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Review Article

Osteopenic mice: Animal models of the aging skeleton

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Abstract

While our understanding of the developmental biology of the skeleton, like that of virtually every other subject in biology, has been transformed by recent advances in human and mouse genetics, we still know very little, in molecular and genetic terms, about skeletal physiology. Thus, among the many questions that are largely unexplained are the following: why is osteoporosis mainly a women's disease? How is bone mass maintained nearly constant between the end of puberty and the arrest of gonadal functions? Molecular genetics has emerged as a powerful tool to study previously unexplored aspects of the physiology of the skeleton. Among mammals, mice are the most promising animals for this experimental work. This has been previously demonstrated e.g. through the tremendous impact of the different osteopetrotic models on our molecular understanding of osteoclastic bone resorption. Until recently the only way of studying bone loss situations and osteoporosis in mice was by using ovariectomy with all its limitations. Today, however, we have access to more sophisticated osteoporotic mouse-models from four different origins: Transgenic mice (HSV-TK), knock-out mice (OPG), inbred-strains (SAMP6), and through physiological modulation (icv application). These new models have already taught us several important lessons. The first is, that bone remodeling is more than just an autocrine/paracrine process. Multiple experimental evidence has demonstrated that the latter regulation exists, but genetics prove that there is no functional cross-control between resorption and formation. The second lesson is, that remodeling is, at least in part, subject to central regulation. Thus, osteoporosis is partly a central or hypothalamic disease. However, the most dramatic change and the most important advantage we feel is, that today we have models to test a new hypothesis regarding the etiology of osteoporosis before it turns to dogma. Taken together, mouse-studies may lead to a shift in our physiological understanding of skeleton biology and to the emergence of novel paradigms. These, in turn, should help us to devise new treatments for degenerative diseases of the skeleton such as osteoporosis and its associated clinical problems.

Keywords: Aging Skeleton, Animal Models, Osteopenia, Mouse Skeleton, Osteoporosis

Introduction

Skeletal aging must be considered in the broad context of the multifaceted issues of aging processes. Thus, the aging of the skeletal system should not be viewed in isolation, however the understanding of complex systems – as the skeleton itself and its control mechanisms respectively – sometimes requires simplifications. Models often provide these simplification of some facets of more complex systems. The skeleton is a multifunctional organ required for locomotion, for protection of inner organs, and as a reservoir of vital ions. While the skeleton in aging retains its ability for providing mineral ions to the organism, the bone stock declines and the bones become fragile and susceptible to fractures. It is likely that skeletal failure that parallels the aging process is due to multifactorial etiologies. It is conceivable that a reduced bone formation, an increased bone resorption, changes in bone regeneration or in mineral composition, an accumulation of microdamage, and genetic determination are some of the possible sources that contribute to functional failure of bone. Thus, to understand the means by which age affects the skeleton, it is necessary to consider the interdependence of distinct etiologies, but it is also appropriate to study them one by one. In this context animal models of the aging skeleton should closely resemble the pathology of human skeletal aging or certain aspects of the latter. As low bone mass, enhanced bone fragility, and increased fracture risk are hallmarks of the aging skeleton, osteopenic animals seem to be an invaluable tool to study important facets of the aging skeleton, even if already by

