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Perspective Article

Hylonome

Transgenic and knock out mice in skeletal research. Towards a molecular understanding of the mammalian skeleton

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Abstract

Our understanding of the biology of the skeleton, like that of virtually every other subject in biology, has been transformed by recent advances in human and mouse genetics. Among mammals, mice are the most promising animals for this experimental work. Because extensive genetic information exists, many mouse mutations are known, and cells from early mouse developmental stages are accessible, scientists have developed transgenic mice - mice in which a gene is introduced or ablated in the germ line. Thus far, we have analyzed more than 100 different transgenic and knock out models with various skeletal phenotypes, covering the major aspects of both skeletal development and skeletal maintenance. Based on these results we here present a first perspective on transgenic and gene knock out animals in skeletal research, including insights in signaling pathways controlling endochondral bone formation, in the regulation of osteoblast function, osteoclastic bone resorption and in bone tumorigenesis, as well as the central control of bone formation. The use of transgenic mice to dissect and analyze regulatory mechanisms in bone cell physiology and the pathogenesis of human bone diseases is an extremely powerful experimental tool. The data presented here demonstrate that the successful convergence of novel genetic approaches with the established and fundamental knowledge of bone biology has made a beginning.

Keywords: Bone, Molecular Genetics, Knock Out, Transgenic Mice, Skeletal Maintenance, Skeletal Development

Introduction

Until recently most of our knowledge about the skeleton was derived from descriptive morphology, histomorphometry, endocrinology, and cellular studies of bone turnover[1-3]. Recent approaches have led to the identification of local factors that regulate skeletal morphogenesis. Molecular and biochemical studies of bone cells in vitro, and most importantly the power of genetics entering the bone field have led us toward the beginning of a molecular understanding of the skeletal system[4]. Indeed the identification of genes responsible for mouse and human skeletal abnormalities, gene inactivation and targeted gene misexpression in mice have documented the importance of specific signaling molecules, receptors, growth factors, matrix proteins, and transcription factors for the development and maintenance of bone. The successful convergence of mouse and human genetics in skeletal biology has been demonstrated several times, e.g. chondrodysplasia in PTHrP receptor mutant mice[5-9] and patients with Jansens metaphyseal dysplasia[10]; mutations in collagen type XI in cho/cho mice[11] and patients with Stickler syndrome[12]; identical phenotypes of Cbfa-1+/- heterozygous mice[13] and patients with cleidocranial dysplasia[14], which lack the expression of one allele of the Cbfa-1 gene; as well as mice with targeted ablation of the second zinc finger of the vitamin D receptor DNA-binding domain as a model for patients with vitamin D-dependent rickets type II[15]. These examples underscore the invaluable importance of transgenic and knock out animals in skeletal research.

One has to keep in mind, however, that so far the results from mouse models have been mixed. Take the mouse in which the retinoblastoma tumor-suppressor gene (RB) was knocked-out. In humans, the lack of RB leads to a cancer in the retina of the eye. But when the gene is inactivated in mice, the animals develop pituitary gland tumors. Mice lacking BRCA1, which were supposed to be a model for human breast and ovarian cancer, don't develop any tumors at all. Thus, it seems that in mice, other genes can compensate

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for a missing gene, such as BRCA1, and that the genetic wiring for growth control in mice and humans is subtly different.

Therefore, to be an important tool toward our further understanding of the skeleton transgenic and knock out animals need to be designed and analyzed based on bone biology and physiology. This background defines the potential targets for specific mutation of defined genes in the germline of mice. Future progress in skeletal research using transgenic animals will thus markedly depend on (i) a bone specific approach which is the choice of a promising target gene, target cell, target function, and target stage, and on (ii) a general approach which is the genetic approach.

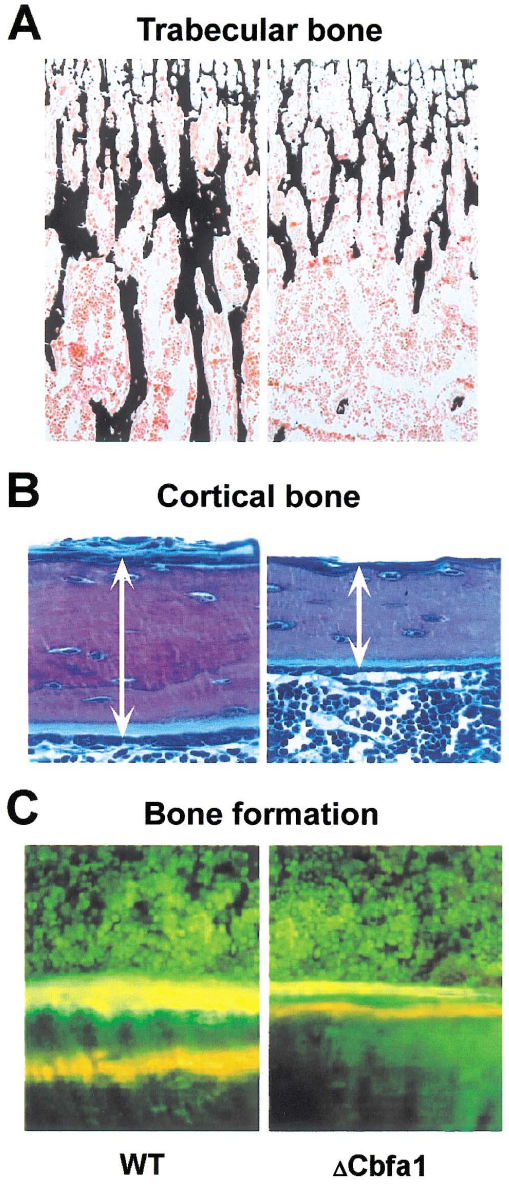

Figure 1. Cbfa-1 controls bone formation. Overexpression of the dominant negative form of Cbfa-1 (ΔCbfa1) in differentiated osteoblasts leads to a decrease in trabecular bone (A, tibia, undecalcified histology; von Kossa staining) and a decrease in cortical thickness (B, tibia, undecalcified histology; toluidine blue staining) due to decreased bone formation (C, tibia, tetracyclin/ calcein- sequence labeling).

Bone biology defines targets in skeletal research

The development and maintenance of the skeleton is ultimately the result of coordinated cellular differentiation, function, and interaction. Four major cell types contribute to the skeleton: 1. chondrocytes, which form cartilage; 2. osteoclasts, the only cell capable of resorbing bone; 3. osteoblasts, which are responsible for bone formation; and 4. osteocytes, representing terminally differentiated osteoblasts embedded in bone matrix and thought to be involved in mechanotransduction.

