REVIEW

The genetic transformation of bone biology

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The skeleton, like every organ, has specific developmental and functional characteristics that define its identity in biologic and pathologic terms. Skeleton is composed of multiple elements of various shapes and origins spread throughout the body. Most of these skeletal elements are formed by two different tissues, cartilage and bone, and each of these two tissues has its own specific cell types: the chondrocyte in cartilage, and the osteoblast and osteoclast in bone. Finally, each of these cell types has its own differentiation pathway, physiological functions, and therefore pathological conditions. The complexity of this organ in terms of developmental biology, physiology, and pathology, along with the multitude of important conceptual advances in our understanding of skeletal biology, are such that it has become impossible to present in a short review an up-to-date summary of both cartilage and bone biology. Thus, this review will concentrate only on bone biology beyond embryonic patterning. The entire bone field is dominated by the impact of degenerative diseases, such as osteoporosis. This mere fact does influence the research in bone biology and will influence the topics presented in this review. It is no surprise that, like for most other organogenesis processes, human and mouse genetic studies have been a major driving force in redefining bone biology. Genetic studies have opened new areas of research, elucidated at the molecular level some known phenomena, and sometimes challenged untested textbook assumptions, thus transforming the field profoundly.

In mammals, bone development is a late embryonic event as it is the last event of skeleton development. Once the mesenchymal condensations prefiguring each future skeletal element have formed, between 10.5 and 12.5 days postcoitum (dpc) in mouse, they can evolve along two different paths. In some skeletal elements, prefiguring part of the skull and the clavicles, the cells of the mesenchymal condensations differentiate directly into osteoblasts that appear at 15.5 dpc of mouse development (Hall and Miyake 1992; Huang et al. 1997). This process is called intramembranous ossification. For the rest of the future skeleton, cells of the mesenchymal

condensations differentiate into chondrocytes forming the "cartilage anlagen" of the future bones. In the periphery of the anlage, cells from the perichondrium differentiate into osteoblasts, while the periphery of the anlage become hypertrophic. Eventually, the matrix surrounding these hypertrophic chondrocytes calcifies and blood vessel invasion of the calcified cartilage brings in osteoblasts ~14.5–15.5 dpc (Horton 1993; Erlebacher et al. 1995). Once a bone matrix is deposited the bone marrow forms and the first osteoclasts appear (Hofstetter et al. 1995). Thus, sequential appearance of a cartilage anlage, calcified cartilage, and then bona fide bone characterizes the endochondral ossification. Regardless of the mode of ossification, osteoblast differentiation precedes osteoclast differentiation.

One peculiar characteristic of bone resides in its physiology. Bone is the only organ that contains a cell type, the osteoclast, whose only function is to constantly destroy the organ hosting it. This destruction, or resorption, of bone occurs throughout life and in healthy individuals is counterbalanced by *de novo* bone formation in a process called bone remodeling (Frost 1969). It is through bone remodeling that bone mass is maintained at a constant level between the end of puberty and gonadal failure, and bone remodeling is the process affected during osteoporosis, a disease characterized at the cellular level by a relative increase of bone resorption over bone formation (Rodan et al. 1996). Recently, we have begun to understand at the molecular level how bone resorption is controlled. It is striking how little we know about the molecular mechanisms governing bone formation.

The first goal of this review is to present an up-to-date perspective on the genetic control of cell differentiation in the skeleton. The second goal is to describe our current understanding of bone physiology and pathology. The last and most elusive goal is to use our current knowledge of bone biology to formulate, along the way, some of the novel questions that need to be addressed. Before going any further in this review a few definitions are important as these words will be commonly used to summarize phenotypic abnormalities. A loss of function of the osteoclast leads to *osteopetrosis*, a gain of function of the osteoblast leads to *osteosclerosis*, and a relative increase of bone resorption over bone formation results in *osteoporosis*.

This paper is dedicated to the memory of Dr. Louis Avioli.

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Regulation of osteoclast differentiation and function

By far, the aspect of bone biology that has made the most progress in the last few years is the genetic control of osteoclast differentiation and function. This is why the biology of the osteoclast, the cell type resorbing mineralized bone matrix, is discussed first in this review even though it is the last specific cell type of the skeleton to appear during development. The systematic and logical study of many mouse mutants generated to study osteoclast differentiation or, more often for other reasons, has led to the establishment of a fairly detailed genetic cascade controlling either osteoclast differentiation or function. Some of this progress has important implications not only for bone resorption but is also, as discussed below, leading to novel hypotheses about the molecular control of bone formation.

The osteoclast belongs to the monocyte/macrophage cell lineage (Teitelbaum et al. 1996). Phenotypically, the osteoclasts are distinct from other cells in this lineage as it is a giant multinucleated cell (1–50 nuclei per cell depending of the species) found in contact with calcified bone surfaces. Only a relatively small number of genes have been shown to be expressed in osteoclasts and not in other monocyte/macrophage cells, yet more regulatory genes have been shown to control osteoclast differentiation than for any other cell type in the skeleton.

Transcriptional control of osteoclast differentiation and function

A recurrent theme in bone biology is that many of the genes that were identified as regulators of cell differentiation or function have been known for a long time. Yet, perhaps because bone cells are more difficult to isolate, or because skeletogenesis appeared less attractive initially than other processes of organogenesis, they were not originally studied in the context of the skeleton. Pu.1 function in osteoclast differentiation is one of the best illustrations of this.

Pu.1 is an ETS domain-containing transcription factor that is expressed specifically in the monocytic and B lymphoid lineages (Klemsz et al. 1990). It has been known for several years that the deletion of Pu.1 results in a multilineage defect in the generation of progenitors for B and T lymphocytes, monocytes, and granulocytes (Scott et al. 1994; McKercher et al. 1996). The fact that Pu.1 was thought to regulate transcription of *c-fms*, the gene encoding the receptor for M-CSF (Sherr et al. 1985), which plays an important role in osteoclast biology (see below), led Tondravi et al. (1996) to explore the possibility that Pu.1 might also control osteoclast development. As hypothesized by this group, *Pu.1*-deficient mice exhibit a classical osteopetrosis phenotype. There are no osteoclasts and no macrophages in the bone marrow of Pu.1-deficient mice. This is a cell autonomous defect that can be corrected by bone marrow transplantation. To date, Pu.1 is the earliest known marker of osteoclast differentiation as it controls both macrophage and osteoclast differentiation. No cell type decides in an auto-

nous manner to differentiate along a particular lineage, thus, one of the challenges in the field will be to identify the extracellular signals that induce the expression of *Pu.1* and of other cell-specific transcription factors involved in osteoclast differentiation.

