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Osteoclast differentiation and gene regulation

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1. ABSTRACT

Osteoclasts, the bone resorbing cells, play a key role both in normal bone remodeling and in the skeletal osteopenia of arthritis, osteoporosis, periodontal disease and certain malignancies. Osteoclast cellular commitment, differentiation and function depend upon the establishment of specific patterns of gene expression achieved through networks of transcription factors activated by osteoclastogenic cytokines. This review is an updated look at the various transcription factors and cytokines that have been demonstrated to play critical roles in osteoclast differentiation and function, along with their known animal models, such as: *PU.1, Mcsf, RANKL, NF- kappaB, AP-1, NFATc1, Mitf, Myc,* and *Src.* Further studies on these transcription factors and cytokines will not only expand our basic understanding of the molecular mechanisms of osteoclast differentiation, but will also aid our ability to develop therapeutic means of intervention in osteoclast-related diseases.

2. INTRODUCTION

Bone is an essential mineralized tissue with critical mechanical and metabolic functions. It has the capacity to adapt to its functional environment in such a way that its morphology is "optimized" for the mechanical demand (1). The combined and balanced activities of bone-forming cells, osteoblasts, and bone-resorbing cells, osteoclasts, in bone tissue result in the continuous turnover of bone, termed bone remodeling. Bone is continuously remodeled throughout life and an imbalance in this process can result in bone disease. As the most commonly occurring bone disease, osteoporosis, caused by elevated osteoclast numbers and activity, affects 28 million adults in the United States alone. Osteoporosis is characterized by a reduction in bone mass leading to an increased risk of fractures following minimal trauma. On the other hand, osteoclast dysfunction or lack of differentiation leads to osteopetrosis, a rare bone disease characterized by increased bone mass and obliteration of the bone marrow

Table 1. Genes and their mouse models associated with impaired osteoclast function

Mutated Gene	Defects in Osteoclasts	Role	Reference Mouse Model
PU.1	Devoid of osteoclasts and macrophages	A transcription factor associated with the m-csf pathway	59
M-csf	Devoid of osteoclasts and macrophages	A critical cytokine in the differentiation of osteoclast precursors	65
RANK	Devoid of osteoclasts and B cells	A critical cytokine in the differentiation and activation of osteoclasts	72
NF-κB 1 and NF-κB 2	Devoid of osteoclasts	A transcription factor in the differentiation and activation of osteoclasts	75
NFATc1	Existence of monocyte/ macrophage precursor cells but devoid of osteoclasts	A transcription factor in the differentiation and activation of osteoclasts	51
c-Fos		A transcription factor in the differentiation and activation of osteoclasts	49,80
c-Jun		A transcription factor in the differentiation and activation of osteoclasts	81
Mitf	Existence of mononuclear osteoclasts but devoid of multinucleate cells	A transcription factor in the differentiation and activation of osteoclasts	53, 89-91
Myc		A regulator associated with the differentiation of osteoclasts	
Src	Existence of osteoclasts but devoid of bone-resorption activity	A regulator associated with the bone resorpton activity in mature osteoclasts	12

cavity. Osteoclasts are derived from monocyte/macrophage cells; they undergo sequential stages of differentiation and then further differentiate to form mature osteoclasts. How these distinct stages of differentiation are regulated in such a precise manner is a central problem in osteoclast biology. We review what is known about the mechanisms of osteoclast function as well as transcription factors and cytokines that regulate the stages of differentiation.

3. OSTEOCLAST FUNCTION

Bone resorption, a function of osteoclasts, forms an essential half of the coupled process of bone remodeling required to maintain normal skeletal homeostasis. Activation of osteoclasts starts with their adhesion to bone matrix. Thereafter, cytoskeletal reorganization, cell polarization, and formation of unique membrane areas for bone resorption take place. During resorption, osteoclast microfilaments form a specific ring-like "sealed zone" that mediates tight attachment of the cell to mineralized bone matrix. The sealed zone surrounds the ruffled border, a convoluted membrane area formed as a result of vesicle insertion and active directional secretion. This compartment is acidified to a pH approximating 4.5 by an electrogenic proton pump (H^+-ATPase) and a Cl^- channel. The acidified milieu mobilizes the mineralized component of bone, exposing its organic matrix, consisting largely of type 1 collagen that is subsequently degraded by the lysosomal enzyme cathepsin K.

