequences evolutionary conserved along species are likely to have critical functional roles (38). This data provides for the first time evidence that Estrogen receptor, androgen receptor, STAT5B, ELK-1 and RUNX2 were found to have transcription factor binding motifs in genes involved in growth plate maturation, suggestive for a role of these transcription factors in pubertal maturation of the human growth plate.

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. In chapter 7 a cross-sectional microarray analysis was performed on 6 female tibial growth plates of various pubertal stages. Results suggested many genes changing 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. The fact that we did not observe large changes is also in line with the senescence theory. This theory suggests that stem-like cells in the resting zone have a finite proliferative capacity, which is gradually exhausted resulting eventually in epiphyseal fusion (27;39). Loss of DNA methylation is suggested to be a biological marker for growth plate senescence and this can not be detected by microarray analysis (40).

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 longitudinal study in chapter 6, however gene profiles were not fully overlapping and sets of other genes were at times in contrast as well.

Epiphyseal fusion

The exact mechanism by which epiphyseal fusion occurs is not yet completely understood. In chapter 8 we investigated apoptosis as the final mechanism by which chondrocytes eventually die and are replaced by bone. At the chondro-osseous junction site of the growth plate, apoptosis is nowadays generally accepted as the mechanism by which terminally hypertrophic chondrocytes die (41;42). It is generally assumed that the same mechanism eventually also results in epiphyseal fusion. We performed a detailed study on apoptosis in terminal hypertrophic chondrocytes in the pubertal female growth plate and found no signs of classical apoptosis. In addition, we studied a unique tissue specimen of a late pubertal human growth plate in the process of epiphyseal fusion and found clear evidence that apoptosis is not likely to be involved in the end phase of growth plate fusion. We did observe a dense border of thick bone surrounding the growth plate remnant, signs

of hypoxia and early necrosis in this fusing growth plate. We hypothesize that the border of dense bone is functioning as a physical barrier for oxygen and nutrients to reach the fusing growth plate resulting in hypoxia and eventually cell death in a non-classical apoptotic way through necrosis or a mixture of apoptosis and necrosis. In line with this new hypothesis White et al. recently demonstrated bridging bone in the center of a distal human tibial growth plate obtained from a 12.9 years old girl which might be an early sign of this shelling process (43).
Interestingly, in chapters 6 and 7 we found a significant increase in expression of the hypoxia-inducible factor 2 alpha gene from prepuberty to early and late stage puberty. The HIF-1alpha gene was also found to be expressed, but no change was seen with maturation. These findings are in line with Stewart et al, who reported an up-regulated expression of HIF-2alpha mRNA during chondrocytes differentiation in vitro, but no change in HIF-1alpha mRNA expression (44). Hif-2alpha knockout mice are small, what might indicate that this gene has an important role in the growth plate and subsequently in the regulation of longitudinal growth (45).
Therefore we believe it could be a hypoxia related process leading eventually to cell death of growth plate chondrocytes. Indeed with our microarray experiments in chapters 6 and 7 we did find many affected genes changing with pubertal maturation that are involved in programmed cell death, e.g. proapoptotic and anti-apoptotic genes, but also genes involved in the regulation of autophagy. However, histological and electron microscopy analyses thus far showed no signs of classical apoptosis and no autophagosomes in the fusing growth plate in chapter 8. Apoptosis and autophagy are closely related and there is an overlap in signaling proteins (46;47). The results of our studies are in line with this and suggestive for a non-classical and perhaps intermediate mechanism of different types of cell death.