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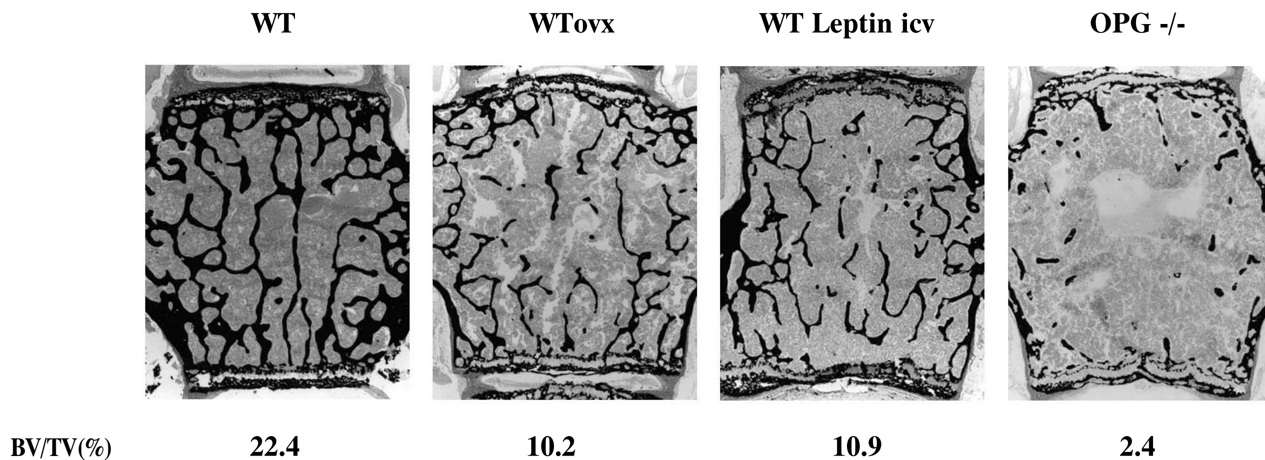

BV/TV(%)	22.4	10.2	10.9	2.4

Figure 1: Osteopenic mouse models. Osteopenia can be induced in mice by targeting hormonal metabolism, osteoblast activity, or osteoclast activity. The normal bone structure of a mouse lumbar vertebral body is shown on the left (WT = wildtype). Ovariectomy (ovx) induced estrogen deficiency results in a significant decrease in bone volume per tissue volume (BV/TV, %) and reduced trabecular interconnection. A similar effect on BV/TV and trabecular connectivity is yielded by decreasing osteoblastic bone formation due to intracerebroventricular application of leptin (leptin icv). Severe osteopenia is found in osteoprotegerin-deficient-mice (OPG-/-) that display an increase in osteoclast number and bone resorption. (lumbar vertebrae, 5 μm undecalcified sections, von Kossa staining, magnification 10x).

definition a single model cannot resemble the complete diversity of contributing causes of bone loss in the elderly.

Several species have been used as models for human age-related bone loss. These include non-human primates, dogs, sheep, pigs, rats and mice[1-4]. The ovariectomized rat is by far the most extensively studied and most widely used animal model of aging bone loss[1]. The rat meets the four characteristics of a good animal model[5]: convenience, relevance, predictability, and appropriateness. The same is true for mice. Mice and rats are common laboratory animals, studies can be carried out easily under standardized conditions in almost any laboratory, they are both relatively inexpensive, and changes in bone mass and bone structure can be induced in a short time. However, with the emergence of molecular genetics as a powerful tool to study previously unexplored aspects of the physiology of the skeleton, the question whether the rat or the mouse is the better animal model has been answered in favor of the mouse. Among mammals, mice are the most promising animals for genetic experimental work. This has been previously demonstrated in skeletal biology through the tremendous impact of different osteopetrotic models on our molecular understanding of bone resorption[6-8] and dwarf models on skeletal development and endochondral bone formation[9-12]. Until recently the only way to study bone loss situations and osteopenia in mice was by using ovariectomy-induced estrogen-deficiency with all its limitations. Today we have access to more sophisticated osteopenic mouse-models due to genetic targeting and physiological modulation of (i) aging, (ii) bone resorption, and (iii) bone formation.

These new models have already taught us several important lessons. The first is, that bone remodeling is more than just an autocrine/paracrine process. Multiple experimental evidence has demonstrated that the latter regulation exists, but genetics prove that there is no functional cross-control between bone resorption and bone formation. The second lesson is that remodeling is, at least in part, subject to central regulation. Thus, age-related bone loss and osteoporosis – the major bone remodeling disease – can be viewed at least in part as a central or hypothalamic disease. Taken together, mouse-studies may lead to a shift in our physiological understanding of skeleton biology and to the emergence of novel paradigms[13]. These, in turn, should help us to devise new treatments for age-related diseases of the skeleton such as osteoporosis and its associated clinical problems.