Matrix degradation requires adhesion of the osteoclast to bone, an integrin $\alpha_v\beta_3$-mediated event that also stimulates signals which polarize the cell and secrete resorptive molecules such as hydrochloric acid and acidic proteases. Definitive proof of the pivotal role that $\alpha_v\beta_3$ plays in the resorptive process came with the generation of $\alpha_v\beta_3$- integrin knockout mice, which develop a progressive increase in bone mass due to osteoclast dysfunction (18). Osteoclasts isolated directly from mice lacking $\alpha_v\beta_3$ also fail to form a sealed zone (18). Protons are extruded by a pump belonging to the vacuolar-type H^+-ATPase (V-H^+-ATPase) class, which is present in a high concentration on the ruffled border (apical surface) of the resorbing osteoclast. We reported Atp6i is a novel subunit of the osteoclast-specific proton pump (2) and Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification (3). ClC-7 is a late endosomal/lysosomal member of the CLC family of chloride channels and transporters. It provides the chloride conductance required for efficient proton pumping by the H^+-ATPase of the osteoclast ruffled membrane. Mice deficient in the ubiquitously expressed ClC-7 Cl^- channel show severe osteopetrosis and retinal degeneration because osteoclasts cannot acidify the extracellular resorption lacuna. Cathepsin K was cloned from human osteoclastomas in our lab (4) and from rabbit and human osteoclasts in other labs (5-8). The important role of *Cathepsin K* in osteoclast function was first suggested by the finding of clinical research that mutations in this gene caused pycnodysostosis. The human disorder pycnodysostosis is a rare, autosomal, recessive, skeletal disorder caused by mutations in *Cathepsin K* at 1q21, which codes for cathepsin K, a lysosomal cysteine protease. Mutation in this gene affects the metabolism of the skeletal system, causing defects in bone resorption and bone remodeling (9). In clinics, pycnodysostosis is characterized by short stature, osteosclerosis, acroosteolysis, spondylolysis, separated cranial sutures with open fontanelles, bone fragility, and loss of mandibular angle (10, 11). The *Cathepsin K* mutation causes unique pycnodysostosis disorders rather than simple osteopetrosis, as seen in other diseases associated with osteoclast genes, such as c-src (12)or Atp6i (3). This feature of the *Cathepsin K* mutation suggests that Cathepsin K may constitute other functions beyond just matrix protein degradation that may result in the unique phenotypes of pycnodysostosis.

4. OSTEOCLAST ORIGIN AND CELL LINEAGE

Osteoclast (OC) lineage, phenotype, and gene expression are associated with OC differentiation. Transplantation studies have demonstrated that the OC precursor is a mononuclear cell that is hematopoietic in origin (13). Earlier studies performed *in vivo* (14) and *in vitro* (15-17) suggested that OCs are derived from cells of the mononuclear phagocyte system. However, more recent evidence indicates that although macrophages and OCs share a common precursor, these lineages diverge upon further differentiation (18). OCs possess a distinct phenotype and functional capabilities compared to cells of

Table 2. Expression of phenotypic markers during osteoclast generation

Stem cell CFU-GM	Osteoclast precursor	Preosteoclast	Quiescent osteoclast	Active osteoclast
CTR	-	+	+	+
Tartrate-resistant Acid Phosphatase	-	+	+	+
Gelatinase B	+	+	+	+
Cathepsin K	-	+	+	+
OC-116kDa	?	?	+	+
Cystatin C	?	?	+	+

the macrophage series, particularly the ability to resorb bone. OC hematopoietic precursors migrate via vascular pathways to the skeleton. Osteoblasts, chondrocytes, and their mineralized matrices, together with stromal and endothelial cells, provide the microenvironment for homing of these precursor cells. Stromal cells produce cytokines, including M-CSF and IL-6, that induce and modulate growth and differentiation of the precursors to mature OCs (19, 20). In addition, the vitamin D metabolite calcitriol and the parathyroid hormone support OC development (21, 22).

The OC precursor cells have multiple Golgi complexes and abundant mitochondria (14), and are positive for non-specific esterase (23) and possibly type IV collagenase. In a suitable microenvironment, OC precursors differentiate into pre-osteoclasts (preOCs). The term "preOC" has been used to describe a direct mononuclear precursor showing morphological and cytochemical features that are similar to those of the multinucleated OC (24-27). They are TRAP-positive cells that express mRNAs for all OC-associated phenotypes (e.g. TRAP, calcitonin receptor (CTR), and cathepsin K), and are poised to fuse into multinucleated OCs upon further differentiation (27). OCs possess a highly specialized proton-generating mechanism for the rapid dissolution of mineral, and secrete collagenases (28), cathepsin K (4), and other hydrolases active in the degradation of bone matrix proteins. OC formation *in vitro* can be studied either in organ culture of murine embryonic metatarsals (19), in a co-culture system in which hematopoietic precursor cells (spleen or marrow cells) are cultured with osteoblasts (29), or in a cloned SV40-transformed OC precursor cell line, i.e. MOCP-5 (30). Soluble RANKL and M-Csf allow spleen or marrow cells to differentiate into OCs without co-culture with osteoblasts (Figure 1, Table 2).