Growth plate models

Most of our knowledge regarding the regulation of the growth plate is based on animal studies. However most animal models only partially correspond to the human situation and many species differences are present. In mouse and rat for example, in contrast to humans, growth plates do not fuse under the influence of estrogen at the end of puberty (48;49). This indicates that rodents are not perfectly representative for studies on growth plate fusion, excluding the use of transgenic approaches.
In chapter 9 we investigated a promising relatively new human model for the growth plate, human mesenchymal stem cells (hMSCs). hMSCs are multipotent and can differentiate into the chondrogenic lineage. Originally MSCs were isolated from bone marrow, however nowadays they can be obtained from various tissue sources, like fetal tissues, placenta, umbilical cord blood and adipose tissue (35;50-54). In chapter 9 we investigated the chondrogenic potential of hMSCs originating from various tissues. Bone marrow derived MSCs appeared to have the best chondrogenic potential, with fetal bone marrow prevailing over adult bone marrow. A passage-dependent decrease in the capacity of cartilage formation was seen in line with previous results in adult MSCs (55).
In chapter 10 we studied in detail the cartilage forming capacity of fetal hMSCs in order to explore in more detail 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.

Final remarks and suggestions for future studies

Patients with growth disorders are frequently presented to pediatricians. The etiology can only be established in a minority of these patients and even in such cases a causal treatment is usually not available. A better understanding of the mechanisms by which longitudinal growth is regulated at the level of the epiphyseal plate is therefore needed. We focused in our studies on the effects of estrogen and growth plate maturation and found interesting new clues for further study in order to design new treatment options.

17β-estradiol treatment is used from the mid-fifties to decrease final height, however there are several potential side-effects like an increased risk for breast and uterus cancer and decreased fertility (3). We showed a growth plate selective estrogenic effect in rats with 2-methoxyestradiol treatment. This could be a potential new treatment option, however more detailed studies on longitudinal bone growth and potential side-effects of 2-methoxyestradiol treatment in the human are needed.

Adult height is achieved at the end of puberty after the growth plate has fused and there is no longitudinal growth potential anymore. The process of epiphyseal fusion in the human is still largely unknown and modulation of this process could potentially lead to new treatment strategies. Delaying epiphyseal fusion is expected to result in an increase in adult height by allowing more time for growth supporting treatments. On the contrary, initiating epiphyseal fusion would help in the treatment of tall stature. In our studies we have shown that estrogen increases VEGF expression in growth plate chondrocytes and suggested that VEGF plays an important role in estrogen-induced growth plate fusion.

We showed that apoptosis is not likely to be involved in the end phase of growth plate fusion, however we did detect signs of hypoxia in the fusing growth plate. Therefore we present a new hypothesis in which hypoxia plays a more important role in growth plate maturation and epiphyseal fusion. Both our morphological and microarray experiments are in line with this assumption. We hypothesize that hypoxia increases during maturation of the growth plate. At the end of puberty a border of dense bone is formed that surrounds the growth plate functioning as a physical barrier for oxygen and nutrients resulting in more hypoxia and eventually cell death in a non-classical apoptotic way. Increasing levels of VEGF might be a result of this process as well, since many studies reported an increase in VEGF expression by hypoxia (56-58). This model needs further investigations. It would be interesting to study Hif-1alpha and Hif-2alpha protein expression in our human growth plate collection. Moreover it would be useful to extend our collection of growth plates for investigations regarding maturation of the growth plate and the proposed hypotheses in more detail. 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.

Without a large collection of human growth plates other methods and models are needed, like MSCs. We recommend using MSCs derived from human fetal bone marrow. These MSCs showed to

be a good model for the human growth plate and have the advantage that they can be genetically modified. Interesting experiments would be over-expression or down-silencing of certain growth factors and receptors like the estrogen-receptor. Ultimately this will help us in understanding in depth the molecular mechanisms that drive growth plate maturation and will help us to develop new strategies for the treatment of cartilage and growth disorders.