In general, two types of bone formation are distinguished: intramembranous and endochondral bone formation. During intramembranous bone formation mesenchymal precursor cells directly differentiate into bone forming osteoblasts. Examples are the formation of flat bones of the skull, the formation of part of the clavicle, and the bone apposition to the periosteal surface of diaphyseal cortex of long bones. In contrast, endochondral bone formation represents the conversion of an initial cartilage template into bone and is responsible for generating the majority of bones. During endochondral ossification, the chondrocytes present in the early cartilaginous model, and later in the growth plate, first proliferate and then progressively differentiate into mature hypertrophic chondrocytes. Once fully differentiated, these hypertrophic cells participate in the mineralization of the cartilaginous matrix and undergo cell death. In normal bone development, this is followed by - and may be the necessary signal for - the local recruitment of blood vessels and osteoclasts into the zone of provisional mineralization, leading to the progressive replacement of cartilage by bone, the homing of the hematopoietic bone marrow and ultimately longitudinal bone growth. During life vertebrates constantly renew bone through remodeling characterized by two successive phases: resorption of existing bone by osteoclasts followed by new bone formation by the osteoblasts. In physiological situations, bone resorption and bone formation are balanced to maintain bone structure and bone volume.

Furthermore the cellular interaction of osteoclasts and osteoblasts connects the skeleton as the major reservoir of calcium to the endocrine regulation of ion homeostasis in man.

Thus, potential targets in skeletal research can be 1.) A specific cell type, 2.) genes which are specifically expressed in one bone cell type, 3.) a special process, either bone formation or bone resorption, 4.) a specific stage during life, either skeletal development or skeletal maintenance, and 5.) control centers, by which the body itself regulates bone mass.

Skeletal development, chondrocyte differentiation, and bone growth

The two major milestones in the recent understanding of skeletal development are the significant molecular insights into the differentiation of the osteogenic lineage and the progress in understanding endochondral bone formation.

Cbfa-1: a master key of osteoblastic bone formation

A series of four papers published in Cell 1997 provided compelling evidence that Cbfa-1 is an essential transcription factor required for osteoblast differentiation and thus is the master key for bone formation. Cbfa-1 belongs to the Runt domain gene family, homologous to the Drosophila melanogaster pair-rule gene runt that plays a role in the formation of the segmented body pattern as well as in the development of the nervous system and in sex determination[16]. In two independent studies, Komori et al.[17] and Otto et al.[13] deleted cbfa1 and observed the complete lack of bone formation in Cbfa-1-deficient mice, while Cbfa1+/- heterozygous mice presented a phenotype paralleling the findings in patients with cleidocranial dysplasia (CCD)[18]. Knowing the findings of Otto et al.[13] in mice, Mundlos et al.[14] were able to demonstrate the lack of expression of one allele of the cbfa1 gene in patients with CCD. Finally, the observation of Cbfa-1 expression in fully differentiated osteoblasts suggested that Cbfa-1 is not only crucial for osteoblast differentiation, but also for functional control of osteoblast activity. Indeed Karsenty and co-workers provided direct evidence that Cbfa1 acts as a transcription factor that controls the expression of all major osteoblast-related genes[19]. Cbfa1 controls its own expression and is required for bone formation postnatally[20]. Osteoblast-specific expression of a truncated form of Cbfa-1 (deltaCbfa-1) that lacks the transactivation domain and acts in a dominant negative fashion leads to osteopenia in mice (Fig.1). In turn overexpression of Cbfa-1 is able to significantly increase bone mass by increasing bone formation. Thus Cbfa1 has a dual function. Besides its role during development it is a positive regulator of bone formation by pre-existing osteoblasts after birth.

Endochondral bone formation

Although the different genetic models with implications for endochondral bone formation result in a huge variety of skeletal phenotypes, their unifying principle is the disturbed timing of chondrocyte differentiation. A delay of chondrocyte differentiation is observed in humans with Jansens metaphyseal dysplasia due to constitutive activation of the PTH/PTHrP receptor[10] as well as in mice after targeted overexpression of PTHrP to chondrocytes[8]. Overexpression of fibroblast growth factor-2 (FGF-2)[21], and targeted deletion of IGF-1[22] causes a phenotype paralleling that of the GH-deficient Snell dwarf mice. At the other end of the spectrum deletion of PTHrP[5,6], or its receptor[7], as well as of Bcl-2 [9,23,24] results in acceleration of this developmental program marked by premature terminal chondrocyte differentiation. Other important factors in growth plate control are the FGF-3 receptor and activating transcription factor-2 (ATF-2). A gain of function mutation in the FGF-3 receptor results in achondroplasia due to decreased chondrocyte proliferation[25,26], as does the lack of ATF-2[27], while the FGF-3 receptor knock out mice show a growth plate with an increased proliferating and hypertrophic zone[28,29].

PTH-related peptide (PTHrP) was first isolated from human carcinomas[30-32] and is the causative agent for the humoral hypercalcemia associated with various malignancies. PTHrP is structurally related to PTH, a hormone of major importance in calcium metabolism. Both peptides share 8 of 13 amino-terminal residues, and bind to and activate the same G-protein-coupled PTH/PTHrP receptor[33]. Unlike PTH, however, PTHrP does not circulate in appreciable amounts in normal subjects but is instead widely expressed in fetal and adult tissues, where it is thought to regulate cell differentiation, cell proliferation and organogenesis as a paracrine or autocrine soluble factor[34-36]. In this context, PTHrP is a mediator of cellular growth and differentiation[5,6] and is involved in mesenchymal-epithelial interactions in several tissues[35,37,38].

The critical role played by PTHrP and its receptor in skeletal development has recently been demonstrated unequivocally by gene targeting and disruption experiments in mice and a natural mutation in humans. Mice homozygous for ablation of the PTHrP gene or the PTH/PTHrP receptor gene die at birth and exhibit skeletal deformities that are due, at least in part, to a decrease in proliferation and the accelerated differentiation of chondrocytes in the developing skeleton[5-7,39]. The endochondral bones of these animals are shorter, wider, deformed, and undergo premature mineralisation. At the other end of the spectrum, striking skeletal deformities are observed in Jansen's metaphyseal chondrodysplasia. This rare human genetic disorder characterized by short-limbed dwarfism with delayed endochondral maturation and agonistic independent hypercalcemia that has been attributed to an activating PTH/PTHrP receptor mutation that results in constitutive cAMP accumulation[10,40,41].

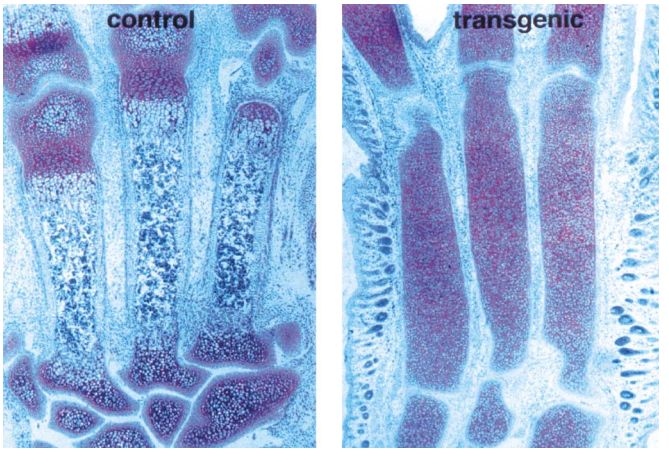

Figure 2. Delayed endochondral bone formation after overexpression of PTHrP in chondrocytes. PTHrP delays the differentiation of chondrocytes and results in an accumulation of prehypertrophic chondrocytes compared to control mice (metacarpals of the midhand; undecalcified histology, semi-thin section, 1mm thick; toluidine blue staining).