5. OSTEOCALST SIGNALING

Osteoblasts, chondrocytes, and their mineralized matrices, together with stromal and endothelial cells, provide the microenvironment for homing of osteoclast precursor cells. Osteoblasts/stromal cells produce cytokines including macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) that induce and modulate growth and differentiation of the precursors to mature osteoclasts. M-CSF binds to its receptor, c-Fms, present on osteoclast precursors, providing the signals for macrophage survival and proliferation. Intracellular RANK signaling by its interaction with RANKL induces recruitment and activation of cytoplasmic tumor necrosis factor receptor-associated factors (TRAFs), leading to the activation of multiple signaling cascades such as MAP, NF-κB, Src, and Akt (31). Of the several TRAF proteins that have been described in conjunction with RANKL, including TRAF-1, -2, -5, and -6, TRAF-6 is indeed an essential adaptor required for RANK-associated signaling. It is necessary for RANK-induced NF-κB activation and *in vitro* osteoclastogenesis and the deletion of TRAF-6 leads to osteopetrosis. By contrast, the contributions of TRAF2 and TRAF5 to osteoclastogenesis are minor (32, 33).

The most notably activated pathway by RANK is NF-κB. NF-κB dimers are normally rendered inactive in the cytosol. Activation of this transcription factor is a consequence of phosphorylation of its inhibitory protein, IκB proteins (IκBa, IκBb, IκBe, IκBg, Bcl-3), by an upstream kinase complex termed IκB kinase (IKK), resulting in the release and translocation of NF-κB to the nuclear compartment. The NF-κB pathway is relevant for RANKL-RANK-regulated osteoclast development and osteoclast function not only in mice, but also in humans as can be seen from patients with X-linked osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia and immunodeficiency (OLEDA-ID syndrome) which carry a X420W point mutation in IKKg and have osteopetrosis (34). Besides NF-κB, TRAF proteins also activate the upstream MAP kinase (MAPK) MEKK1, which is followed by activation of a large number of MAP kinase mediators, eventually leading to induction of three distinct MAP kinase families, JNK/c-Jun, ERK1/2, and p38 kinases. Dominant-negative forms of various MAP kinases and selective inhibitors of the MAP kinase pathways inhibited osteoclastogenesis or reduced osteoclast survival (35-37). Activated p38-MAPKs downstream of RANK can directly phosphorylate signal transducer and activator of transcription 1 (STAT1) and hence control expression of a variety of target genes (38). JNKs and their direct upstream kinase MKK7 have been shown to be involved in osteoclastogenesis through *in vitro* cell culture (39, 40). JNK activation facilitates the phosphorylation of c-Jun and increases its transcriptional activity, leading to activator protein-1 (AP-1) activation. ERK, which activates the other component of AP-1, c-Fos, is also activated upon RANK stimulation. Inhibition of MEKs (ERK kinases) by PD98059 or U0126 does not, however, attenuate osteoclast differentiation, but rather increases osteoclastogenesis.

Recently *Takayanagi et al.* reported that NFATc1 plays an essential and sufficient role in osteoclastogenesis. They demonstrated that RANKL induces and activates NFATc1 through calcium signaling. Both the transient initial release of Ca^{2+} from intracellular stores and the influx through specialized Ca^{2+} channels control the dephosphorylation of the cytoplasmic components (NFATc1 proteins) and lead to their nuclear localization which is followed by the activation of osteoclast-specific genes (41). They reported that inhibiting NFATc1 activity

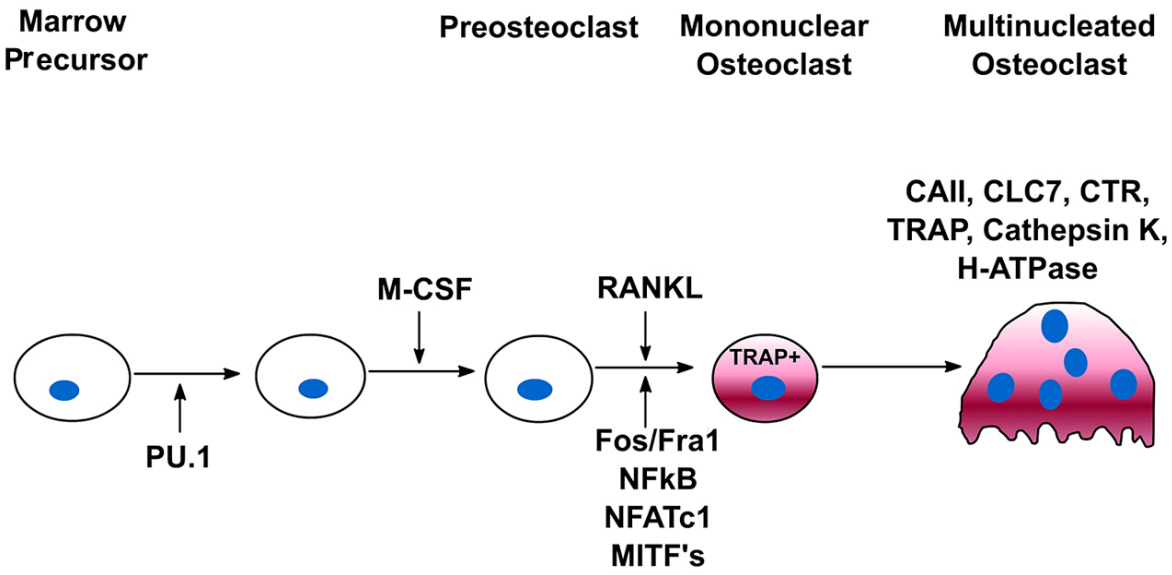

Figure 1. Stages of osteoclast differentiation. The proposed sites for cell and transcription factors involved in osteoclast differentiation.

