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Antler development and coupled osteoporosis in the skeleton of red deer Cervus elaphus: expression dynamics for regulatory and effector genes

Theses of PhD dissertation

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Introduction

An extremely intensive form of skeletal osteoporosis, termed cyclic physiological osteoporosis developed in deer stag. It is associated with antlerogenesis where the most robust bone development of the animal kingdom takes place. The pair of these two contrasting processes, antler ossification and skeletal osteoporosis, contributes to the reproductive success of the individual. Antlers are male secondary sexual characteristics and used for display and in combat. They are bony appendages of the deer's head, which are cast and re-grown each year. The development of antlers is a modified endochondral ossification process. After casting, antler re-growth is initiated at the distal rim of the pedicle, where cells de-differentiate into embryonic stem cells. These mesenchymal cells sequentially proliferate and differentiate into chondroblasts and chondrocytes associated with the formation of cartilage. The transition from undifferentiated cells to chondrocytes is gradual. The longitudinal growth proceeds proximal to distal from the pedicle, while the differentiation points at the opposite direction, from the tip to the base. The matrix of the antler cartilage is biochemically similar to that of other hyaline cartilages, however, it is well vascularised, and ossified bony trabeculae are formed on the cartilage scaffold by mineral deposition. Calcification and ossification gradually occludes the blood supply of the antlers. When growth stops, transformation to bone is completed, the velvet skin is shed, the antler is polished and ready for the rutting season. (For a comprehensive overview see a recent review by. Since the growth rate of the antler may reach over 100 g per day between May and July, enormous bone mass - generally 7-9 kg, occasionally 13-15 kg, as reported from the Danube-Drava-Gemenc-Bilje National Park in Hungary and Croatia - develops within 100-120 days (the skeletal mass is estimated to be 15% of the live weight of the stags, about 30 kg in our case). The demand for mineral precursors surmounts the dietary intake by browsing. As a consequence, the gap is filled by mobilizing minerals from the skeleton, thus causing a temporary bone loss termed cyclic physiological osteoporosis. This phenomenon resembles human (pathological) osteoporosis both in its visual appearance and in its genetic background. Before the rutting season, during the fitness recovery period in July and August, the process is reversed and bone mineral density (BMD) is restored. Mineral resorption is the highest, reaching 23%, in the ribcage and after reaching maximum antler growth it falls to less than 3%.

Previously we identified a set of genes which are differentially expressed during the rapid but controlled tissue proliferation and another set which are involved in the skeletal physiological osteoporosis.

Main objectives

In this work we developed an antler specific cDNA microarray in order to compare gene expression levels in the tissues of the mineralizing velvet antler and the skeleton (ribs and vertebra) of deer stags. Our results demonstrate the robust expression of genes *col1A1, col1A2, col3A1, ibsp, mgp, sparc,* and *osteocalcin* in the ossified part of the velvet antler. Results also suggest that overexpression of the transcription factor genes *runx2* and *osx* are key elements in the upregulation of the above genes. We attempt to integrate the gene expression data and GC-MS metabolite analyses to explain the bone mineral "traffic" between the antler and the skeleton. We also draw the attention to the potential use of deer genes in human osteoporosis research.

Materials and methods

Collection of tissue samples of a red deer stag: Velvet antler and skeletal bone samples were collected from a red deer stag shot at the Deer Farm of Pannon Equestrian Academy, Bőszénfa, Hungary. Tissue collections were performed according to the Hungarian Animal Rights Law (243/1998, XII. 31). The time of tissue collection was during the period of active mineralization of the antler, in the beginning of June, when skeletal osteoporosis takes place. Deer fetuses were collected from hinds shot for selection purposes in late December and early January. Samples were stored under liquid nitrogen within 30 min after shooting.

Velvet antler samples: The velvet antler, grown for 90 days before the stag was shot in the beginning of June, was removed (kept in ice) and was dissected. Positions of soft tissue layers (reserve mesenchyme, precartilage, cartilage) of the growing antlers were determined as described by Molnar *et al*. 2007. The slices (~0.5 cm thick) containing the "pure" tissues were cut in two parts, one of them was fixed, sectioned, stained and used as a histological control, the other part was stored in liquid nitrogen and kept for mRNA preparations (Molnár *et al.*, 2007). Bone samples of the velvet antler were taken from the section between the developing trez and brow tines. It is worth mentioning that the stag (Stag1) was an 8-year-old capital individual.