Another transcription factor that plays a critical role during osteoclast differentiation is *c-fos* (for review, see Grigoriadis et al. 1995). *c-fos* is the cellular homolog of the *v-fos* oncogene and is a major component of the AP-1 transcription factor. The first indication that *c-fos* might play a role in bone cell differentiation came from the observation that *v-fos*-containing constructs injected into rodents led to the appearance of osteosarcomas (malignant tumors of mesenchymal origin with the ability to form bone tissue) (Ward and Young 1976). Likewise, transgenic mice expressing high levels of *c-fos* in multiple tissues and cell types eventually developed only one type of tumor: chondroblastic osteosarcoma, and clonal cell lines derived from these transgenic mice have altered osteoblastic gene expression (Grigoriadis et al. 1993). Given the results of these overexpression types of studies it came as a surprise that the deletion of *c-fos* in mice led to an early arrest of osteoclast differentiation without any overt consequences on osteoblast differentiation. As a result of this block in osteoclast differentiation, the main phenotype of the *c-fos*-deficient mice is osteopetrosis (Johnson 1992; Wang et al. 1992). The presence of a large number of macrophages in *c-fos*-deficient mice places *c-fos* downstream of *Pu.1* in the genetic pathway controlling osteoclast differentiation (Fig. 1). The osteopetrotic phenotype of the *c-fos*-deficient mice was rescued by bone marrow transplantation and by ex-

Figure 1. Genetic control of osteoclast differentiation. Osteoclasts differentiate from a myeloid progenitor cell common with macrophages. This process is under the control of the transcription factor Pu.1 and of a growth factor, M-CSF. The latter is also involved in the maturation of differentiating osteoclastic cells, along with other secreted molecules such as RANKL/ODF, or transcription factors such as NF κ B, mi or c-Fos. OPG/OCIF is a negative regulator of this process. Once maturation is achieved, factors such as c-Src, c-Cbl, Pyk2, and cathepsin K maintain the mature cells in a functional state. Transcription factors are in red, secreted factors are in blue, membrane receptors are in green, and signal transduction molecules are in orange.

pression of a *c-fos* transgene (Grigoriadis et al. 1994). Although other *c-fos*-related proteins are expressed in osteoclasts, *c-Fos* fulfills a unique function as no other proteins can substitute in vivo for its loss of function. Yet, *c-fos* may not be the only member of the *fos* gene family to contribute to osteoclast differentiation, as the Fos-related protein Fra-1 can favor osteoclast differentiation in osteoclast macrophage precursor cell lines (Owens et al. 1999).

A third transcription factor, NF- κ B, appears to play a role early during osteoclast differentiation. NF- κ B is a dimer composed of various combinations of proteins: p50, p52, p65, c-Rel, and RelB (Verma et al. 1995). These proteins are all related by the Rel homology domain that contains the DNA-binding motif. Through genetic analysis in mice it was shown that each of these proteins play an essential and unique role in the immune response, although no defects in bone cell differentiation was observed (Franzoso et al. 1997; Gerondakis et al. 1998). In contrast, mice deficient in both *p50* and *p52* harbor an osteopetrotic phenotype because of an arrest of osteoclast differentiation (Franzoso et al. 1997; Iotsova et al. 1997). Those findings are important also because NF- κ B seems to be one of the targets of recently identified growth factors that regulate osteoclast differentiation (Lomaga et al. 1999) (see below and Fig. 2).

The last transcription factor found to play a role in osteoclast differentiation was identified by searching for the gene mutated in the mouse mutant *microphthalmia* (*mi*). Heterozygous *mi* mice have the following defects: loss of pigmentation, reduced eye size, and failure of secondary bone resorption (Hertwig 1942). The gene mutated in these mice encodes a basic helix-loop-helix (bHLH) transcription factor called *mi* (Hodgkinson et al. 1993). In *mi* mice, osteoclasts differentiate normally, but

they fail to resorb bones, thus placing *mi* downstream of the other transcription factors mentioned above (Fig. 1).

The wealth of transcription factors controlling osteoclast differentiation contrasting with the apparent paucity of known osteoclast-specific factors was unexpected. It suggests that many of the genes involved in bone resorption and expressed specifically in osteoclasts may not have been identified yet. Another observation common to most of these osteopetrotic mutants is that, although bone resorption was arrested, bone formation was not halted, otherwise the osteopetrosis phenotype would not exist. This is a genetic indication that bone formation is not regulated by bone resorption in vivo.

Control of osteoclast differentiation and function by secreted molecules

The requirement for secreted molecules to control osteoclast differentiation was first demonstrated with the genetic elucidation of a classical mouse mutation called *op/op* (Marks and Lane 1976). Mice homozygous for this recessive mutation lack osteoclasts and macrophages. The osteopetrotic phenotype of the *op/op* mice is not cured by bone marrow transplantation, indicating that it is not a cell autonomous defect (Marks et al. 1984). The gene mutated in osteopetrosis (*op/op*) mice encodes the growth factor macrophage colony-stimulating factor (M-CSF) (Yoshida et al. 1990) whose receptor synthesis is thought to be regulated by Pu-1 (Zhang et al. 1994). Forced expression of *Bcl2*, a gene that prevents apoptosis in monocytes, can partially reverse the osteopetrosis of the *op/op* mice, suggesting that m-CSF function may be to favor survival of osteoclast progenitors (Lagasse and Weissman 1997).