The patterning gene Indian hedgehog (Ihh) regulates the expression of the central signaling molecule PTHrP[7,42], which exerts, at least in part, control of chondrocyte maturation by stimulating expression of Bcl-2, a protein that controls programmed cell death in several cell types. Bcl-2 also delays terminal differentiation and apoptosis in chondrocytes[9,43-47].

Consequently targeted overexpression of PTHrP in chondrocytes using the mouse collagen II promoter was found to result in overexpression of Bcl-2 and a striking form of chondrodysplasia characterized by an accumulation of chondrocytes in their prehypertrophic stage and severely delayed endochondral bone formation[8] (Fig. 2).

Bcl-2 is indeed directly involved, and is not just a bystander protein, in endochondral bone formation as demonstrated by premature maturation of chondrocytes in Bcl-2 knock out mice, where Bcl-2 levels are regulated independently of PTHrP or any other molecule. These observations have led to a new model for the control of chondrocyte differentiation. Preliminary data analyzing the coexpression of PTHrP and Bcl-2 in human chondrosarcomas confirms further the importance of the PTHrP/Bcl-2 pathway, at least in chondrogenic tumors, where the level of coexpression seems to be correlated with the degree of malignancy of the tumor[48].

BMI-1: Skeletal patterning and tumorigenesis

Interestingly the availability of transgenic models yields insights into the complexity of gene functions, which are often unexpected. An example for the convergence of bone tumorigenesis and skeletal patterning is the Bmi-1 proto-oncogene. The human bmi-1 gene encodes a nuclear protein of 326 amino acids and is homologous to certain members of the Polycomb family of proteins that regulate homeotic gene expression through alteration of the chromatin structure in Drosophila. By initially using a differential display approach we identified Bmi-1 as one of the genes that is overexpressed in high-grade versus low-grade osteosarcoma. Bmi-1 over-expression in osteosarcoma was then further confirmed by western blot analyses of a variety of primary bone tumors and bone tumor cell lines. Indeed Bmi-1 was found to be specifically overexpressed in osteosarcomas and in addition showed a specific speckled subnuclear localization pattern[49]. By analyzing animals models, it became clear that Bmi-1 is also involved in skeletal patterning during embryogenesis as manipulation of Bmi-1 results in skeletal phenotypes. Transgenic mice overexpressing Bmi-1 exhibit a dose-dependent anterior transformation of vertebral identity along the complete antero-posterior axis, while at the other end of the spectrum mice with targeted deletion of the Bmi-1 gene show a posterior transformation. This regulation is mediated by repression of specific hox genes caused by interaction of Bmi-1 with other members of the mammalian Polycomb complex during development[49-51]. Insights gained from such studies should hasten our understanding of both skeletal development and tumorigenesis, and finally open the way to new forms of therapy.

Skeletal maintenance, bone structure, and remodeling

Through the tremendous progress in developmental biology during the last 5 years, much attention in the bone field has been drawn to the mechanisms of skeletal development. However, the period in which skeletal development takes place is a relatively short time in the life of man. The major part of the life span is characterized by skeletal maintenance. It is during this period in which almost all major metabolic osteopathies, including osteoporosis, develop and become clinically manifest. Therefore, the cellular mechanisms of bone remodeling and continuous renewal and reconstruction of the trabecular microarchitecture and bone volume are of major interest[1, 52].

There are four major targets which may be useful in studying the mechanisms of remodeling responsible for skeletal maintenance: (i) the osteoclast, (ii) hormone receptors, (iii) bone matrix proteins, and (iv) the osteoblast.

Osteoclasts

Almost all of the major bone diseases are associated with an increase in osteoclastic bone resorption, which is the primary cause of bone loss. Since the osteoclast is the only cell that is capable of resorbing bone, it is implied that it is the main cellular target for studying the mechanisms of bone resorption[3,53].

As another consequence, successful therapies for the most common bone diseases are dependent on our understanding of the molecular mechanisms that regulate osteoclast differentiation and function. Four major animal models have influenced our current understanding of osteoclast biology. The op/op mouse, the c-fos-/- mouse, and

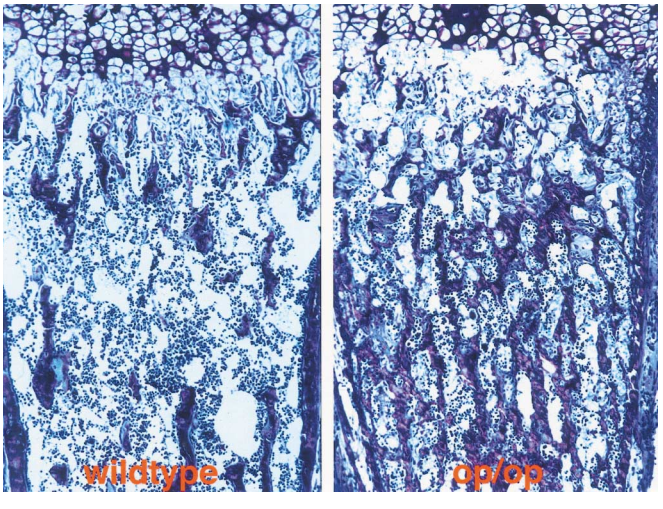

Figure 3. Osteopetrosis in op/op-mice. The gain of bone mass in any osteopetrotic model indicates that bone formation continues in the absence of bone resorption. Thus, bone formation is independent of bone resorption in vivo. (Tibia, undecalcified histology; toluidine blue staining).

the src-/- mouse, all present with osteopetrosis of varying severity and the opg-/- mouse which develops severe osteopenia. Both op/op mice, which lack circulating Colony Stimulating Factor 1 (CSF-1) and exhibit reduced numbers of osteoclasts and macrophages, and c-fos-/- mice, which lack osteoclasts but not macrophages, demonstrate the essential role of CSF-1 and c-Fos for osteoclastogenesis / osteoclast differentiation. On the other side of the spectrum, src-/- mice have increased osteoclast numbers, thus osteoclast differentiation does take place in the absence of c-Src, however, src-/- osteoclasts are essentially non-functional and not able to form a ruffled border[54]. Opg-/- mice also present increased osteoclast numbers, but these are functional, so bone resorption is strongly increased leading to the most severe form of osteopenia known.

It was Wiktor-Jedrzejczak et al.[55] who first reported the possibility that a defect in op/op mice is due to the failure of hematopoietic stromal cells to release CSF-1 (Fig. 3).

Evidence for this was confirmed unequivocally by two independent studies: Yoshida et al.[56] who demonstrated an extra thymidine insertion at base pair 262 in the coding region of the CSF-1 gene in op/op mice resulting in a stopcodon TGA, 21 basepairs downstream. At the same time Felix et al.[57] reported that osteoblastic cells from op/op mice could not produce CSF-1 activity. Subsequently, it was reported by Kodama and co-workers[58], that administration of recombinant CSF-1 restored the impaired bone resorption of op/op mice in vivo.