Skeletal bone samples: Ribs and vertebras were removed from the killed Stag1, were extensively washed in PBS for eliminating blood and marrow contamination and then immediately frozen in liquid nitrogen. Both the inner part of this ribs and the body of

the vertebras displayed an extremely porotic and fragile bone structure in the stag having velvet antlers. In stags shot in the velvet shedding phase and in the late autumn dwell the bones were no more porotic, their BMD and bone structure was restored (see Borsy *et al.* 2009 and Steger *et al.* 2010).

Foetal cartilage samples: Foetal cartilages were collected with the help of the professional hunter employee of the farm, where the balance between males and females should be maintained, and sorting out is unavoidable. The ~4-month old fetuses were dissected, and the epiphyseal growth plates of long bone precursors were collected (Molnar *et al.* 2007).

Human bone tissue samples and Real-Time RT-PCR: Gene expression profiles in bone samples were determined in seven postmenopausal, unrelated, consecutive, Hungarian, Caucasian women suffering from age-related osteoporosis (PP group). The control group included ten bone tissue samples from postmenopausal non- osteoporotic, healthy women (PNP group). Sampling and measuring gene expressions were described in Borsy *et al.* 2009.

Total RNA purification from bone samples of red deer stags: Deer bone samples were cryogrinded under liquid nitrogen, then extracted with acidic phenol-chloroform. Total RNAs were precipitated from the aqueous phase with LiCl and dissolved in 50 µl RNase-free water following DNase treatment.

Construction and screening of lambda cDNA libraries: cDNA libraries were constructed from the three antler tissue zones using the SMART cDNA Library Construction Kit (Clontech).

"Antler" microarrays, probe preparation and hybridization: The SMART cDNA Library were converted to plasmid clones (SMART™ protocol) in the *Escherichia coli* BM25.8. After colony PCR amplification 3200 cDNAs were selected randomly for microarray construction. cDNA inserts from the lambda cDNA libraries generated from foetal growth plate, from velvet antler tissues mesenchyme, precartilage, cartilage were amplified by PCR, purified with MultiScreen-PCR plate (Millipore, Billerica, MA, USA), resuspended in 50% dimethyl sulfoxide, and arrayed on FMB cDNA slides (Full Moon BioSystems, Sunnyvale, CA, USA). Scanning and data analysis were done as describe earlier (Gyurjan *et al*. 2007).

Northern blot analysis: Tissue samples (approximately 300 mg) were homogenized in guanidinium-thiocyanate and extracted with acidic phenol-chloroform. RNAs were precipitated from the aqueous phase with 3M LiCl and dissolved in 30 μl RNase-free water. RNA concentrations were determined by measuring the absorbance at 260 nm.

Five microgram aliquots of total RNA were separated by electrophoresis in 1.2% formaldehyde agarose gels. RNA was blotted to Hybond N+ filter (Amersham). Fifty nanograms of cDNA fragments were labeled with [α^{32}P] dATP using random hexamers and *E. coli* DNA Polymerase I Large (Klenow) Fragment. The hybridization was performed at 65°C in the Perfecthyb™ Plus buffer (Sigma). After hybridization, the filters were washed at the same temperature. The signals obtained were evaluated using a PhosphorImager™ and quantified with STORM™ imaging system (Molecular Dynamics) and by the GeneTools program from Syngene.

Affymetrix microarray analysis: Expressions of deer genes (antler bone versus vertebrae) were analyzed on *Bos taurus* 24K cDNA microarrays (GeneChip® Bovine Genome Array Affymetrix). (See details, Steger *et al.* 2010).