In the last 3 years a group of secreted molecules regulating osteoclast differentiation positively or negatively has been identified. The cloning and the functional analysis of these molecules have modified our understanding of bone resorption profoundly. In a systematic genomic screen for novel secreted molecules, a group at Amgen identified a novel member of the tumor necrosis factor (TNF) receptor superfamily (Simonet et al. 1997). This novel molecule contained no hydrophobic transmembrane-spanning sequence, indicating that it is a soluble receptor. Overexpression in transgenic mice of this molecule called osteoprotegerin (OPG) resulted in osteopetrosis due to an arrest of osteoclast differentiation. This molecule is identical to osteoclastogenesis inhibitory factor (OCIF) that has been purified and subsequently cloned by a group at Snowbrand Pharmaceuticals using a different approach (Yasuda et al. 1998). The identification and the functional study of OPG/OCIF showed that in addition to steroid and known peptide hormones, novel secreted molecules could act systemically to control osteoclast differentiation. The specificity of the function of OPG/OCIF in inhibiting osteoclast differentiation was illustrated by the phenotype of OPG/OCIF-deficient mice. These mice develop an osteoporosis due to an increase in osteoclast number (Bucay et al. 1998; Mizuno et al. 1998).

Figure 2. Regulation of osteoclast differentiation/function by TNF α family members. RANKL/ODF is produced by bone marrow stromal cells and T lymphocytes. It can be cell bound or secreted in the bone microenvironment. Its binding to the RANK receptor present on osteoclastic cells activates a transduction cascade involving TRAFs, NF- κ B, and JNK, which triggers osteoclast maturation toward a functional state. By binding to RANKL/ODF, OPG/OCIF can inhibit this process.

Obviously the identification of a soluble receptor with such a powerful inhibitory effect on osteoclast differentiation suggested that it might be titrating out an osteoclast differentiation activity. This factor was cloned by the same two groups nearly at the same time. The Amgen group used recombinant OPG/OCIF to screen for OPG/OCIF ligand on the surface of various cell lines (Lacey et al. 1998). This time they replaced the criteria of absolute novelty by the criteria of specificity of interaction with OPG. The protein they isolated had previously been cloned and called TRANCE or RANK ligand. Hereafter, I will call it RANK ligand (RANKL) or osteoclast differentiation factor (ODF), although this nomenclature controversy is not settled. RANKL/ODF is present on the membrane of the osteoblast progenitor but can also be found as a soluble molecule in the bone microenvironment. Systemic administration of RANKL/ODF leads to increased bone resorption. In vitro RANKL/ODF has all the attributes of a real osteoclast differentiation factor: It favors osteoclast differentiation in conjunction with M-CSF, it bypasses the need for stromal cells and 1,25(OH)₂ vitamin D₃ to induce osteoclast differentiation, and it activates mature osteoclasts to resorb mineralized bone (Burgess et al. 1999). Consistent with these cell and molecular biology data, RANKL-deficient mice lack osteoclasts and develop a severe osteopetrosis besides immunological defects (Kong et al. 1999). At the same time Yasuda and colleagues, pursuing their biochemistry and molecular biology effort, purified to homogeneity ODF and showed that it was RANKL (Yasuda et al. 1998). Two observations are of potential interest. First, RANKL/ODF is secreted by the osteoblasts and other cells; conceivably the secreted form of OPGL/ODF could be the active form and the membrane-bound RANKL/ODF could be a reservoir of active molecule. Second, RANKL/ODF is also expressed and synthesized by T cells, cells that can, in vitro, induce osteoclastogenesis (Horwood et al. 1998; Rifas et al. 1998). The production and secretion of RANKL/ODF systemically or locally by T cells may explain some of the bone abnormalities observed in autoimmune disorders (Jones and Bhalla 1993; Piepkorn et al. 1997).

As mentioned above, ODF is also called RANKL because it binds to a receptor present on T cells and bone marrow stromal cells called RANK (Dougall et al. 1995; Anderson et al. 1997). Transgenic mice expressing a soluble form of RANK develop an osteopetrosis similar to the one observed in RANKL/ODF-deficient mice and addition of a polyclonal antibody against RANK extracellular domain promote osteoclastogenesis in bone marrow culture suggesting that RANK activation mediates the effect of OPGL/ODF (Hsu et al. 1999). The signal transduction pathway initiated following binding of RANKL/ODF to RANK has also been partly elucidated, thus establishing a cascade from extracellular signals to nuclear effectors. RANK's intracellular domain contains two binding sites for members of a family of proteins called TNF receptor-associated factors (TRAFs) (Wong et al. 1998). TRAFs have been implicated in mediating signals induced by a subset of TNF receptor family mem-

bers. RANK contains a binding site for TRAF6 in its intracellular region (Darnay et al. 1999). Importantly TRAF6-deficient mice exhibit an osteopetrotic phenotype due to defective osteoclast function, thus providing the beginning of a signal transduction cascade leading to osteoclast terminal differentiation (Lomaga et al. 1999). This observation is even more important as TRAFs seem to control the activation of NFκB, a transcription factor required for osteoclast differentiation (Franzoso et al. 1997; Iotsova et al. 1997). Given all the information available it is now possible to summarize in a cartoon form the role of each component of this signal transduction pathway (see Fig. 2).

What we learned from the OPG–RANK–RANKL story is obviously of critical importance for the control of osteoclast differentiation and function but it goes beyond that. The existence of such a tight system controlling bone resorption implies that another system of systemic regulation using proteins of the same family or of other families must control bone formation. Such a regulatory loop would be of critical importance as a target for therapeutic interventions in degenerative bone diseases such as osteoporosis.

Other cytokines such as IL₁, IL₆, oncostatin-M, and IL₁₁ that all transduce their signals through a composite receptor containing a gp130 subunit, can induce osteoclast differentiation in vitro (Suda et al. 1995; Manolagas et al. 1996; Mundy et al. 1996). Their role in vivo has been difficult to establish possibly because of functional redundancy. Although unpublished observations from several laboratories indicate that signal transduction through the gp130 protein plays an important role in osteoclast and osteoblast physiology, osteoclasts are present in gp130-deficient mice (Kawasaki et al. 1997).