using dominant negative alleles blocks osteoclastogenesis, whereas overexpression of the wild-type protein stimulates osteoclast development from embryonic stem cells in a RANKL-independent manner. Despite the importance of the calcium-NFAT pathway, it remained unclear how RANKL activates calcium signals leading to the induction of NFAT2. Most recently, *Koga et al.* reported that mice lacking immunoreceptor tyrosine-based activation motif (ITAM)-harboring adaptors, Fc receptor common γ subunit (FcRγ) and DNAX-activating protein (DAP) 12, exhibit severe osteopetrosis owing to impaired osteoclast differentiation. In osteoclast precursor cells, FcRγ and DAP12 associate with multiple immunoreceptors and activate calcium signaling through phospholipase Cγ (PLCγ). Thus, ITAM-dependent co-stimulatory signals activated by multiple immunoreceptors are essential for the maintenance of bone homeostasis (42). Besides these pathways, c-Src interaction with TRAF6 was found to enhance the kinase activity of c-Src, activating the Akt/PI3K pathway, skeletal rearrangement and cell motility. The molecular adapter Grb-1-associated binder-2 (Gab2) was also found recently to associate with RANK and mediate RANK-induced activation of NF-κB, Akt, and Jnk (43) (Figure 2).

6. OSTEOCLAST GENE TRANSCIRPATION

The transcription of eukaryotic genes is regulated by specific DNA binding proteins (transcription factors) that assemble on cis-acting DNA sequences in promoters and enhancers. Many of these DNA binding proteins are ubiquitous in their expression and serve a general role in gene transcription. Others are restricted in expression to one or a few cell types. Cell-specific transcription factors have been identified which activate lineage-specific gene expression in skeletal muscle, neuronal, erythroid, myeloid and lymphoid lineages (44-48).

Gene-targeted disruption studies have revealed that various transcription factors are essential at different stages of osteoclast differentiation, activation and survival. PU.1 is responsible for the earliest established event in osteoclastogenesis and PU.1 disruption in embryonic stem (ES) cells can make ES cells fail to differentiate into macrophages *in vitro*. Several other transcription factors have been found crucial for osteoclast differentiation downstream of M-CSF and RANKL/RANK signaling. Development of macrophages is preserved in NF-κB null mice, suggesting that NF-κB functions later than PU.1 during osteoclast differentiation. A deficiency in c-fos causes severe osteopetrosis with lack of osteoclasts (49, 50). NFATc1 is a transcription factor that plays an essential and sufficient role in osteoclastogenesis. It is a master switch for regulating terminal differentiation of OCs, functioning downstream of RANKL (51). Overexpression of a constitutively active form of NFATc1 in c-fos null cells restores expression of osteoclast-specific genes, demonstrating that NFATc1 is a critical transcriptional regulator downstream of c-fos during osteoclast differentiation (52). Microphthalmia-associated transcription factors (MITF), transcription factor E (TFE) 3, TFEB, and TFEC, are essential for differentiation of mononuclear precursors into multinucleated osteoclasts (53). Most of these transcription factors are involving in both the RANK-associated signaling and the expression of typical osteoclastic genes, including calcitonin receptor, TRAP, cathepsin K, osteoclast-associated receptor (OSCAR), and $\alpha_v\beta_3$ class of integrins.

7. TRANSCRIPTION FACTORS AND CYTOKINES THAT REGULATE OSTEOCLAST DIFFERENTIATION

How a multipotential cell chooses a single pathway of OC differentiation is a central problem in OC biology. Spontaneous and genetically engineered osteopetrotic