In situ hybridization: The velvet antler tip was dissected and fixed in 4% (w/v) paraformaldehyde in PBS. Blocks were embedded in low-melting-point paraffin (Paraplast, Sigma) and sectioned longitudinally. Sections were mounted on slides coated with (3-aminopropyl) triethoxy-silane. Sections were then dewaxed, rehydrated and digested with Proteinase K and acetic anhydride, and hybridized overnight. RNA probes were labeled with digoxigenin-UTP (Roche) by in vitro transcription of the T7 RNA polymerase (cDNA template was cloned in TriplEx2 vector in the antisense orientation behind T7 promoter). The hybridization temperatures chosen were specific for the probes. After hybridization, the sections were washed in 2xSSC, 50% formamide, 2xSSC, for 30 min each at the hybridization temperature. Then the sections were treated with RNase H for 30 min at 37°C and then washed twice in 0.2xSSC. Digoxigenin was detected using sheep anti-digoxigenin alkaline phosphatase Fab fragments (Roche), followed by an alkaline phosphatase reaction using nitrotetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrate. Sections were mounted using Kaiser's glycerin gelatine.

Extraction and analysis of metabolites by GC-MS: For GC-MS analysis, polar metabolite fractions were extracted from 60 mg of each frozen material. Sample preparation, derivatization and metabolite analysis: see details in Steger *et al.* 2010. Ribitol was added for internal standardisation. Samples were injected in splitless mode (1 µl/sample) and analysed in a quadrupole-type GC-MS system (Finnigan Trace/DSQ, Thermo Electron Corp.). The chromatograms and mass spectra were evaluated by using the XCALIBUR software (Thermo Electron Corp.) and the NIST 2.0 library.

Bioinformatics: All sequence manipulations were done in UNIX environment using standard BASH and PERL scripts. Promoter sequences were obtained from ENSEMBL and DOOP databases: see details in Steger *et al.* 2010. Motif searching and other sequence analysis tasks were carried out using the programs from the EMBOSS package.

Statistical Analyses: The differential expression of 15 human orthologs of identified deer genes (*col1A1, col1A2 col2A1, col3A1, col10A1, mgp, sparc, eno1, fabp3, serf2, anxa1, tmsb4x, tmsb10, oc/BGLAP, runx2*) in osteoporotic versus non-osteoporotic patients were detected with Relative Quantitative Real-Time RT-PCR analysis as described in Borsy et al 2009. Gene expression data were evaluated by multivariate statistical analyses according to Borsy *et al.* (2009), Principal Components Analysis (PCA) and Canonical Variates Analysis (CVA or discriminant analysis) using the SYNTAX 2000 program package of Podani 2001, see more details in Steger *et al.* 2010.

Results and Discussion

Regulation of bone metabolism in antlerogenesis: In this paper we aimed to get insight into the epigenetic regulation bone resorption and deposition in red deer stags during antlerogenesis. Changes in bone metabolism during this period are associated with two contrasting processes, the robust ossification in the developing velvet antler and the extremely intensive osteoporosis in the skeleton, which is termed cyclic physiological osteoporosis. We assumed that identification of genes which are related to the known histological characteristic of the antler cartilage, namely its intensive pre-osteogenesic calcification, brings us closer to the answer. Therefore we developed an “antler cDNA microarray” which allowed efficient identification of deer genes upregulated in the cartilagineous zones of the developing velvet antler (28 genes). The genes obtained belonged to different functional groups. In this work we paid particular attention to those genes which belong to a group which can be characterized as “bone reference marker or matrix genes”. We found that these genes are highly expressed already in the (antler) chondrocytes and chondroblasts, 2-10 fold over the foetal growth plate cartilage level. The expression patterns of these genes are in agreement with the contrast of the robust ossification of the antler versus the osteoporosis in the skeleton. Their expression level was much higher in the bony part of the velvet antler compared to the skeletal samples, ribs and vertebras. We calculated 7-17 fold higher *col1A1* expression in the antler bone over the skeletal level when using various internal controls for computing (i.e. 18S rRNA, 28S rRNA, *gapdh*) and two independent approaches (Northern blot

analysis combined with Syngene program, Affymetrix measurements). However, we note that the fold numbers must be interpreted with caution because the modified endrochondrial type bone development (i.e. antler) was compared here with the skeletal process. Even more robust expression differences were recorded for the other mineralization genes. It would be interesting to see to what extent these figures are adaptable for the female osteoporosis developing during gestation (for example, the severe sternal and iliac bone loss of the cow at calving has been known for long).