Another regulatory gene whose functional importance in skeletal biology was revealed by mouse genetics is *c-src*, the cellular homolog of the *v-src* oncogene. *c-Src*, which is not a secreted protein but a membrane protein, belongs to a large family of tyrosine kinases that has been reviewed recently (Lowell and Soriano 1996). *c-src* is expressed ubiquitously although its highest level of expression is in platelets and neurons (Golden et al. 1986; Brugge et al. 1987). Yet its deletion led to an osteopetrotic phenotype (Soriano et al. 1991). In *c-src*-deficient mice, osteoclasts are present but they fail to resorb bone (Boyce et al. 1992). Consequently, the continuing bone formation in the face of impaired bone resorption leads to progressive osteopetrosis. It was later shown that the inherent defect in the *Src*-deficient mice was within osteoclasts and autonomous of the bone marrow microenvironment (Lowe et al. 1993). The mechanisms by which *c-Src* controls osteoclast function have been, and still are, the object of investigations. Schwartzberg et al. (1997) using a transgenic approach showed that rescue of the phenotype was not dependent of the kinase domain. This is one of the best illustration of the power of mouse genetics because for a long time it was thought that *Src* was primarily a kinase. In other lines of investigation Tanaka et al. (1996) have shown that *c-cbl* is a downstream gene of *c-src* in a signaling pathway re-

quired for bone resorption. Duong et al. (1998) have presented evidence suggesting that phosphorylation of Pyk2 a cytoplasmic kinase activated by c-Src is required for osteoclastic bone resorption.

The list of regulatory genes controlling osteoclast differentiation and function is already surprisingly long, yet it may not be complete as there are two classical mouse mutations causing osteopetrosis for which the mutated gene has still not been identified: *osteosclerotic* (*oc/oc*) and *gray lethal* (Suda et al. 1996).

Regulation of osteoblast differentiation and function

The osteoblast is a cell type of mesenchymal origin that is responsible for bone matrix deposition, or bone formation. As will be discussed below, the osteoblast has another function, sometimes forgotten, and that is to be required for longitudinal bone growth. We know much less about the molecular control of osteoblast differentiation and function compared to what we know about osteoclast and chondrocyte differentiation. To date, only two genes have been shown to control osteoblast differentiation directly or indirectly, *Cbfa1* and *Indian hedgehog* (*Ihh*). This relatively limited knowledge is partly attributable to the fact that the osteoblast could be viewed, from a genetic standpoint, as a sophisticated fibroblast. The osteoblast expresses all the genes expressed in a fibroblast, a cell type for which little is known in terms of differentiation, plus only a few more (Aubin and Liu 1996). Of these few extra genes one is osteoblast-enriched (*Bone sialoprotein*) but only one is truly osteoblast-specific in vivo (*Osteocalcin*).

Transcriptional control of osteoblast differentiation and function

Cbfa1 is the earliest and most specific marker of osteoblast differentiation known. Functional studies have shown that it is a central regulator of osteoblast differentiation and function (see Fig. 3). The names given to this gene have changed several times since it was isolated. It was originally called *Pebp2a1*, then *Aml3*, and then *Cbfa1* (Ogawa et al. 1993; Levanon et al. 1994; Speck and Stacy 1995). In 1995, the gene nomenclature committee decided to use the *Cbfa* nomenclature for all the genes of this family (Speck and Stacy 1995), and this will be used in this review. *Cbfa1* is one of the mammalian homologs of the *Drosophila* transcription factor Runt (Gergen and Wieschaus 1986). Three different lines of evidence reinforcing each other demonstrate that *Cbfa1* plays a role apparently not redundant with the function of any other gene during osteoblast differentiation. Following a molecular biology approach using initially the promoter of *Osteocalcin* as a tool to search for osteoblast-specific *cis*-acting elements (OSEs), *Cbfa1* was identified as the factor binding to one OSE present in the promoter of most genes expressed in osteoblasts (Ducy et al. 1995, 1997; Banerjee et al. 1997; Ji et al. 1998; Jimenez et al. 1999; Porte et al. 1999). Its expression dur-

ing development and after birth is highly osteoblast-specific, and is regulated by the bone morphogenetic proteins (BMPs, see below) and other growth factors. More importantly, its ectopic expression in nonosteoblastic cells leads to the expression of osteoblast-specific genes such as *Osteocalcin* in these cells. All these molecular biology experiments demonstrated that *Cbfa1* was an osteoblast differentiation factor (Ducy et al. 1997). The demonstration that this is its only function and that this function is not redundant with the function of other proteins in vivo came from mouse and human genetics experiments.

In a genetic approach, deletion of *Cbfa1* in mice leads to mutant animals in which the skeleton was made only of chondrocytes producing a typical cartilaginous matrix without any evidence of bone (Komori et al. 1997; Otto et al. 1997). Because there was no bone in these mice, there was no bone marrow. One of the two groups who performed *Cbfa1* deletion in mice explored *Cbfa1*^{+/-} mice and realized that they had hypoplastic clavicles and delay in the suture of some cranial bones (fontanelles), two bone structures that formed through intramembranous ossification (Otto et al. 1997). These abnormalities are identical to the ones observed in a classical mouse mutation called cleidocranial dysplasia (CCD) (Sillence et al. 1987). *Cbfa1* maps at the same location as does *CCD* and the two mutations are allelic (Otto et al. 1997). The third line of evidence demonstrating the role of *Cbfa1* in osteoblast differentiation came from human genetics. Two groups, one looking for a gene causing human CCD and one having elucidated *Cbfa1* biologic function and looking for a disease in which *Cbfa1* was mutated, identified at the same time deletions, stop codon insertions, and missense mutations in *CBFA1* in patients affected with CCD (Lee et al. 1997a; Mundlos et al. 1997).

In short, these different lines of investigation can be summarized as follows: *Cbfa1* is an osteoblast-specific gene whose expression precedes the appearance of the osteoblasts; its deletion leads to a skeleton made of chondrocytes (including hypertrophic chondrocytes) in most but not all skeletal elements but is devoid of osteoblasts; and its function is dominant during osteoblast differentiation. Recently, it has been shown that *Cbfa1*-deficient mice lack hypertrophic chondrocytes in a few skeletal elements (Inada et al. 1999; Kim et al. 1999). This may suggest that *Cbfa1* is expressed in some hypertrophic chondrocytes. The significance of this observation is currently under study. As is the case for the osteoclast lineage, we do not know yet what turns on *Cbfa1* expression in vivo. Although the BMPs can induce its expression in vitro, the time course needed for this induction is too long to be a direct effect and other signaling molecules must be involved. Moreover, evidence that the BMPs control the late stage of skeletogenesis, that is, osteoblast differentiation, in vivo are still missing.