The role of c-Fos as an essential transcription factor for the differentiation of early osteoclast precursors was impressively demonstrated by the phenotype of the c-fos-/- mouse[59-61]. Moreover the increased number of bone marrow macrophages in c-fos-/- mice indicates that c-Fos also affects a related cell type, and is perhaps involved in the lineage determination of putative macrophage-osteoclast progenitors[61].

C-Src was first identified as the normal cellular counterpart of the transforming protein of Rous sarcoma retrovirus, v-Src[62,63]. In an effort to elucidate the physiological role of c-Src, Soriano et al.[64] generated transgenic mice lacking the c-Src gene. Surprisingly, c-Src deficient mice were able to survive and did not show any gross abnormalities in the cell types that were known to express high levels of c-Src, such as platelets and neurons. Unexpectedly, the only phenotype observed in the c-Src deficient mice was that of osteopetrosis. The skeletal abnormalities in the src-/- mice include a failure of teeth eruption, increased osteoclast number, the absence of a ruffled border, and in aging mice progressive osteopetrosis and the development of odontomas[54] (Fig. 4).

Osteoclasts express high levels of the c-Src protein[65,66] and the defect responsible for the osteopetrotic phenotype of the c-Src-deficient (src-/-) mouse is cell autonomous and occurs in mature osteoclasts[67,68]. The specific signaling pathways that require c-Src expression for normal osteoclast activity have, however, not been fully elucidated. We have shown that the proto-oncogene product c-Cbl is tyrosine-phosphorylated in a Src-dependent manner in osteoclasts, where the two proteins co-localize on vesicular structures[69-71]. In vitro bone resorption by osteoclast-like cells (OCLs) is inhibited by both c-Src and c-Cbl antisense oligonucleotides[70].

Furthermore, tyrosine phosphorylation of c-Cbl and the localization of c-Cbl-containing structures to the peripheral cytoskeleton are impaired in resorption-deficient src-/- OCLs as well as in wild-type OCLs that have been treated with c-Src antisense oligonucleotides[70].

Thus, both c-Cbl and c-Src expression are necessary for bone resorption, while c-Src expression is also necessary for c-Cbl phosphorylation. We therefore conclude that, in osteoclasts, c-Cbl is downstream of c-Src in a signaling pathway that is required for bone resorption. Although c-Cbl may not be the only substrate that is not phosphorylated in the absence of c-Src, the disruption of this pathway may be involved in the osteopetrotic phenotype of the src-/- transgenic mouse, leaving other cells not detectably affected[70].

Simonet et al. were the first to describe osteoprotegerin (OPG)[72]. OPG is a glycoprotein with a molecular weight of 60 kDa capable of inhibiting the late stages of differentiation of mononuclear precursor cells into osteoclasts. Cloning of the cDNA of OPG showed that OPG is a soluble member of the tumor necrosis factor receptor (TNFR) superfamily. In contrast to the other members of the TNFR-family, OPG lacks a transmembrane domain suggesting it is a soluble cytokine-receptor. Overexpression of OPG in transgenic mice leads to an osteopetrotic phenotype and prevents bone loss in the estrogen-deficient state caused by ovariectomy. Lack of OPG on the other hand, leads to severe osteopenia in opg-/- mice[73] (Fig. 4). One year after the discovery of OPG, two independent groups found at the same time, the ligand for OPG (OPGL) by screening OPG-binding cell-

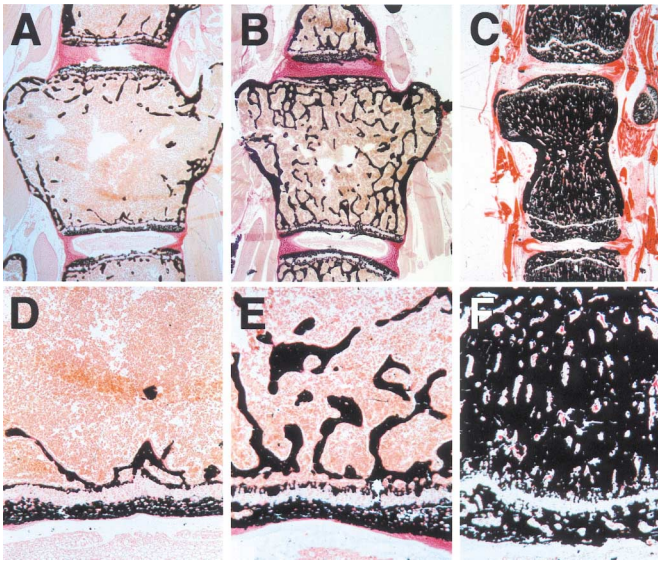

Figure 4. Different bone phenotypes in mutant mice. Model of osteopenia (A,D opg-/- mouse), control (B,E wildtype mouse) and osteopetrosis (C, F src-/- mouse) (Lumbar vertebra, undecalcified histology, von Kossa staining, A-C low magnification, D-F high magnification)

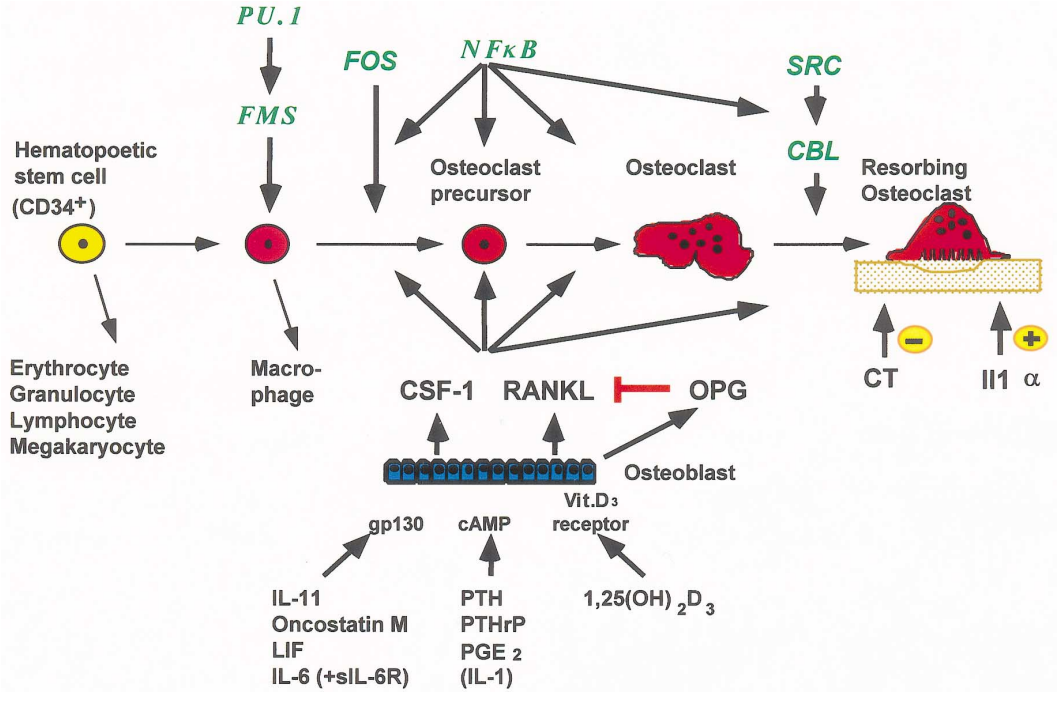

Figure 5. Model of osteoclast differentiation from mononuclear precursor cells. Osteoclast differentiation is dependent on multiple genes acting at different stages of differentiation (green), as well as on endo- and paracrine regulation. Most known endo- and paracrine effectors of osteoclast differentiation act indirectly via osteoblasts. They produce factors like osteoclast differentiation factor (RANKL) and colony stimulating factor-1 (CSF-1), which are necessary to promote osteoclastic differentiation after binding to their specific receptors on mononuclear osteoclast-precursors. Osteoprotegerin (OPG) can block this step by competitive inhibition of the binding of RANKL.