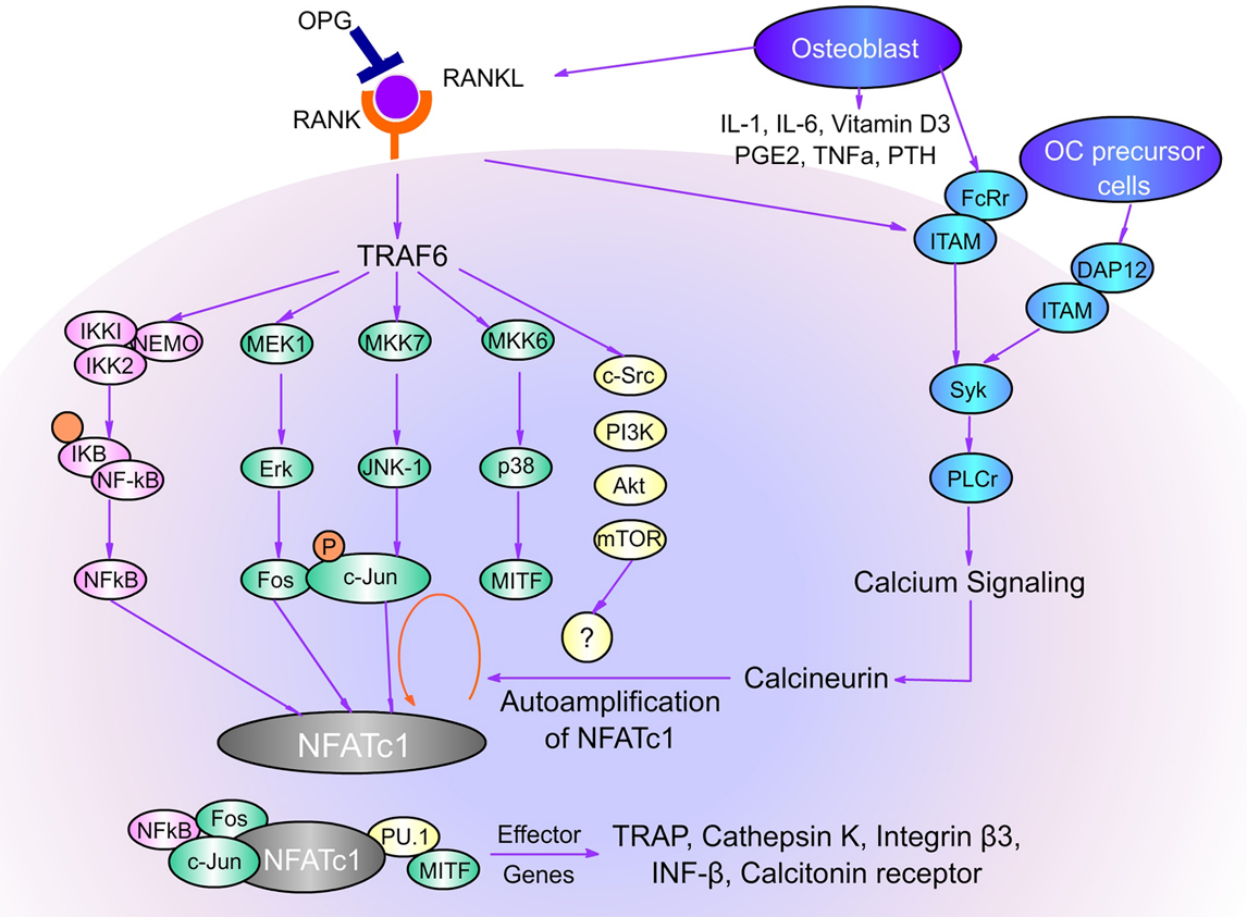

Figure 2. RANK signaling network of osteoclast differentiation. Binding of RANKL to its receptor RANK induces various intracellular signaling cascades, such as MAP (ERK, p38, JNK), NF-κB, Src and NFATc1. Several transcription factors have been found crucial for osteoclast differentiation downstream of RANKL/RANK signaling such as: NF-κB, NFATc1, c-fos, c-Jun, Mitf. They are essential for differentiation of mononuclear precursors into multinucleated osteoclasts and are essential for the transcription of osteoclast–specific genes.

mutant mice have yielded important insights into the regulation of OC differentiation.

7.1. PU.1

PU.1 is an EST-domain transcription factor essential for the development of myeloid and lymphoid lineage cells. Mice homozygous for a null mutation in the PU.1 gene die during fetal development by 18.5 days post coitum (d.p.c.) and lack lymphoid and myeloid lineages (54). PU.1$^{-/-}$ ES cells fail to differentiate into macrophages *in vitro* (55). This failure is complemented by a PU.1 transgene, providing evidence that PU.1 promotes macrophage differentiation from pluripotent ES cells (56). The role of PU.1 in macrophage differentiation was further determined by the discovery that commitment to the monocytic lineage occurs in the absence of PU.1 and a low percentage of monocytic precursors are produced in the PU.1 null mice (57). The major role of PU.1 in lymphoid and myeloid development is thought to be the regulation of lineage-specific cytokine receptor genes, such as M-CSF receptor (c-fms), GM-CSFRα, G-CSFR, and IL-7Rα (58). The combined data show that PU.1 is absolutely required for macrophage development during the differentiation from pro-monocyte to the monocyte stage.