Cbfa1 function in osteoblasts is not limited to cell differentiation (Fig. 3). At least one in vivo study has shown that beyond development *Cbfa1* regulates the

Other osteoblast-specific *cis*-acting elements have been described in several genes that are expressed exclusively or mostly in osteoblasts suggesting that other osteoblast-specific transcription factors have yet to be identified [Ducy and Karsenty 1995; Rossert et al. 1996; Dodig et al. 1996]. One of these transcription factors, provisionally termed *Osf1*, has been partially characterized [Schinke and Karsenty 1999]. The identification and functional characterization of these other osteoblast-specific transcription factors is needed to improve, in the future, our understanding of the molecular control of osteoblast differentiation.

Ihh is one member of the Hedgehog family of growth factors that is expressed in the developing skeleton (Bitgood and McMahon 1995). Misexpression experiments in chick first identified *Ihh* as a regulator of chondrocyte differentiation (Vortkamp et al. 1996). *Ihh*-deficient mice that survive to birth have a disorganized growth plate as expected but they also have no osteoblasts in bone formed through endochondral ossification, whereas osteoblasts appear to be present in bones of the skull, the

Another family of growth factors that has received, legitimately, a tremendous amount of attention are the BMPs. These secreted molecules are members of the transforming growth factor β (TGF β) superfamily of growth factors that can, when applied locally, induce de novo bone formation by recapitulating all the events that occur during skeletal development (for review, see Hogan 1996; Karsenty 1998). The analysis of their functions *in vivo* has relied mostly on gene deletion experiments. Although several BMPs affect skeletal patterning and joint formation, it has not been possible yet using this approach, to show that they affect osteoblast differentiation. It may be attributable to functional redundancy; however, this functional redundancy does not seem to affect the function of other BMPs in organs where several of them are expressed. Alternatively, it may be that, physiologically, they act earlier during skeletal development. One approach that has not been tried at all with the BMPs, would be to overexpress them in the liver postnatally to increase their serum concentration or locally. This approach that has been so informative, for OPG, for instance, that it would help define their range of possible actions *in vivo*. Interestingly, one of the BMPs, BMP1, does not belong to the TGF β superfamily but rather has homology to Tolloid, a gene product thought, based on genetic data, to be a protease in *Drosophila* (Shimell et al. 1991). In mouse, *Bmp1* has been demonstrated to encode the protease releasing the carboxy-terminal propeptide from the type I procollagen molecule, the most abundant protein of the bone extracellular matrix (Kessler et al. 1996). Deletion of *Bmp1* and other experiments have revealed the existence of other Bmp1-like or Tolloid-like proteins that may also be involved in type I collagen processing (Suzuki et al. 1996; Lee et al. 1997b).

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phenotype, characterized by an overall increase in bone resorption, a large increase of osteocyte numbers (osteoblasts embedded in matrix), and the presence of an hypomineralized matrix (Erlebacher and Derynck 1996). An increase in osteocyte number was also reported by the same group in a mouse model overexpressing a dominant negative forms of the type II TGF β receptor in osteoblasts, suggesting that TGF β could control the steady-state rate of osteoblast differentiation (Erlebacher et al. 1998). Additional mouse models overexpressing dominant negative forms of TGF β receptors or affecting the level of expression of TGF β binding proteins should allow to further define the role of TGF β on osteoblast function.

Other growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor 1 (IGF1) have been shown in vitro to favor osteoblast progenitor proliferation and differentiation, respectively (Canalis and Raisz 1980; Rodan 1987; Canalis 1993). The role of these factors in vivo has been difficult to establish because they have major functions in other developmental and physiological processes.

Endocrine regulation of bone development and physiology

Superimposed on the regulation by transcription and growth factors presented above, is an endocrine regulation of osteoblast and osteoclast differentiation and function. I discuss here only the hormones whose major sites of actions include the skeleton.

Bone is the reservoir of 99% of the extracellular calcium pool. Hormones regulating calcium levels influence cell function. Parathyroid hormone (PTH) is a hypercalcemic hormone: it prevents calcium elimination through the kidneys, and it favors the synthesis of another hypercalcemic hormone, 1,25(OH) $_2$ vitamin D $_3$, as well as bone resorption (Chorev and Rosenblatt 1996). There is no PTH-deficient mouse available yet, but there are mice and human harboring inactivating mutations of one PTH receptor. This PTH receptor is also the receptor for another ligand called PTH-related peptide (PTHrP), which controls chondrocyte differentiation (Jüppner et al. 1991; Karaplis et al. 1994). In mice and humans deficient in this receptor, there is besides the cartilage phenotype, a major increase of bone mass (Jobert et al. 1998; Lanske et al. 1999). Likewise in a mouse model of hypoparathyroidism there is also a low turnover (low number of cells) high bone mass phenotype, indicating that the overall action of PTH in the skeleton might be to favor a normal turnover and to limit the bone volume (Chisaka and Capecchi 1991).

As mentioned above, the other major hypercalcemic hormone is 1,25(OH) $_2$ vitamin D $_3$, a steroid hormone that favors intestinal absorption of calcium (Dusso and Brown 1998). Deletion or inactivation of the vitamin D receptor (VDR) in mice and in humans leads to rickets (decreased calcification of the bone), a phenotype completely reversible in both organisms by treatment with calcium (Balsan et al. 1986; Li et al. 1997, 1998;

Yoshizawa et al. 1997; Amling et al. 1999). Osteoclast differentiation is normal in VDR-deficient mice, indicating that in vivo 1,25(OH) $_2$ vitamin D $_3$ is not required for this process in vivo. The role of 1,25(OH) $_2$ vitamin D $_3$ in osteoblast differentiation is still a matter of debate. There is no overt defect of osteoblast differentiation in VDR-deficient mice. However, humans with kidney failure can develop a particular type of bone phenotype called renal osteodystrophy (Slatopolsky and Delmez 1998). Treatment of these patients with 1,25(OH) $_2$ vitamin D $_3$ has been shown to inhibit osteoblast differentiation (Goodman et al. 1994). These observations imply that 1,25(OH) $_2$ vitamin D $_3$ is an inhibitor of osteoblast differentiation. This is in agreement with the fact that treatment of primary osteoblast cultures with 1,25(OH) $_2$ vitamin D $_3$ inhibits *Cbfa1* expression (Ducy et al. 1997).