This review will summarize and briefly discuss advantages and disadvantages of the six established osteopenic mouse models including the ovariectomized mouse model, the inbred SAMP6 aging mouse, the osteoprotegerin deficient high resorption model, and three models of decreased or abolished bone formation (Fig. 1).

The mouse skeleton: A few general implications

The mouse skeleton has some important attributes to be considered. The growth plate tends to fuse relatively early. Ornoy and Katzburg report that fusion occurs at about 3 to 4 months of age[14], while Kimmel reports that he observes well sealed epiphyses by 6 to 8 months of age[15]. These differences most likely reflect strain, background, and gender specific variations. This is in line with the reports of Beamer and colleagues, who report that in some strains linear bone growth is still evident at 12 months of age[16]. Epiphyseal closure can be used as one measure of adulthood, which is in fact later in life than sexual maturity; this is important, as we

suggest to perform ovariectomy in adult mice for bone study purposes. Thus, ovariectomy should never be performed in mice younger than 3 months for the latter studies. It is probably better and advisable to use even older animals as peak bone mass in mice is also reached around 6 months of age. In mice, physiologic senescent bone changes begin around 12 months of age, and physiologic osteopenia increases with advancing age[14]. Age-related bone changes occur in both trabecular and cortical bone and are therefore similar to the situation observed in humans.

When it comes to characterization of skeletal phenotypes, there should be a minimal basic consensus as to the profile that is required to make any valid statements. To account for the skeletal heterogeneity - that exists in humans and in mice[17] - histomorphometrical analysis should be performed on long bones and the spine respectively. We routinely subject both tibiae and the lumbar spine to histological processing while the femurs are taken for biomechanical analysis in a 3-point bending test. If structural histomorphometry is performed by means of a microCT scanner that has a sufficient resolution like, e.g., the μCT 40, one can argue that the distal femur is a more appropriate sampling site in long bones, as it allows direct correlation with the results of subsequent biomechanical testing and easier standardization of microCT analysis. Regarding the use of pDEXA measurements it has been demonstrated that the size of mouse skeletal elements is too small for these techniques to yield meaningful results. One has to keep in mind that even the best non-invasive device – CT or MRI – will provide us with structural parameters only; characterization of a skeletal phenotype however requires both dynamic and cellular parameters as much as structural indices.

Ovariectomized mice: osteopenia through E2-deficiency

Ovariectomy in mice, as in rats, is followed by accelerated bone loss through estrogen-deficiency, paralleling the situation in humans after menopause. This rapid bone loss is prevented by substitution of estrogen, e.g., administered via subcutaneous pellets[18, 19]. As in humans the skeletal response to estrogen is dose-dependent. At low doses bone resorption is decreased, while higher doses of 17-beta estradiol also stimulate bone formation. For any experimental work it is however important to respect the biological dynamics after ovariectomy. Experience from some ten years of work with mice shows that it takes about 4 weeks after ovariectomy before a new steady state within the skeleton develops. This means that dynamic measurements (urine and serum crosslinks, sequence labeling, etc.) and any external therapeutic modulation of bone turnover should not be performed within the first 4 weeks after ovariectomy. If bone mass rather than turnover is the measure of interest it seems appropriate to allow development of absolute osteopenia and keep an interval of 8 weeks from ovariectomy before the bones are subjected to structural histomorphometry.