surface antigens and identified it as the long-time postulated osteoclast differentiation factor (ODF)[74,75]. ODF is a type II transmembrane protein consisting of 137 amino acids. It exists as a membrane-bound and as a soluble C-terminal form. Comparison with known sequences showed, that it is identical to TNF-related-activation-induced-cytokine (TRANCE) and the receptor-activator for NFkappaB ligand (RANKL)[75], known to be essential for the activation of T-cells and dendritic cells. ODF is now referred to as RANKL. Without activation of this receptor by RANKL no osteoclastic differentiation takes place. Other studies showed, that the effect of $1,25(OH)_2$vitamin D_3, PTH, PTHrP, PGE_2, oncostatin M, Il-1, Il-6 and Il-11 on osteoclasts is mediated by regulation of mRNA for OPG and RANKL in osteoblasts[76]. In conclusion: OPG competes with RANKL for binding to RANK[77] on the hematopoietic osteoclast precursor, thus regulating bone resorption by influencing the terminal differentiation and activity of osteoclasts (Fig. 5).

Hormone receptors: Rickets type II with alopecia in VDR knock out mice

1,25 dihydroxyvitamin D is the major steroid hormone that plays a role in mineral ion homeostasis. Its actions are thought to be mediated by a nuclear receptor, the vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor and interacts with specific DNA sequences on target genes. The VDR is evolutionarily well conserved and is expressed early in development in amphibians, mammals, and birds. As well as being expressed in the intestine, the skeleton, and the parathyroid glands, the VDR is found in several tissues not thought to play a role in mineral ion homeostasis. Its precise functions in these tissues, as well as its developmental role remain unclear. Although the VDR is widely expressed early during embryonic development, no major developmental abnormalities are observed in the VDR knock out mice or in humans with vitamin D dependent rickets type II (VDDR II). VDR knock out animals are normal at birth, however, they develop hypocalcemia, hyperparathyroidism, and alopecia within the first month of life[15].

Although VDR-/- animals are normocalcemic until day 21, they become progressively hypocalcemic after that point. Concomitant with hypocalcemia, a progressive increase in serum immunoreactive PTH levels was observed from day 21 in VDR-/- mice, and the animals became hypophosphatemic by day 21. The time of the onset of the hypocalcemia in the VDR-/- mice is not unexpected in view of the observation that intestinal calcium absorption in rats occurs by a nonsaturable 1,25 Vit.D-independent mechanism the first 18 days of life[78]. Interestingly, the growth plate abnormalities in VDR-/- mice precede the development of disordered mineral ion homeostasis, which is observed as early as 15 days of age. These data suggest that although the receptor

dependent actions of 1,25 dihydroxyvitamin D_3 are not necessary for normal embryogenesis, they may play a role in the maturation of chondrocytes during longitudinal growth, even in the setting of normal mineral ion homeostasis. By 5 weeks of age, however, both a lack of osteoid mineralisation (15-fold increased osteoid volume) and profound abnormalities in the growth plate were observed.

Interestingly, this phenotype also demonstrated that in the setting of a whole animal, the VDR is not indispensable for osteoclast differentiation and function, as functionally active osteoclasts were detectable on the bone surface of the knock out animals. The critical role of calcium is further documented by the fact that the phenotype could drastically be shifted toward normal by a high calcium diet (Fig. 6).

An independent study by Kato et al.[79] was entirely consistent with these findings, leaving the data on the shorter lifespan of the Japanese mice disregarded.

Bone matrix proteins: Different effects on bone volume by osteonectin and osteocalcin

Bone consists of matrix proteins and the cells that make, mineralize, resorb, renew, and maintain them. The matrix of bone, which is physiologically mineralized with hydroxyapatite is composed of a multitude of proteins that determine its unique characteristics and functions. While 90% of total bone proteins consist of type I collagen, noncollagenous proteins (NCP) account for the other 10% of bone protein. However, the physiological roles of most individual bone proteins remains undefined. Recently knock out experiments shed some light on at least two of the most abundant NCPs: osteocalcin (bone gla-protein) and osteonectin/SPARC (Secreted Protein, Acid and Rich in Cysteine).

Karsenty and co-workers[80] reported that osteocalcin-deficient mice develop a phenotype marked by higher bone mass. This suggested that osteocalcin is a negative regulator of bone formation without impairing bone resorption or mineralization. The molecular mechanism by which osteocalcin controls bone matrix deposition remain however unknown.

The results of SPARC-deficient mice suggest that in the absence of osteonectin, mice develop osteopenia[81]. Together, these findings indicate that a further understanding of the biological role of these abundant bone matrix proteins is needed to clarify the complex regulatory pathways of bone.

Osteoblasts: Reversible ablation of osteoblasts - an animal model of osteoporosis

One assumption in the theory of bone metabolic units (BMU's) is that bone formation and bone resorption are mechanistically coupled during skeletal maintenance and remodeling. However, the existence of a functional link between bone formation and bone resorption has never been demonstrated conclusively in vivo. To define the role of bone formation in the regulation of bone resorption in vivo we generated an inducible osteoblast ablation model. We used an emerging strategy for cancer gene therapy which involves the transfer of the herpes simplex thymidine kinase gene (HSV-TK) in target cells[82,83]. Transgenic mice were generated in which a 1.3 kb fragment of the osteocalcin gene 2 (OG2) was used to drive expression of the HSV-TK. The OG2 promoter is sufficient to achieve osteoblast-specific expression of HSV-TK in vivo. Since dividing cells expressing the HSV-TK die upon treatment with ganciclovir (GCV), HSV-TK expression in dividing osteoblasts allows inducible osteoblast ablation in vivo. In transgenic mice, osteoblast ablation leads to an arrest of skeletal growth and to the development of osteopenia (Fig. 7). Serum levels of osteocalcin are dramatically decreased, while calcium and phosphate levels remain unchanged. Histologically, the bones were denuded of osteoblasts and the bone formation rate was zero. Upon withdrawal of GCV, there was a complete reversal of the phenotype. Most interestingly, the number of osteoclasts remained unchanged and the bone volume was decreased after osteoblast ablation. Indeed, in the absence of bone formation bone resorption occurred both in vivo and in vitro. These results indicate clearly that bone resorption is not controlled by and not coupled to bone formation. Furthermore, this animal model is amenable to