Much less is known about the function in vivo of calcitonin, the last major hormone regulating serum calcium. Calcitonin is a hypocalcemic hormone secreted by the thyroid. The functional importance of calcitonin is not really understood, as thyroidectomized patients that lack calcitonin have no major metabolism abnormalities and no major bone phenotype (Hurley et al. 1987). Study of calcitonin-deficient mice should help to establish the function of this hormone (R. Gagel, pers. comm.). Calcitonin is related to two other polypeptide hormones, calcitonin gene-related product (CGRP) and amylin (Wimalawansa 1997). Amylin appears in vitro and in vivo to favor osteoblast function and to inhibit bone resorption (Cornish et al. 1998). It is the only identified secreted molecule able to act in opposite directions in both aspects of bone remodeling.

The most important hormones in bone physiology and physiopathology are the sex steroid hormones, particularly estrogens. In vivo experiments performed daily in humans have shown their importance. Osteopenia (bone loss) and osteoporosis are obligatory consequences of the estrogen deprivation characterizing the menopause, and treatment of menopausal women with estrogen prevents or corrects osteoporosis (Pacifi 1998). There are relatively few estrogen receptors in osteoblasts and even fewer in osteoclasts (Bodine et al. 1998; Oursler et al. 1998; Oreffo et al. 1999). Although the number of investigations trying to elucidate the mechanisms of action of estrogens on bone are countless, we still know virtually nothing about how they control bone remodeling. This may suggest that their mode of action on bone may be indirect via another target organ. A reader not fully versed in all the arcane and nuances of bone biology should make no mistake about it; in terms of priority and because of the major public health problem osteoporosis represents, understanding how estrogens control bone resorption is the one most important issue in the field of endocrine control of bone biology.

Genetic analysis of skeletal function

Cells of the skeleton are also involved in specific functions, such as bone growth, bone mineralization, and bone remodeling. We know surprisingly little about the

molecular mechanisms regulating these critical functions.

Longitudinal bone growth

The osteoblast contributes, along with the chondrocyte of the growth plate, to the longitudinal growth of the skeleton. This was first proposed based on histological analysis (Warszawsky 1982). Chondrocytes from the growth plate cartilage produce cartilaginous extensions, termed spicules, that enter the region of junction between cartilage and bone, or metaphysis. The osteoblasts then adhere to the spicules and eventually add a bony matrix to the cartilage, forming what is called mixed spicules. Mixed spicules form over the entire metaphyseal surface of the growth plate. The peripheral mixed spicules are thought to anchor the metaphysis to the epiphysis. These peripheral spicules lengthen and their lengthening would act as a "hydraulic jack" to move the growth plate centrifugally. The validity of this model has been verified in vivo in multiple mouse mutant strains and in human genetic diseases.

Osteogenesis imperfecta (OI) is a genetic disease characterized by synthesis and secretion by the osteoblasts of defective type I collagen molecules, the most abundant bone extracellular matrix protein (Chu et al. 1983; Byers and Steiner 1992). In essence this is a functional deletion of the osteoblasts. There are several forms of various severity of OI but all patients affected with severe forms of OI are shorter than expected given the existence of fractures (P. Byers, pers. comm.). Likewise, in a mouse model of inducible osteoblast ablation, it has been documented that in the absence of osteoblasts there is a complete arrest of growth demonstrating that osteoblasts are required for longitudinal growth of the skeleton (Corral et al. 1998). Remarkably in this mouse model, as the osteoblasts repopulate the bones, the mice reached rapidly the exact same size as their littermates and their rate of longitudinal growth slows down. Such a tightly tuned regulation of bone growth and formation is consistent with the existence of systemic or endocrine regulators of bone formation similar to the one recently identified regulating bone resorption. Likewise, mice overexpressing a dominant-negative form of *Cbfa1* have no functional osteoblasts and are shorter than their littermates (Ducy et al. 1999). The fact that genetic analyses showed that mouse and human bone physiology are extremely similar is important in a broader sense as it shows that mouse is a good model to study skeleton physiology. This is important as the future of bone biology resides probably more in elucidating physiologic processes.

Bone mineralization

Bones and teeth are the only tissues that mineralize under physiological conditions, whereas mineralization or calcification in any other tissue is pathologic. Thus, the question could be asked as follows: Is bone mineraliza-

tion an active function requiring one or multiple genes, or rather is the absence of calcification in every other tissue an active function genetically controlled? It has long been proposed that in the bone matrix there is/are protein(s) or phosphoprotein(s) that could serve a function of nucleation at the beginning of the mineralization process (Glimcher 1989; Hunter et al. 1996). Many of the genes encoding these proteins, such as bone sialoprotein and osteopontin, have been deleted in mice so far without any overt effects on bone mineralization, indicating that these proteins do not alone control bone mineralization in vivo (Aubin et al. 1996; Liaw et al. 1998; Hunter et al. 1998). The current lack of success in identifying through genetic means initiators or activators of bone mineralization does not mean that they do not exist. Nevertheless, it raises the possibility that bone mineralization may also be explained by the absence of inhibitors of mineralization in the bone matrix. Work from Glimcher and collaborators >40 years ago showed that calcium and phosphate can precipitate passively on collagen, and this was viewed as an indication that collagen molecules are the initiators of mineralization (Glimcher et al. 1957). A similar ability was later shown for elastin and fibronectin (Seligman et al. 1975; Daculsi et al. 1999). If this is the case, because these molecules are not present only in bone, some proteins must prevent calcification from occurring in nonskeletal tissues. These latter proteins should be virtually absent from the bone matrix. One genetic experiment lends credence, indirectly, to the hypothesis that bone mineralization is largely a passive process. Matrix GLA protein (MGP) is a mineral-binding protein present in the extracellular matrix of arteries and cartilage but not of bone (the fact that it was isolated from demineralized bone powder could be explained by the large number of arteries present in bone) (Luo et al. 1997). Deletion of MGP in mice leads to massive calcification of arteries and cartilage demonstrating that inhibition of calcification is genetically controlled in at least these two tissues (Luo et al. 1997). Given this function of MGP, it will be now possible to test whether its ectopic expression in osteoblasts will impair bone mineralization. While the search is still on for an activator of bone mineralization, these experiments should help define the extent to which bone mineralization is a passive phenomenon.