References

1. **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

2. **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

3. **Venn A, Bruinsma F, Werther G, Pyett P, Baird D, Jones P, Rayner J, Lumley J** 2004 Oestrogen treatment to reduce the adult height of tall girls: long-term effects on fertility. Lancet 364:1513-1518

4. **Kennedy J, Baris C, Hoyland JA, Selby PL, Freemont AJ, Braidman IP** 1999 Immunofluorescent localization of estrogen receptor-alpha in growth plates of rabbits, but not in rats, at sexual maturity. Bone 24:9-16

5. **Kusec V, Virdi AS, Prince R, Triffitt JT** 1998 Localization of estrogen receptor-alpha in human and rabbit skeletal tissues. J Clin Endocrinol Metab 83:2421-2428

6. **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

7. **van der Eerden BC, Gevers EF, Lowik CW, Karperien M, Wit JM** 2002 Expression of estrogen receptor alpha and beta in the epiphyseal plate of the rat. Bone 30:478-485

8. **McDonnell DP** 1999 The Molecular Pharmacology of SERMs. Trends Endocrinol Metab 10:301-311

9. **Turner RT, Evans GL** 2000 2-Methoxyestradiol inhibits longitudinal bone growth in normal female rats. Calcif Tissue Int 66:465-469

10. **Sibonga JD, Sommer U, Turner RT** 2002 Evidence that 2-methoxyestradiol suppresses proliferation and accelerates apoptosis in normal rat growth plate chondrocytes. J Cancer Res Clin Oncol 128:477-483

11. **Batsi C, Markopoulou S, Kontargiris E, Charalambous C, Thomas C, Christoforidis S, Kanavaros P, Constantinou AI, Marcu KB, Kolettas E** 2009 Bcl-2 blocks 2-methoxyestradiol induced leukemia cell apoptosis by a p27(Kip1)-dependent G1/S cell cycle arrest in conjunction with NF-kappaB activation. Biochem Pharmacol 78:33-44

12. **Sutherland TE, Anderson RL, Hughes RA, Altmann E, Schuliga M, Ziogas J, Stewart AG** 2007 2-Methoxyestradiol--a unique blend of activities generating a new class of anti-tumour/anti-inflammatory agents. Drug Discov Today 12:577-584

13. **Karimian E, Chagin AS, Gjerde J, Heino T, Lien EA, Ohlsson C, Savendahl L** 2008 Tamoxifen impairs both longitudinal and cortical bone growth in young male rats. J Bone Miner Res 23:1267-1277

14. **Nilsson O, Falk J, Ritzen EM, Baron J, Savendahl L** 2003 Raloxifene acts as an estrogen agonist on the rabbit growth plate. Endocrinology 144:1481-1485

15. **Rey JR, Cervino EV, Rentero ML, Crespo EC, Alvaro AO, Casillas M** 2009 Raloxifene: mechanism of action, effects on bone tissue, and applicability in clinical traumatology practice. Open Orthop J 3:14-21

16. **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

17. **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

18. **Aronica SM, Katzenellenbogen BS** 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol Endocrinol 7:743-752

19. **Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS** 1992 Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. Proc Natl Acad Sci U S A 89:4658-4662

20. **Kazi AA, Jones JM, Koos RD** 2005 Chromatin immunoprecipitation analysis of gene expression in the rat uterus in vivo: estrogen-induced recruitment of both estrogen receptor alpha and hypoxia-inducible factor 1 to the vascular endothelial growth factor promoter. Mol Endocrinol 19:2006-2019

21. **Mekraldi S, Lafage-Proust MH, Bloomfield S, Alexandre C, Vico L** 2003 Changes in vasoactive factors associated with altered vessel morphology in the tibial metaphysis during ovariectomy-induced bone loss in rats. Bone 32:630-641

22. **Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR** 2004 VEGFA is necessary for chondrocyte survival during bone development. Development 131:2161-2171

23. **Zelzer E, Olsen BR** 2005 Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 65:169-187

24. **Dai J, Rabie AB** 2007 VEGF: an essential mediator of both angiogenesis and endochondral ossification. J Dent Res 86:937-950

25. **Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N** 1999 VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5:623-628

26. **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

27. **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

28. **Drissi H, Hushka D, Aslam F, Nguyen Q, Buffone E, Koff A, van WA, Lian JB, Stein JL, Stein GS** 1999 The cell cycle regulator p27kip1 contributes to growth and differentiation of osteoblasts. Cancer Res 59:3705-3711