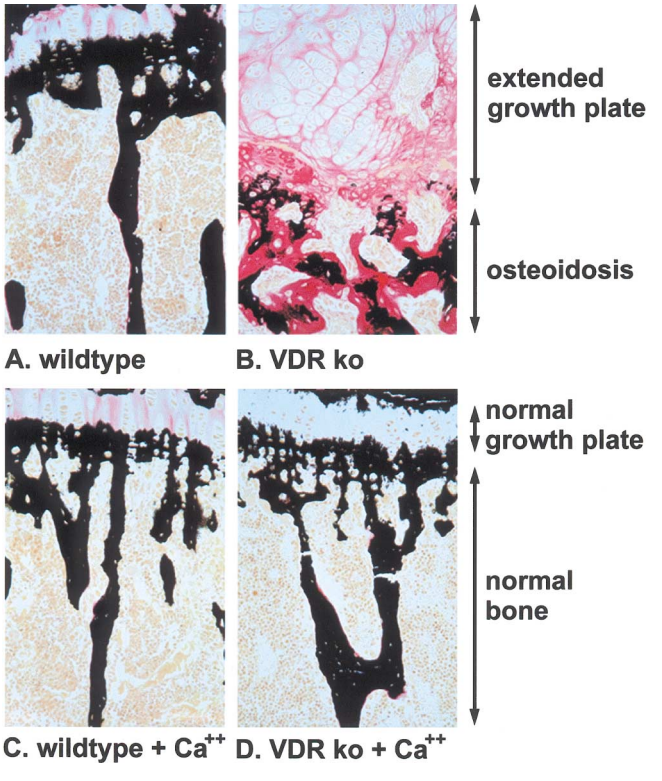

Figure 6. Rickets due to absence of vitamin D signaling can be rescued by a high calcium diet. Vitamin D receptor (VDR) deficient mice develop rickets with severe osteoidosis and deformation of the growth plate (A,B). Feeding of a high calcium diet to these mice leads to normalisation of the growth plate and prevents development of osteoidosis (C,D). This demonstrates, that bone can develop normally in the absence of vitamin D signaling, in face of a balanced serum calcium homeostasis (tibia, undecalcified histology, von Kossa staining).

modulation with respect to the severity of the phenotype. In addition, bone resorption can be maintained in the absence of bone formation for even longer periods of time. Consequently OG2 HSV-TK mice can be used to mimic osteoporosis of variable degrees marked by continuing bone resorption in the face of little or no bone formation[84].

This animal model provides a new tool to address several questions regarding osteoporosis that could not be addressed previously, including the role of peak bone mass, the efficacy of antiresorptive drugs, and the feasibility of novel approaches to treatment of osteoporosis such as gene therapy.

Central control of bone formation via a hypothalamic relay

One of the major issues in bone physiology is the question of how bone formation and bone resorption are balanced. The bone remodeling process is controlled in a way that guarantees maintenance of optimal bone structure from puberty to the end of gonadal function. Until recently the most favored hypothesis was that the function of osteoblasts and osteoclasts is exclusively regulated by each other[85].

There are many experiments favoring this hypothesis, but two of the observations discussed above cannot be explained by local regulation of bone mass alone: 1. In src-/- mice like in all other models of osteopetrosis, bone formation is normal in the absence of osteoclastic bone resorption, so there is no regulation of formation by resorption; 2. In the OG2-HSV-TK-mice bone resorption is unaffected by the complete absence of osteoblasts, so there is no regulation of resorption by formation. These observations, in combination with the tightly regulated bone mass under physiological conditions, suggest the existence of other mechanisms controlling bone formation and resorption, than the known local bone cells.

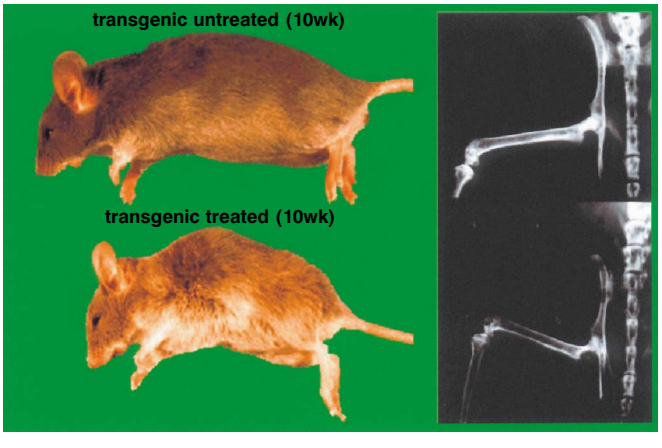

Figure 7. Ablation of differentiated osteoblasts in the HSV-TK-mouse. Expression of the herpes-simplex-virus-thymidine-kinase under the control of the osteoblast-specific osteocalcin-promoter enables specific ablation of differentiated osteoblasts by administration of ganciclovir. While the transgene has no effect on untreated mice, it converts ganciclovir in treated mice selectively in osteoblasts to its toxic metabolite, leading to ablation of differentiated osteoblasts. Without osteoblasts, these mice develop a short stature and an osteopenic phenotype due to continued osteoclastic resorption in face of abolished bone formation. This establishes that bone resorption is independent of bone formation in vivo.

Searching for new centers of bone regulation

To understand why bone formation is so tightly regulated physiologically, one can ask the question What is deregulated in pathological situations? The most frequent disease affecting bone remodeling is osteoporosis[86]. It is characterized by a lower bone mass with an increased risk of fractures following minor trauma[87].

Osteoporosis is the most prevalent disease in developed countries, a fact that emphasizes the importance of understanding in molecular terms the regulation of bone remodeling and in particular of bone formation. Moreover, the incidence of osteoporosis is only going to increase with the aging of the population. Multiple clinical, epidemiological features characterize osteoporosis[88]. Two clinical features that have been known for a long time suggest a molecular basis to explain the regulation of bone mass. These two features are that osteoporosis is often triggered or enhanced by gonadal failure[89,90] and that obesity protects from bone loss[91-93].

This latter observation was for a long time poorly understood, as illustrated by the following citation: "*Heavier people generally have stronger bones as well as a lower risk of suffering from osteoporotic fractures, and our studies have shown that this is mainly due to the greater proportion of body fat. Increased weight bearing does stimulate further bone growth, and, in women, estrogen is produced by fat cells. However, these facts do not adequately explain the relationship between body weight and bone density, so it is likely that other mechanisms are operative*" [94].

Translated into a molecular vocabulary, these two observations may be viewed as suggesting that bone mass, body weight, and gonadal function may be regulated by the same secreted molecules. This can be assumed without attempting yet to decipher their mode of action on their target organs. How could such a molecule be identified? Two possible approaches can be used.

The first approach is to use large genomic screens looking only for novel genes. This approach has been and will be successful, although it may be a bit ambitious for an average academic laboratory. This approach is also based on the assumption that we already know all the functions and certainly the main functions of all the genes already cloned. This, however, is almost certainly not true, although the approach led to the discovery of RANKL, which was identified and initially studied without any knowledge of its critical function during osteoclast differentiation.

The second approach is a candidate gene approach. This is the one we took, that was made possible by the recent advances in molecular endocrinology.