Biology is the domain of compromises. Therefore, the notion that bone mineralization can be a passive phenomenon is neither contradictory nor exclusive with the existence of proteins favoring it. One such a protein is tissue-nonspecific alkaline phosphatase (TNSALP). *TNSALP* is expressed ubiquitously but particularly abundant in liver, kidney, and bone (Weiss et al. 1986). Mutations of *TNSALP* in humans lead to hypophosphatasia, a rare disease of various severity characterized by defects in skeletal mineralization (Caswell et al. 1989; Whyte 1994). These defects could result directly from the loss of function of the protein in the context of the bone matrix or be secondary to increased levels of circulating pyrophosphate, an inhibitor of mineralization (Whyte 1994). The lethality of the *TNSALP*-deficient

mice, due to a defective metabolism of vitamin B₆ has prevented the complete demonstration of one or the other cause (Waymire et al. 1995; Narisawa et al. 1997).

Bone remodeling

Cells of the osteoblast lineage are required for osteoclast differentiation, although T lymphocytes can do the job (Horwood et al. 1998; Rifas et al. 1998), and in vivo bone formation follows bone resorption during a bone remodeling cycle. Do these two facts necessarily imply that osteoblast and osteoclast functions are regulated by each other? This has not been demonstrated in vivo yet. As mentioned earlier, there are numerous examples provided by mouse and human genetics showing that in the absence of functional osteoclasts the osteoblasts deposit bone matrix normally (see above). This is precisely because the osteoblasts are functional, in that an osteopetrosis phenotype can develop in the absence of functional osteoclasts. In some cases, such as in the mice overexpressing OPG, the osteopetrosis was not present at birth but developed over a long period of time, clearly demonstrating that improper bone resorption does not affect bone formation (Simonet et al. 1997). On the other hand, are the osteoblasts controlling osteoclast function? This does not appear to be the case either. Transgenic mice in which the *Osteocalcin* promoter drives the expression of the thymidine kinase suicide gene have proven to be useful in addressing this question (Corral et al. 1998). When bone formation was completely arrested in these mice bone resorption continued unaffected. This result indicates that the bone-forming cells do not control the function of the osteoclasts although it does not exclude that the osteoblast progenitors do so. These genetic observations are not surprising. They are consistent with the identification of molecules such as OPG, and OPGL affecting bone resorption but not bone formation. They suggest that a systemic regulation of bone formation must exist.

Genetic analysis of bone extracellular matrix biology

Besides collagens, multiple proteins were found in bone matrix first and for that reason were called noncollagenous bone matrix proteins. Very often, these proteins have been given names that suggest a tight specificity of expression and a function that was not necessarily verified genetically. For the bone field, as for any field, the following statement is true: "I urge that we control our tendency to give these proteins specific names either on the basis of molecular weight or on the basis of a proposed function (not on experimentally proven function)" (Glimcher 1989).

Bone is not made only of cells; it also contains an extracellular matrix (ECM). The bone ECM contains mostly type I collagen, which account for 90% of the protein content and a slew of noncollagenous proteins and proteolytic enzymes. Mouse and human genetics have shown that most of these proteins and proteases are

required for the integrity of the bone tissue, although it is not always understood how they achieve their functions at the molecular level. The first protein of the bone ECM whose function was elucidated genetically is type I collagen, a heteromeric protein made of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain (Chu et al. 1983). Osteogenesis imperfecta (OI) is an autosomic-dominant disease of variable severity marked by decreased bone mineralization, spontaneous fractures, and short stature (Byers and Steiner 1992). In its worst forms the disease is lethal perinatally. The vast majority of the cases of OI are caused by stop codons, missense mutations, and deletions in the genes coding for either the $\alpha 1(I)$ or the $\alpha 2(I)$ chain (Prockop et al. 1994). Mutations decreasing synthesis of normal type I procollagen molecules result in mild phenotype, whereas mutations leading to abnormal type I procollagen molecules result in increased severity. Recent mouse models of OI have also been generated to study several manifestations of OI, such as short stature, which cannot be ascribed to defective type I collagen molecules (Bonadio et al. 1990; Khillan et al. 1991; Chipman et al. 1993). It has been proposed that the short stature of the OI patients, which cannot be accounted for only by their fractures, could be a result of a growth hormone deficiency (Marini et al. 1993). As mentioned above, in light of the growth-arrest phenotype of osteoblast-depleted transgenic mice, it may reflect the fact that the osteoblasts must deposit a bone ECM of good quality to support longitudinal growth.