OCs are bone-resorbing cells of hematopoietic origin. Hematopoietic transcription factor PU.1 is also critical for osteoclastogenesis; PU.1 expression is detected at all stages of OC differentiation and PU.1 mRNA increases 3-fold as cells differentiate into OCs (59). PU.1$^{-/-}$ knockout mice are osteopetrotic and are devoid of both OCs and macrophages, indicating a differentiation block at a common MO-CSF precursor stage (59). PU.1 also binds to the corresponding sequences in the promoters and enhancers of many OC-specific genes. RANK gene, which has been shown to be crucial for osteoclastogenesis, is a transcriptional target of PU.1. The PU.1$^{-/-}$ progenitor cells failed to express the RANK gene and reconstitution of PU.1 in these cells induced RANK expression (60). RANKL-induced cathepsin K gene expression is cooperatively regulated by the combination of PU.1 and NFATc1 (61). The

transcription factors micropthalmia associated factor (Mitf) and PU.1 interact with the tartrate-resistant acid phosphatase (TRAP) gene promoter and activate TRAP gene expression in OCs (62). Thus, PU.1 appears to be a master regulator, critical for the development of a common progenitor for the lymphoid myeloid cell lineages in the hematopoietic system.

7.2. MCSF

After commitment to the OC lineage, the entrance of mononuclear cells into the early preOC pathway is a response to macrophage colony-stimulating factor (M-CSF). M-CSF is required for the proliferation, differentiation, and survival of hematopoietic cells in the monocytic lineage (20, 63, 64). Treatment with M-CSF alone results in the formation of macrophage-like cells. It also contributes to their differentiation and regulates the cytoskeletal changes that accompany bone resorption. The absence of a functional M-CSF in the op/op mouse causes not only a macrophage deficiency but also a lack of OCs, resulting in an osteopetrotic phenotype (65, 66). These deficiencies can be restored with injections of M-CSF (66, 67). Op/op marrow stromal cells can support the differentiation and proliferation of OC progenitors from inoculated stem cells, as shown by experimental evidence that M-CSF is not essential for the early stages of OC development (68). Furthermore, MCSF, although necessary for entry of precursors into the early preOC pathway, was found to inhibit osteoclastogenesis at high doses.

Binding of M-CSF to its receptor c-Fms recruits adapter proteins and cytosolic kinases, thereby activating a variety of intracellular signals. Tyrosines 559 and 807 in the cytoplasmic tail of c-Fms play distinct roles in OC differentiation and function. Changes in M-CSF-receptor expression appear to modulate the final lineage selection of the pluripotent monoblastic progenitor (69). M-CSF-induced genes are necessary for a direct response to RANKL and interleukins. *Cappellen et al.* (70) reported that M-CSF induced the receptor for RANKL (RANK) and other RANK/NF-κB pathway components (TRAF2A, PI3-kinase, MEKK3, and RIPK1), providing a molecular explanation for the synergy of M-CSF and RANKL. Interleukins, interferons, and their receptors (IL-1α, IL-18, IFN-β, IL-11Rα2, IL-6/11R gp130, IFNγR) were also induced by M-CSF (70). Furthermore, M-CSF acts as a survival factor for the OC precursors through Bcl-X(L)-induced inhibition of caspase-9 activation, which inhibits apoptosis of OC precursors (71).

7.3. RANKL

Receptor activator of NF-κB ligand (RANKL), also called TNF-related activation-induced cytokine (TRANCE), osteoprotegrin ligand (OPGL), or OC differentiation factor (ODF), is a member of the tumor necrosis factor (TNF) family. $RANK^{-/-}$ mice demonstrated profound osteopetrosis resulting from an apparent block in OC differentiation, revealing that RANK provides critical signals necessary for OC differentiation (72). RANK connects to its extracellular signal factor RANKL and induces recruitment and activation of its adaptor TRAF6, leading to multiple downstream cascades. There are six known main signal pathways: MAP (ERK, p38, and JNK), NF-κB, Src, and Akt, shown by recent research results. OPG, encoded by Tnfrsf11b, as a decoy receptor of RANKL, is also expressed by OCs and preOCs. It is secreted and competes with RANK by binding to RANKL. Overexpression of OPG causes osteoclast-deficient osteopetrosis, while deletion of OPG leads to osteoporosis due to increased OC number and activity.

7.4. NF-κB

NF-κB is a family of five transcription factors that are expressed in most cell types and play an essential role in immune and inflammatory responses by regulating the expression of a variety of proinflammatory cytokines and other inflammatory mediators (73). The five transcription factors p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel, and RelB each contain a Rel homology domain that allows these factors to dimerize and bind DNA. Unlike most transcriptional activators, this family of proteins resides in the cytoplasm and must therefore translocate into the nucleus. Once in the nucleus, they attach to κB binding sites in the promoter region of a large number of genes including IL-1, IL-6, TNFα-, and granulocyte macrophage colony-stimulating factor (GM-CSF) and, after stimuli-induced IκB protein degradation in response to extracellular signals, induce their transcription (74).