29. **Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday AC, Frohman LA, Koff A** 1996 Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell 85:721-732

30. **Teixeira LT, Kiyokawa H, Peng XD, Christov KT, Frohman LA, Kineman RD** 2000 p27Kip1-deficient mice exhibit accelerated growth hormone-releasing hormone (GHRH)-induced somatotrope proliferation and adenoma formation. Oncogene 19:1875-1884

31. **Ballock RT, Zhou X, Mink LM, Chen DH, Mita BC, Stewart MC** 2000 Expression of cyclin-dependent kinase inhibitors in epiphyseal chondrocytes induced to terminally differentiate with thyroid hormone. Endocrinology 141:4552-4557

32. **Beier F, Taylor AC, LuValle P** 1999 The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. J Biol Chem 274:30273-30279

33. **Lin J, la-Fera MA, Li C, Page K, Choi YH, Hartzell DL, Baile CA** 2003 P27 knockout mice: reduced myostatin in muscle and altered adipogenesis. Biochem Biophys Res Commun 300:938-942

34. **Boersma B, Wit JM** 1997 Catch-up growth. Endocr Rev 18:646-661

35. **Marino R, Hegde A, Barnes KM, Schrier L, Emons JA, Nilsson O, Baron J** 2008 Catch-up growth after hypothyroidism is caused by delayed growth plate senescence. Endocrinology 149:1820-1828

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

37. **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

38. **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

39. **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

40. **Nilsson O, Mitchum RD, Jr., Schrier L, Ferns SP, Barnes KM, Troendle JF, Baron J** 2005 Growth plate senescence is associated with loss of DNA methylation. J Endocrinol 186:241-249

41. **Hunziker EB** 1994 Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 28:505-519

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

43. **White JR, Wilsman NJ, Leiferman EM, Noonan KJ** 2008 Histomorphometric analysis of an adolescent distal tibial physis prior to growth plate closure. J Child Orthop 2:315-319

44. **Stewart AJ, Houston B, Farquharson C** 2006 Elevated expression of hypoxia inducible factor-2alpha in terminally differentiating growth plate chondrocytes. J Cell Physiol 206:435-440

45. **Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA** 2003 Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet 35:331-340

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

47. **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

48. **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

49. **van der Eerden BC, Gevers EF, Lowik CW, Karperien M, Wit JM** 2002 Expression of estrogen receptor alpha and beta in the epiphyseal plate of the rat. Bone 30:478-485

50. **Bieback K, Kern S, Kluter H, Eichler H** 2004 Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625-634

51. **Friedenstein AJ** 1980 Stromal mechanisms of bone marrow: cloning in vitro and retransplantation in vivo. Haematol Blood Transfus 25:19-29

52. **Im GI, Shin YW, Lee KB** 2005 Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis Cartilage 13:845-853

53. **in 't Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, Beekhuizen W, Willemze R, Kanhai HH, Fibbe WE** 2003 Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. Haematologica 88:845-852

54. **in 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH** 2004 Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338-1345

55. **Sekiya I, Colter DC, Prockop DJ** 2001 BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun 284:411-418

56. **Cramer T, Schipani E, Johnson RS, Swoboda B, Pfander D** 2004 Expression of VEGF isoforms by epiphyseal chondrocytes during low-oxygen tension is HIF-1 alpha dependent. Osteoarthritis and Cartilage 12:433-439

57. **Kim HK, Bian H, ya-Ay J, Garces A, Morgan EF, Gilbert SR** 2009 Hypoxia and HIF-1alpha expression in the epiphyseal cartilage following ischemic injury to the immature femoral head. Bone

58. **Nishida T, Kondo S, Maeda A, Kubota S, Lyons KM, Takigawa M** 2009 CCN family 2/ connective tissue growth factor (CCN2/CTGF) regulates the expression of Vegf through Hif-1alpha expression in a chondrocytic cell line, HCS-2/8, under hypoxic condition. Bone 44:24-31