Multiple noncollagenous proteins, proteoglycans, and enzymes have been deleted from the mouse genome. Here, one has to contend with the growing number of mutant mouse strains and highlight their major phenotypic abnormalities. For most of them the molecular bases of the phenotype of the mutant mice generated have not been elucidated. Osteocalcin is the only known osteoblast-specific protein (Hauschka et al. 1989). It owes its name to the presence of γ -carboxylated glutamate residues called gla residues, which can bind, with high affinity, calcium and other mineral ions. However, osteocalcin-deficient mice have no mineralization defects, and in other in vivo assays osteocalcin has no mineral-binding properties (Ducy et al. 1996; Boskey et al. 1998; T. Schinke and G. Karsenty, unpubl.). Instead, osteocalcin-deficient mice have an increased osteoblast function resulting in a high bone mass phenotype (Ducy et al. 1996). The mechanism of action of osteocalcin is for now unknown. The term osteopontin was chosen for a protein thought to act as a bridge between the cell surface and the hydroxyapatite crystals (Oldberg et al. 1986). Osteopontin-deficient mice do not have mineralization defects either. The major defect, in terms of bone biology, of the osteopontin-deficient mice is an alteration of the osteoclast formation (Liaw et al. 1998; Rittling et al. 1998). Here again, the molecular basis of this action is under study. Bone sialoprotein is another phosphoprotein of the bone matrix. Its absence in mice does not affect overtly bone mineralization but results in an increase in bone mass phenotype (J.E. Aubin, A.K. Gupta, R. Zirngibl, and J. Rossant, 1996, unpubl.). Proteo-

glycans are thought to be required for matrix organization. Of all the proteoglycan-encoding genes that have been deleted from the genome, biglycan is one of the proteoglycans enriched in the bone matrix. Biglycan-deficient mice displayed an osteoporotic phenotype that becomes more pronounced over time (Xu et al. 1998). Finally, the function of two enzymes highly expressed in osteoclasts has been analyzed genetically. Cathepsin K is a lysosomal enzyme expressed at high levels in osteoclasts. Through a classical gene search approach it was shown to be the gene responsible for pycnodysostosis (Gelb et al. 1996). Nonsense, missense, and stop codon mutations of cathepsin K cause a disease characterized by short stature and osteosclerosis (Gelb et al. 1996; Hou et al. 1999). Mice deficient in cathepsin K have a very similar phenotype (Saftig et al. 1998). Osteoclasts express band 5 isozyme of tartrate-resistant phosphatase (TRAP), which is considered to be the most osteoclast-specific gene. Like most of the structural proteins expressed by osteoblasts, osteoclast function has been addressed by gene targeting. TRAP-deficient mice have a mild osteopetrotic phenotype (Hayman et al. 1996).

Multiple proteases are present in the cartilaginous and bone matrix. The function of one, gelatinase B, has been genetically studied (Vu et al. 1998). This study is important for two reasons: First, of all the structural proteins and proteases of the bone matrix whose function has been studied genetically, it is the only one for which a molecular mechanism of action has been proposed; second, it affects a key event during endochondral bone formation, namely angiogenesis. Gelatinase B also called MMP-9 is a protease highly expressed during development by osteoclasts that can cleave several collagens and elastin (Vu et al. 1998). The major phenotype of MMP-9-deficient mice was that their long bones were slightly shorter (Vu et al. 1998). Growth plates of the animals had a major lengthening of the zone of hypertrophic chondrocytes. Beyond 3 weeks of age, apoptosis, vascularization, and ossification remodeled this giant growth plate to produce bones of normal appearance. The critical cells whose lack of MMP-9 leads to this phenotype reside in the bone marrow as bone marrow transplantation can rescue the phenotype. These cells were called chondroclasts. Finally, growth plates from MMP-9-deficient mice show a delayed release of an angiogenic factor. A second set of experiments showed that inactivation of vascular endothelial growth factor (VEGF) completely blocked blood vessel invasion and at the same time there was an increase in the hypertrophic zone of the growth plate (Gerber et al. 1999). According to these results, VEGF made by the hypertrophic chondrocytes would be present in the cartilaginous matrix, the action of MMP-9 in the matrix would release VEGF that would then bind to its receptor on chondroclasts and activate cartilage replacement by bone (Fig. 4). In a similar line of work Liu et al. (1995) generated mice that are resistant to collagenase cleavage of type I collagen. Although the main phenotype of these mutant mice is a slow fibrosis subsequent studies showed that the bone resorption activity of PTH was strikingly diminished in these mice (Zhao et al.

Figure 4. Role of VEGF in the control of longitudinal bone growth. VEGF is synthesized by hypertrophic chondrocytes and embedded in the cartilaginous matrix. It is released from the matrix by the action of MMP-9 secreted by chondroclasts and then binds to its receptors on endothelial cells, triggering vascular invasion, and bone matrix deposition by osteoblasts.

1999). These examples are probably not the only examples of protease involvement in bone remodeling. The generation of more mouse strains will define the role of the multiple proteases present in the cartilaginous and bone matrix.

By identifying a role for these proteins and others that are present in the bone matrix, mouse genetics has certainly been very useful. Yet the challenges that lie ahead are nearly as enormous as before. Mouse genetics will be useful in defining domains in these proteins that are responsible for their function, but eventually we will have to rely on cell biology and biochemical assays to clearly define the mechanism of action of these gene products.

Perspective

Our understanding of bone development has advanced tremendously in the past 10 years, mostly through genetic means. Yet there is probably still much more to be learned about osteoblast differentiation. It is likely that other osteoblast-specific transcription factors besides *Cbfa1* must exist. Their identification is required to define the genetic cascade controlling osteoblast differentiation. Likewise, the link between growth factors, such as the BMPs and others acting early during skeletal development, has still to be understood. Beyond development, our understanding of bone physiology has made important progress in the last 5 years through large screen approaches and also mouse genetics. However, we still know very little about bone physiology compared to what we know about bone development. In that respect, again the discovery of RANKL/ODF and of OPG/OCIF is of fundamental importance. It demonstrated that novel secreted molecules acting systemically and/or locally are involved in the control of bone remodeling. As mentioned above, the identification of these molecules

affecting mostly bone resorption suggest that other molecules, yet to be recognized, must control the bone formation arm of bone remodeling. This in turn may lead to a better understanding of the mechanisms of action of the steroid hormones on bone physiology. By integrating the action of these various secreted molecules, it may be possible to define in the future several parallel pathways regulating bone physiology, one acting mostly locally and another acting systemically.

In terms of the approaches used, the history of bone biology can be divided into two parts. Initially bone biology, like most fields, was dominated by cell biology, however, in the past 10 years it has been ruled by mouse genetics. Mouse and human genetics are here to stay and rightly so. However, no single approach, whether it is cell biology, genetics, or biochemistry, will have all the answers for any field of biology. Thus, the main challenge in the short term will be to understand at the biochemical level how many of the new gene products that have been identified fulfill their functions.

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