Leptin is the most powerful endocrine inhibitor of bone formation

From the beginning, the best candidate to fulfill this triple regulatory function was leptin. Leptin, the product of the ob gene, is a polypeptide hormone, whose activity is missing in ob/ob mice, a mouse model of obesity[95,96]. Obesity is the most visible phenotype of the ob/ob mice, but it is not the only one. Another prominent phenotype is that these mice are sterile. Clearly leptin, like most known hormones, has a broad range of action on multiple target organs[97,98]. Another mouse mutant strain, the db/db mouse has a mutation in the leptin receptor and the same series of phenotypes as the ob/ob mice[99,100]. Likewise, the fa/fa rats have an inactivating mutation in the gene encoding the leptin receptor and are also obese and hypogonadic. Importantly, the obesity and the sterility phenotypes of these two mouse and rat mutant strains are recessive. Normally, the absence of gonadal function and the presence of the obesity in ob/ob and db/db mice on bone integrity should antagonize each other and result in a mild-low bone mass phenotype. Yet, surprisingly both ob/ob and db/db mice have a massive increase of their bone mass[101] (Fig. 8). The ob/ob and db/db mice are the only known animal models, in any species, in which hypogonadism and high bone mass (HBM) coexist. Thus, in the context of bone physiology, they are an invaluable resource to study bone remodeling and its diseases. This HBM phenotype is even more surprising since these mice are hypercortisolic, a condition usually leading to a decrease in osteoblast functions and to osteoporosis[98].

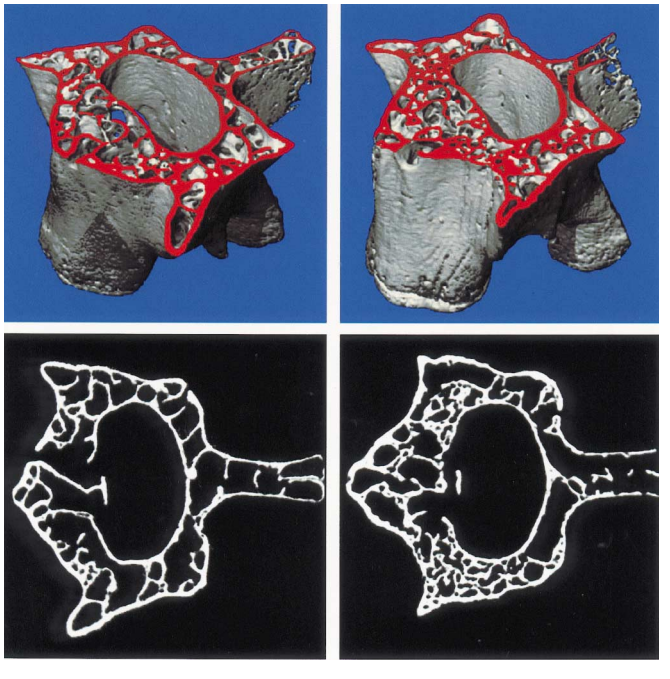

Figure 8. Leptin deficiency leads to a high bone mass phenotype. ob/ob-mice (right) in which leptin signaling is absent have a marked increase in trabecular bone volume, despite their hypogonadic and hypercortisolic state, compared to wildtype controls (left) (lumbar spine, micro-CT image, Scanco Medical, CH)

The HBM phenotype of the ob/ob and db/db mice, which is caused by an increase in bone formation, is not secondary to their obesity as it is observed in young ob/ob mice before they become obese[101]. More importantly, this phenotype is dominant, being observed in heterozygous mice (ob/+ and db/+) and is specific in the absence of leptin signaling since it is not observed in non-ob mouse models of obesity. The fact that the HBM phenotype is dominant whereas the obesity phenotype is recessive, demonstrates genetically that the control of bone mass by leptin is not an accidental function of a body weight regulating hormone. It indicates rather, that control of bone formation is a function of leptin, which is as important as the control of body weight. It also demonstrates that the HBM phenotype is not secondary to any endocrine abnormalities observed in ob/ob and db/db mice, since ob/+ and db/+ mice have none of them. The observation that the high bone mass phenotype of the ob/ob and db/db mice develops despite two osteoporosis-favoring conditions such as hypercortisolism and hypogonadism demonstrates the great importance of leptin regulation in bone formation. Indeed, no other animal model has been identified so far harboring a HBM phenotype despite the coexistence of these two conditions.

Linking bone to brain

How does leptin act to control bone formation? Does it act through an autocrine, paracrine, or endocrine mechanism? Does it require the presence of fat? Or simply does it control bone formation as it controls body weight following its binding to its hypothalamic receptor? Before summarizing what is known about the existence of a central mode of action of leptin in the control of bone mass, one has to summarize what is the phenotype of the ob/ob and db/db mice and the critical implications that this phenotype conveys. These mutant mouse strains do make more bone with a normal number of osteoblasts. In other words, this is a functional phenotype, not a differentiation phenotype. This implies that if leptin acts locally it has to do so through the presence of functional receptors in differentiated primary osteoblasts, not on osteoblast progenitors. This is an important point as there is clear evidence that one can observe the presence of the leptin receptor on immortalized multipotential stromal cell lines in vitro[102].

A multiplicity of experiments, biochemical, molecular, and genetic failed to detect any expression of leptin or of a signal transducing receptor in osteoblasts, thus virtually ruling out an autocrine, paracrine or endocrine mechanism of regulation, at least in vivo. It was conceivable that in the absence of leptin, adipocytes release a molecule that favors bone formation. Studies using a transgenic mouse strain deprived of white fat, however, proved the contrary[103]. These mice, that are called fat-free mice, have a very low level of leptin since they have virtually no adipocytes[103]. Nevertheless, they had a HBM phenotype, thus ruling out the formal possibility that in the absence of leptin, adipocytes

release an activator of bone formation. The remaining possibility to be tested was the most simple and yet the most novel, namely that leptin controls bone formation following its binding to hypothalamic nuclei where the leptin receptor is particularly abundant. This was the simplest explanation because this mode of action is also the one used by leptin to control body weight. It is the most unusual because a central regulator of bone remodeling has never been formally demonstrated in vivo. Indeed, intracerebroventricular (ICV) infusion of leptin in ob/ob mice led to a massive and rapid decrease of their bone mass. Similarly ICV infusion of leptin in wild-type mice led to the development of a severe osteopenic phenotype, demonstrating that bone remodeling or at least its bone formation aspect is under the control of the hypothalamus. No leptin could be detected in the serum of these ICV-treated animals, this latter control demonstrates unambiguously and in the entire animal that leptin can regulate bone formation without contacting directly the osteoblast. These findings, in line with the mode of regulation of body weight and gonadal function, do not close the door to any other possible mode of action of leptin yet to be demonstrated in vivo. Rather they should be viewed as providing investigators in the bone field with a new conceptual framework to better understand bone physiology.