It has often been said or implied that the bones of the ovariectomized mouse and mice in general are more difficult to handle as compared to the larger rat bones, that the rat model is far more established and studied and therefore the mouse is not a real alternative to the rat model. The like of this has often been said but it is sheer nonsense: histological processing, histomorphometry, biomechanical testing, *in vitro* analysis of bone cells, serum and urine analysis can all be done equally well in rats and mice. In contrast, mice have several advantages compared to rats: due to their smaller size drug testing requires smaller amounts; the multitude of available inbred strains with differences in peak bone mass allow genetic studies; and most importantly, the power of molecular genetics that allows us to alter the mouse genome almost at will is an invaluable advantage of the mouse compared to the rat. These facts allow us to study aspects of skeletal physiology in mice that have never been studied before in any other model. Indeed, today the mouse skeleton is in several aspects already better studied than the rat.

SAMP6 Mice: osteopenia and aging in inbred-strains

In 1975, Hosokawa, Takeda and colleagues at Kyoto University, Japan, started to establish the senescence-accelerated mouse (SAM) strain of mice as an animal model of senescence acceleration and age-associated disorders[20, 21]. Five litters with early senescence were selected as progenitors for a total of nine strains of accelerated-prone, short-lived mice (SAMP). In addition, three strains of accelerated senescence-resistant, long lived mice (SAMR) were selected. The observation of spontaneous leg fractures in a few aged SAM mice led to a more systemic skeletal screening. Indeed the SAMP6 strain was identified as a model of senile osteoporosis characterized by a low peak bone mass at their maturation[22]. A decrease in bone formation due to a paucity of osteoblast progenitors[23, 24], and an increase in bone resorption due to the enhanced maturation of osteoclasts have been suggested as the causes of low peak bone mass. Cross-mating studies have indicated that the low bone mass phenotype of the SAMP6 was controlled polygenetically by a relatively small number of genes, probably located on chromosome 11, 13, and on the X chromosome. The polygenetic etiology of osteopenia in SAMP6 is – for a model system – at the same time an advantage, as osteoporosis in man is most likely also not a monogenetically determined disease, and a disadvantage, as the cause of the bone phenotype remains undefined.

OPG-deficient mice: osteoporosis through increased bone resorption

As a decrease in bone mass can result from (i) decreased osteoblastic bone formation, (ii) increased osteoclastic bone

resorption, or (iii) a combination of both, the first cellular target to devise an *in vivo* model for osteoporosis is the osteoclast. In 1997, Simonet and co-worker described a glycoprotein, named osteoprotegerin (OPG), with a molecular weight of 60 kDa capable of inhibiting the late stages of differentiation of mononuclear precursor cells into osteoclasts[25]. Cloning of the cDNA of OPG demonstrated 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 osteoporosis in opg-/- mice[26] (Bucay et al. 1998). 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-surface antigens and identified it as the long-time postulated osteoclast differentiation factor (ODF)[27, 28]. 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) [28], 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 have demonstrated 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[29]. In conclusion, OPG competes with RANKL for binding to RANK[30] on the hematopoietic osteoclast precursor, thus regulating bone resorption by influencing the terminal differentiation and activity of osteoclasts.

As a model system for osteoporosis the use of opg-/- mice is limited due to the severity of the phenotype and an early onset of multiple osteoporotic fractures. The osteoporotic phenotype even requires housing in separate cages to avoid mechanical stress due to direct contact between the mice and consequent numerous fractures. Furthermore, any rescue strategies, like increasing bone formation genetically, are limited by the lack of bony scaffolds as these mice demonstrate an almost complete absence of trabecular bone.