Mice lacking NF-κB1 and NF-κB2 (double-knockout mice) developed osteopetrosis because of a defect in OC differentiation, suggesting redundant functions of NF-κB1 and NF-κB2 proteins in the development of this cell lineage (75). The complete absence of OC precursors in these mice indicates that OC differentiation was arrested at a relatively early stage. Further studies have demonstrated that NF-κB1 and NF-κB2 expression is not required for formation of RANK-expressing OC progenitors but is essential for RANK-expressing OC precursors to differentiate into $TRAP^{+}$ OCs in response to RANKL and other osteoclastogenic cytokines (76).

7.5. AP-1

Activator protein-1 (AP-1), which is composed mainly of Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) proteins, is important in the osteoclastogenic process (77). Differentiation of the common precursors into either bone or immune lineages is determined by ligands binding to cell-surface receptors, RANK for OCs or Toll-like receptors (TLRs) for mononuclear phagocytes. Both RANK and TLRs activate the dimeric transcription factors NF-κB and AP-1. Yet, c-Fos/AP-1 plays a positive role in OCs but a negative role in macrophages and dendritic cells (78).

Disruption of the c-fos proto-oncogene also leads to an osteopetrotic phenotype (49, 66, 79, 80). However, the number of F4/80 and Mac-2 positive cells, presumably macrophages, is increased, and cells positive for the 92-kD type IV collagenase, proposed as a relatively early marker

of the OC lineage, are evident at the capillary invasion front of the metaphysis. Thus, the absence of c-fos blocks OC development at the point of divergence from the common MO-CSF precursor, prior to the expression of TRAP-positivity. RANKL signaling to the cell interior also leads to Fos-dependent transcription of target genes such as Fra-1 and NFATc1. NFATc1 seems to be the critical downstream target gene of Fos in osteoclastogenesis, because differentiation of Fos-deficient bone marrow monocytes (BMMs) into OCs is rescued by ectopic expression of NFATc1 (52).

Transgenic mice expressing dominant-negative c-Jun specifically in the OC lineage manifested severe osteopetrosis due to impaired osteoclastogenesis (81). c-Jun is clearly RANKL activated via TRAF6 by a process involving JNK1, but not JNK2. JNK1 appears to modulate osteoclastogenesis through both c-Jun phosphorylation-dependent and -independent mechanisms (82, 83). Recently, c-Jun signaling was found to be in cooperation with NFAT in RANKL-regulated OC differentiation (81). When transcriptionally active, c-Jun associates with members of the Fos family. Several cytokine genes are cooperatively regulated by NFATs and AP-1, which bind to a composite recognition element in their promoter regions.

7.6. NFATc1

Nuclear factor of activated T-cells cytoplasmic (NFATc) is a family of transcription factors originally identified in T-cells. The gene family is currently known to have four members (NFATc1 through NFATc4), which have roles both within and outside the immune system. NFATc1 is the major induced NFATc in human OCs, with expression greatly exceeding that of NFATc2 through NFATc4 (84). NFATc1-deficient embryonic stem cells fail to differentiate into OCs in response to RANKL stimulation and ectopic expression of NFATc1 causes precursor cells to undergo efficient differentiation without RANKL signaling, indicating that NFATc1 is a master switch for regulating terminal differentiation of OCs, functioning downstream of RANKL (51). RANKL induces and activates NFATc1 through calcium signaling, and calcineurin inhibitors such as FK506 and cyclosporin A strongly inhibit osteoclastogenesis (51). FK506-mediated inhibition of NFATc1 activity results in a defective induction of the mRNA of NFATc1, indicating that NFATc1 induction is dependent on its own activity: NFATc1 autoamplifies its own gene, possibly by binding to its own promoter (85). Transcriptional activation of NFATc1 in OCs is mediated by a RANKL/TRAF6/Fos signaling pathway because NFATc1 rescues osteoclastogenesis in precursors lacking c-Fos (51, 52). c-Jun signaling in cooperation with NFAT is crucial for RANKL-regulated OC differentiation. Osteoclastogenic activities of NFAT were abrogated by overexpression of dominant-negative c-Jun and overexpression of NFAT in transgenic mice expressing dominant-negative c-Jun rescues differentiation of OC precursor cells into TRAP-positive multinucleated OC-like cells even in the absence of RANKL (81). At the final stage of OC differentiation, NFATc1 cooperates with Fos and Jun proteins to induce OC-specific genes such as TRAP, calcitonin receptor, cathepsin K and β_3 integrin gene (61, 81, 86-88).