To date we still do not know whether there is a single linear genetic or biochemical pathway explaining leptin's role in body weight control following its binding to its hypothalamic receptor[104-106]. Likewise, we do not yet know what are the gene products that convey to the osteoblasts the information that leptin delivers into the hypothalamus. Nevertheless, leptin action on body weight and on bone mass seems to use different pathways. Indeed, ICV infusion of neuropeptide Y (NPY), which is an orexigenic peptide that antagonizes leptin's action on body weight[107], has the same osteopenic effect as leptin itself[104,105]. This finding suggests that NPY may have a different function in the control of body weight and of bone mass. Clearly one of the challenges ahead of the field will be to identify genes downstream of leptin. But maybe the more important aspect will be to define the end-point molecules regulating leptin's action on bone. Is leptin the only systemic regulator of bone formation? Most likely not: Besides the molecules downstream of leptin itself, the existence of a negative regulation of bone mass suggests that positive regulators of bone formation may exist and await to be identified.

Towards an understanding of the central control for bone mass

If we go back to our original hypothesis that bone mass, body weight and reproduction may share common regulatory molecules and mechanisms, how do these findings relate to the observation that gonadal failure favors osteoporosis and obesity protects from it? It is well known in the obesity community that obese individuals display a state of leptin resistance. The molecular basis of this leptin resistance is not well understood but it results in a partial functional deficiency of leptin, a situation similar to the one of ob/+ and db/+ mice. In that respect, this mouse study is simply the continuation of clinical investigation of the protective role of obesity on bone mass by other means.

The in vivo analysis of the role of leptin during bone remodeling has taught us several important lessons. The first, most general and we feel most important one, is that there is a lot of important information or suggestive evidence to be found in terms of molecular hypothesis for many physiological processes in the classical clinical literature. The second, and in fact, critical lesson, is that bone remodeling is as much a centrally controlled process as it is a local remodeling one. This central regulation is of paramount importance since its disruption is the only known biological setting in which the deleterious consequences of hypogonadism on bone metabolism are overcome. An implication of this genetic finding is that the most typical and frequent bone remodeling disease, namely osteoporosis, is partly, a central or hypothalamic disease. As such this study may be viewed as establishing a novel paradigm in our understanding of bone remodeling. This does not mean however that we now understand everything about bone remodeling. In particular, these findings cannot explain the bone loss observed in anorexic patients. On the contrary, this shift of concepts raises far more questions than it answers. In terms of potential therapeutics, the identification of leptin as a powerful inhibitor of bone formation also has important potential implications for treatment of low bone mass. Conceivably, since the HBM phenotype is dominant whereas the obesity phenotype is recessive, it should be possible to design drugs acting on this pathway that would have a protective effect on skeleton integrity without leading to obesity.

The genetic perspective

Although this article focuses on the bone specific aspects of gene targeting, the general perspective should be mentioned briefly. Protocols using transgenesis[108] and homologous recombination in embryonic stem cells (ES)[109,110] permit inactivation, overexpression and modification of genes almost at will. These technologies are invaluable in assessing the role of genes in complex processes such as development, tumorigenesis, and cell signaling and function. However, there are several limitations, in addition to the above mentioned, mice with the same genetic mutation as humans do not always mimic the human symptoms. For example, nullizygosity appears to be lethal in many instances or causes complex pleiotrophic effects and, therefore, does not permit the development of an in vivo model system in which gene inactivation is restricted to a defined subset of cells[111].

To overcome these limitations, strategies for conditional, cell type-specific gene targeting[112], inducible gene disruption[113], and cell type specific ablation have recently been developed. Some of these systems take advantage of site-specific recombinases such as the Cre/loxP recombination system of bacteriophage P1.

Only two components are required: the 38 kDa Cre (causes recombination) recombinase from bacteriophage P1, which belongs to the integrase family of recombinases. Cre catalyses site specific recombination between specific DNA target sites of 34 bp each termed loxP (locus of crossing over) (reviewed by Plück, 1996 [114]). The utility of this system has been shown by both the generation of conditional transgenic mice and the production of conditional gene knock outs[115,116]. In the latter case, a target construct flanked by two loxP sites (flox) was used to modify the cognate gene by homologous recombination in ES cells. The expression level of the floxed allele is expected to be the same as that of the wildtype and should therefore not lead to phenotypic changes. Crossing of the floxed mice with transgenic mice carrying the Cre recombinase gene under the control of a cell type-specific promoter or an inducible promoter leads to excision of the intervening sequences. This strategy has been shown to work in a number of settings[113, 117, 118]. This new technology is a milestone in the field of mouse reverse genetics, and will have significant impact on skeletal research. However, full exploitation of this system requires further improvements including the use of adeno Cre viruses[119]. In this respect the control of Cre expression appears critical. Problems to be solved are tissue specificity of expression, background activity, level of induction, control over fraction of cells in which expression can be induced and the timing of expression.

Recent studies of humans and mice with skeletal defects (dysplasias, metabolic disorders, and tumors) have pointed to many genes important in skeletal development and skeletal maintenance. Our understanding of skeletal morphology is starting to extend by insights into the molecular mechanisms controlling bone cell differentiation and function. The questions which can be addressed by further development of strategies like the Cre/loxP system, are generating novel forms of genetic analyses in bone. Our future progress towards a better understanding of bone physiology will depend on the successful convergence of these novel approaches with established and accurate knowledge about bone pathology. Indeed histology, endocrinology, histomorphometry, cell biology, and genetics together should yield new clues and will lead to the development of new therapeutic strategies for major bone diseases.

Summary

Today our morphological understanding of the skeleton is being extended by some insights into the molecular mechanisms controlling bone cell differentiation and function. Genetic analyses and recent studies of humans and mice with skeletal defects have pointed to many genes important in skeletal development and skeletal maintenance. Among mammals, mice are the most promising animals for this experimental work. Scientists have developed transgenic mice - mice in which a gene is introduced or ablated in the germ line, through the use of extensive genetic information, known mouse mutations, and cells from early mouse developmental stages. Thus far, we have analyzed more than 100 different transgenic and knock out models with various skeletal phenotypes, covering the major aspects of both skeletal development and skeletal maintenance. Based on these results, we presented our perspective on transgenic and gene knock out animals in skeletal research, including insights in signaling pathways controlling endochondral bone formation, in the regulation of osteoblast function, in the regulation of osteoclastic bone resorption, in bone tumorigenesis, and the central control of bone formation. Furthermore, these data demonstrate that the successful convergence of novel genetic approaches with the established and fundamental knowledge of bone pathology is only the beginning. A wealth of detail about the skeletal system is available. Still, the successes do not amount to a complete or even very profound understanding. On the contrary, current ignorance is vaster than current knowledge. There remains to be discovered mechanisms and concepts that no one has yet even imagined. In some instances, we have learned enough to identify important areas of ignorance. However, the challenges are great, and the use of transgenic mice to dissect and analyze regulatory mechanisms in bone cell physiology and the pathogenesis of human bone diseases remains an extremely powerful experimental tool. Indeed, we can be certain of one thing: histology, endocrinology, histomorphometry, cell biology, and genetics together will yield new concepts in skeletal biology[120,121] - like one of the greatest conceptional discoveries in our field, namely, the proof of bone being controlled by the brain - that consequently will lead to the development of new therapeutic strategies for the major bone diseases.

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