We assumed that the bone reference marker and matrix genes upregulated in the developing antler respond to the same transcriptional regulators, therefore by analyzing their promoter regions we can predict which transcription factors are involved in the regulation of mineralization. The high similarity of the deer and *Bos taurus* genes allowed PCR amplification of the promoter region of the deer *col1A1* gene. Sequence analysis of the amplified regulatory region unearthed a large number of the binding sites for the transcription factors Runx2 and Osx. We suggest that the genes *runx2* and *osx* function in the antler as master regulators of the antler upregulated genes. This view is supported by several lines of the experimental evidences, see details in Steger *et al.* 2010. (i) As mentioned above, the first intron and the 5' upstream sequence of the deer *col1A1* gene is rich in binding sites for Runx2 and Osx, alike in *Bos* and human. Moreover, their array and often their spacing are conserved (co-linear/commensurable) in the three species. (ii) The 175 bp long sequence spanning over the Osx binding site *sp1* in the *col1A1* first intron is fully conserved in the ruminant and the man, except two positions. This region contains the SNP site which serves as a diagnostic marker for osteoporosis-susceptibility in human, that is, the +1245G or T nucleotide position, characterizing the *S* and *s* alleles of the *sp1* site, is linked to bone density phenotypes (the *s/s* genotype is osteoporotic). (iii) The *osteocalcin* gene, the primary target of *runx2* regulation in mammalian model systems, is also upregulated in the antler bone samples. (iv) All the orthologs of the upregulated antler genes in *Bos* (and human) carry numerous Runx2 and Osx binding sites. Although the deer genomic sequences are not yet available, we assume that the promoters and cis-regulatory regions of the above deer genes show similar evolutionary conservation as we found in the case of *col1A1* (93%).

Antler tip: runx2 pathway: Runx2 is essential for human osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. We detected the expression of *runx2* as well as its downstream target *osx* in the mesenchyme of the antler tip, which indicated that these

cells are already committed toward chondrogenesis and osteogenesis. From the upstream signals, which play determinant role in initiating the positive autoregulation of the *runx2* gene, strong expression of PTHrP (Parathyroid hormone-related protein) has been demonstrated in the antler mesenchyme, precartilage and prechondrium (see details in Steger *et al.* 2010). A candidate for the upstream-most morphogene could be the calcitonin gene-related peptide (CGRP, see argumentation below). In the vascularized antler cartilage additional upregulating stimuli may also reach the cells by the blood stream, keeping the activity of the Runx2-Osx pathway high. Expression of the Runx2 activated genes encoding mineralization proteins (like *col1A1*) exhausts Ca and Mg from the blood. After the apoptosis of the chondrocytes, a prefabricated mineralized matrix is left behind, which is protected from proteolytic decay due to the mineral deposition. This space is then occupied by osteoblasts to form the ossified part of the developing antler. Our results are compatible with this scenario and are supported by both the gene expressions and the histological investigations (see details in Steger *et al.* 2010): (i) large mineralized islands were identified in the antler cartilage, (ii) strong expressions of the mineralization genes were detected in the cells of the antler cartilage, (iii) we calculated 2-7 fold overexpressions in the antler cartilage versus the foetal growth plate cartilage for matrix genes like *col1A1*, *col1A2*, *sparc* as well as their "upregulators" *osx* and *runx2*.

Antler: on the robust bone development: In contrast to the cartilage we find more complexity (and more gaps) when attempting to envisage a path toward the local upregulation of *runx2* in the antler bone. One possible scenario is that a diffusible morphogen leaves the sensory nerves which innervate the velvet dermis and the periosteum and these molecules trigger and establish the enhanced synthesis of receptors and/or ligands in osteoblasts (eg. the PTH/PTHrP, TGFβ/BMP, ECM, and FGF2 containing complexes, which are all positive regulators of *runx2* expression). The best candidate for a such diffusible neuropeptide, acting as a local morphogen for the antler bone, is the calcitonin gene related peptide (CGRP), which is known to be abundant in the sensory nerve terminals, including those which infiltrate bone. The anabolic role of CGRP in bone metabolism has been demonstrated by its enhancing effect on Osteocalcin and PTH expressivity, both involved in bone matrix formation. The regulatory role of CGRP in *runx2* expression has been reported recently in osteoblasts. The (very rich) sensory innervation of the antler comes from the *nervus trigeminus*, where the putative antler growth center (AGC) is located. This anatomical