ΔCbfa1 mice: osteopenia through decreased osteoblastic bone formation

A different approach to induce osteopenia would be by targeting osteoblast function. And, indeed, Ducy and Karsenty have developed such a model in their search for a physiologic role of Cbfa1 beyond embryogenesis[31]. Cbfa1, a transcriptional activator of osteoblast differentiation during embryonic development, is also expressed in differentiated osteoblasts postnatally. To determine if Cbfa1 plays a role during bone formation, Ducy and co-workers generated transgenic mice overexpressing the Cbfa1 DNA-binding domain (ΔCbfa1) in differentiated osteoblasts only postnatally. ΔCbfa1 has a higher affinity for DNA than Cbfa1 itself, has no transcriptional activity on its own, and can act in a dominant negative manner in DNA cotransfection assays. ΔCbfa1-expressing mice have a normal skeleton at birth but develop an osteopenic phenotype thereafter. Dynamic histomorphometric studies show that this phenotype is due to a major decrease of the bone formation rate in the face of a normal number of osteoblasts, thus indicating that once osteoblasts are differentiated, Cbfa1 regulates their function. Molecular analyses revealed that the expression of the genes expressed in osteoblasts and encoding bone ECM proteins, like osteocalcin, osteopontin, and collagen I, is nearly abolished in transgenic mice, and *ex-vivo* assays demonstrated that ΔCbfa1-expressing osteoblasts were less active than wild-type osteoblasts. This mouse model demonstrates that beyond its differentiation function Cbfa1 is a transcriptional activator of bone formation and illustrates that developmentally important genes control physiological processes postnatally. On a side track for Ducy but as the reason to be listed in this review, ΔCbfa1 mice are osteopenic due to decreased osteoblast activity. The same study has demonstrated that Cbfa1 positively regulates the activity of its own promoter, which has the highest affinity Cbfa1 binding sites characterized. Here is the reason for the transient osteopenic phenotype of this model. ΔCbfa1 not only shuts down the expression of the bone matrix proteins through binding to their promotor regions, but also auto-down-regulates its own expression, as the transgene is driven by the osteocalcin promoter. Thus, the value of this model is to demonstrate that isolated functional modulation of osteoblast activity can induce osteopenia. The transient nature of the osteopenic phenotype with severely decreased bone density in 2-week-old animals that returns to normal in 8-week-old animals probably makes this model less attractive for studies with external modulation of bone mass.

HSV-TK mice: osteopenia through conditional and reversible ablation of osteoblasts

One assumption of the theory of bone multicellular units (BMUs) is that bone formation and bone resorption are mechanistically coupled during skeletal maintenance and remodeling[32, 33]. 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,* Dr. Karsenty generated an inducible osteoblast ablation model. Corral et al. used an emerging strategy for cancer gene therapy, which involves the transfer of the herpes simplex thymidine kinase gene (HSV-TK) in target cells[34, 35].

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[36]. 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. 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 clearly indicate that bone resorption is not controlled by and not functionally coupled to bone formation. Furthermore this animal model is amenable to 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[36]. This animal model provides a new tool to address several questions regarding osteoporosis that could not be addressed previously. This includes 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.

ICV Infused mice: osteopenia through hypothalamic modulation of bone mass

It is likely that the most appealing model of inducible osteoporosis today is the *in vivo* modulation of the central axis controlling bone mass. This is possible through intracerebroventricular application of molecules acting as central hormones and/or neurotransmitters. This model system makes use of the fact that besides the well-characterized and critical local regulation of bone remodeling, recent genetic studies have shown that there is a central control of bone formation, one aspect of bone remodeling. This central regulation involves leptin, an adipocyte secreted hormone that controls body weight, reproduction and bone remodeling following binding to its receptor located on the hypothalamic nuclei[19, 37, 38]. Indeed, it is the experimental setting that formally demonstrated the existence of a hypothalamic regulation of bone formation that can be used to generate osteopenia in mice. 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 directly contacting 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. Finally, it provides a great model system to study low bone mass situations in mice.

Conclusion

A model is a model and not the truth. It is essential that we approach all the models presented within this review with a strong sense of their limitations. Each of these models focuses on certain aspects of a complex problem, in this case that of the aging skeleton and osteoporosis. Therefore they might help us to get a better understanding of the respective facets they aim to display. In the case of the osteopenic mouse models presented here it is necessary to keep in mind that it is more than bone mass / bone density that counts when we try to understand osteopenia. However, from a genetic perspective the high degree of homology across mammals is a reasonable basis to believe that mouse models of human bone disorders can provide important insights in the pathophysiology of the aging skeleton.

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