7.7. Mitf

Microphthalmia-associated transcription factor (Mitf) is a member of the basic/helix-loop-helix/leucine zipper (b-HLH-ZIP) transcription factor subfamily, which also includes Tfe3, Tfeb, and Tfec. Mitf is required for the proper development of several cell lineages including OCs, melanocytes, retinal pigment epithelial cells, mast cells, and natural killer cells. Mutations in Mitf result in osteopetrosis in several organisms due to defective OC development (53). Mononuclear OCs can be detected in mi/mi mice but these cells are incapable of fusing to form multinucleate cells, lack a distinct ruffled border, and are defective in bone resorption (89-92). Compared with many other osteopetrotic mouse models, such as c-fos, PU.1, and NF-κB mouse knockout models, in which earlier steps of OC differentiation are affected resulting in lower numbers of OCs *in vivo* (59, 80, 93), defects in the OCs of mi/mi mutant mouse models appear to occur late in differentiation.

The interaction of MITF with either PU.1 or PU.1-interacting protein allows efficient induction of an OC-specific marker of the TRAP gene in a synergistic manner (62). Through three consensus elements in the cathepsin K promoter, Mitf and TFE3 could regulate the expression of cathepsin K on the transcriptional level. PU.1 and MITF transcription factors also synergistically activate the expression of the osteoclast-associated receptor (OSCAR) gene, which has two Ig-like domains and functions as a bone-specific regulator of OC differentiation (94). Furthermore, MITF is a target for the RANKL signaling pathway in OCs and phosphorylation of MITF leads to an increase in OC-specific gene expression (95). M-CSF could induce phosphorylation of Mitf and TFE3 via a conserved MAPK consensus site, thereby triggering their recruitment of the coactivator p300 (96).

7.8. Myc

Genes of the Myc family contribute to the genesis of many human tumors. In mammals, there are four related genes in the Myc family, c-Myc, N-Myc, L-Myc, and S-Myc. They are key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumors (97). The number of target genes that are regulated by Myc is surprisingly large. C-Myc was also found to be strongly up-regulated in RANKL-induced osteoclast-like cells (OCLs) but was absent in undifferentiated cells. Dominant negative Myc in RAW 264.7 cells was able to block RANKL-induced OCL formation. Therefore, c-Myc is a downstream target of RANKL and its expression is required for RANKL-induced osteoclastogenesis (98). It was also found that transcription from the TRAP promoter could be negatively regulated by Myc (99).

7.9. Src

Src is a member of a family of nine NRTKs that associate with the cytoplasmic surface of cellular membranes (100). Deletion of the gene encoding c-Src

produces an osteopetrotic skeletal phenotype that is the consequence of the inability of the mature OC to efficiently resorb bone. $Src^{-/-}$ OCs exhibit reduced motility and abnormal organization of the apical secretory domain (the ruffled border) and attachment-related cytoskeletal elements that are necessary for bone resorption. A key function of Src in OCs is to promote the rapid assembly and disassembly of the podosomes, the specialized integrin-based attachment structures of OCs and other highly motile cells (101). The absence of Src affects the bone-resorbing activity of mature OCs, but does not affect OC formation (12, 102). In fact, the number of OCs in bones of $Src^{-/-}$ mice is more than twice that in normal mice, suggesting that in vivo OC differentiation and/or survival is enhanced in the absence of Src. Recently, it was reported that c-Src kinase activity, not only on the plasma membrane but also within mitochondria where it phosphorylates cytochrome c oxidase (Cox), is essential for the regulation of osteoclastic bone resorption (103). Of the several signaling mechanisms that are activated downstream of RANK, only the sequential activation of PI3K and Akt is known to involve Src and the Cbl proteins.

8. SUMMARY AND PERSPECTIVES

Osteoclasts are the principal, if not exclusive, bone-resorbing cells, and their activity has a profound impact on skeletal health. A more complete understanding of the mechanisms by which OCs differentiate from their precursors is therefore critical to developing therapies for these often debilitating diseases. In this review, we have summarized the transcription factors and cytokines that have been shown through genetic studies to be important in bone development. The $PU.1^{-/-}$ mouse lacks both OCs and macrophages; it represents the earliest developmental form of osteopetrosis yet described. PU.1 is known to be required for macrophage development during differentiation from pro-monocyte to monocyte. Recently, it was also reported that Tal-1 may lie upstream of PU.1 in a regulatory hierarchy during osteoclastogenesis (104). Differentiation of mature OCs from committed OC progenitors was blocked by disruption of the Mitf, NF-κB, c-fos, and NFATc1 genes. Although these transcription factors play an important role in OC differentiation, the expression of these transcription factors in various cell types suggests that they are unlikely to be the switch that dominates the OC differentiation process. Further study is needed to determine which OC-specific transcription factors that function together with ubiquitous transcription factors (i.e. c-fos, PU.1 and NF-κB) control OC differentiation. Cytokines including M-CSF and RANKL also induce and modulate growth and differentiation of the precursors to mature osteoclasts. M-CSF binds to its receptor to provide the signals for macrophage survival and proliferation. RANKL interaction with RANK induces recruitment and activation of TRAFs, leading to the activation of multiple signaling cascades. Taken together, osteoclastogenesis is likely to be a complicated process, controlled by several regulatory mechanisms at several differentiation transition points.

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