separation could explain why a CGRP initiated morphogenic activity should be localized in the antler, including its bony part. Downstream the initiating step, in a later stage the osteoblasts can maintain those gene expressions which finally would stimulate the *runx2* self enhancements. The synthesis of various BMP-s, FGF and TGFβ-s has convincingly been demonstrated (see details and references in Steger *et al.* 2010). The upregulated *runx2* activates its downstream targets, the mineralization and matrix genes, as well as the *osteocalcin* gene. The expected final results of the above events are the very high energy consumption (due to the very intensive protein synthesis) and the exhaustive depletion of the minerals from the bloodstream by either Col1A1 or by the other mineral binding matrix proteins. To maintain the homeostasis of the calcium level in the circulation, the extracted calcium and the other minerals are supplemented from the dietary intake (which is insufficient to support antler growth) and from the skeletal bone resorption. (All details and original references are in Steger *et al.* 2010).

Our observations and experimental data support the above notion at several points (detail in Steger *et al.* 2010). (i) The conical shape of the calcification density at the upper regions of the already ossified antler, as shown in the sagittal cross section of the antler may be the "footprint" of a gradient of a morphogen factor (e.g. CGRP), which diffuses inward from the velvet dermis. (ii) The expression of *runx2*, *osx* and their downstream regulated mineralization genes was much higher in the antler bone than in the skeletal samples. (iii) Our GC-MS experiments were consistent with the high energy requirement of the high expression genes in the antler bone, as was shown by the high glucose content of the antler bone versus the vertebrae (nearly 5 fold). (iv) This high glucose consumption was in accordance with the high expression of the gene *osteocalcin* (*oc*). The activity of *oc* was known to ensure the elevated insulin synthesis and sensitivity required for glucose utilization. Further quantitative GC-MS measurements provided additional supporting data for our suggestion. (v) The hydroxyproline contents indicated much less collagen decay in the antler bone than in the vertebra, which was in harmony with the intensive *col1A1* expression in the antler. (vi) The much lower ethanolamine phosphate and the reduced free phosphate content in the antler bone versus the vertebra and rib bones were concordant with the unidirectional ("antlerward") flow of minerals from the skeleton and deposition of the minerals in the antler bone matrix. Considering our results and the previous findings we suggest that the high (10-30 fold or more) activity of the mineralization genes and the resulting high mass of the antler organic matrix is sufficient to govern the flow toward

the antler and there is no need for the active inhibition of mineral deposition in the skeletal bones. Genes and regulatory mechanisms which are revealed in my work support vigorous and rapid bone tissue deposition. We believe that studying the robust bone development of deer could facilitate and revive biomedical research on the field of osteoporosis treatment and bone replacement therapies. An example was shown in the article of Steger *et al.* 2010: when we compared the expression pattern of the human orthologous genes of the deer genes upregulated in the antler bone, in osteoporotic and non osteoporotic groups of patients, a very unambiguous segregation (of the groups) was achieved.

Summary

Antlers of deer display the fastest and most robust bone development in the animal kingdom. Deposition of the minerals in the cartilage preceding ossification is a specific feature of the developing antler. We have cloned 28 genes which are upregulated in the cartilaginous section (called mineralized cartilage) of the developing ("velvet") antler of red deer stags, compared to their levels in the foetal cartilage. Fifteen of these genes were further characterized by their expression pattern along the tissue zones (i.e. antler mesenchyme, precartilage, cartilage, bone), and by in situ hybridization of the gene activities at the cellular level. Expression dynamics of genes *col1A1, col1A2, col3A1, ibsp, mgp, sparc*, *runx2,* and *osteocalcin* were monitored and compared in the ossified part of the velvet antler and in the skeleton (in ribs and vertebrae). Expression levels of these genes in the ossified part of the velvet antler exceeded the skeletal levels 10-30 fold or more. Gene expression and comparative sequence analyses of cDNAs and the cognate 5' *cis*-regulatory regions in deer, cattle and human suggested that the genes *runx2* and *osx* have a master regulatory role. GC-MS metabolite analyses of glucose, phosphate, ethanolamine-phosphate, and hydroxyproline utilizations confirmed the high activity of mineralization genes in governing the flow of the minerals from the skeleton to the antler bone. Gene expression patterns and quantitative metabolite data for the robust bone development in the antler are discussed in an integrated manner. We also discuss the potential implication of our findings on the deer genes in human osteoporosis research.

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