Chapter 2
Cellular and Molecular Aspects of Bone Repair

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Bone healing is characterized by a series of molecular, cellular, and tissue transformations consisting of resorption and formation of hard and soft tissues. Mineralized tissue remodeling in fracture repair involves the activity of various cells, inter alia, chondroblasts, osteoblasts, osteocytes, and osteoclasts (Fig. 2.1).

Bone and cartilage are produced through a concerted generation of molecular signals that act on lineage-specific stem cells. The stem cells differentiate into various cellular phenotypes. Signal conduction via hormones, growth factors, and mechanical regulation ensures subsequent remodeling of bone and cartilage [1]. Progenitor cells are recruited from periosteal and bone marrow tissues and differentiate into matrix producing mature cells at the injured bone site. Bone is essentially a type of hard connective tissue. It is involved in the regulation of body size and height and provides structural support for skeletal muscles and physical protection of vital organs. Concomitantly, bones serve as a principal depot for calcium and phosphate minerals and the essential site of marrow tissues serving as continuous source for hematopoiesis. Bone formation by osteoblasts and resorption by osteoclasts regulate skeletal remodeling throughout the life. Osteoclasts are derived from hematopoietic stem cell (HSC) of the monocyte/macrophage lineage typically located in bone marrow and blood [1]. Bone-resorbing cells have a key role in the

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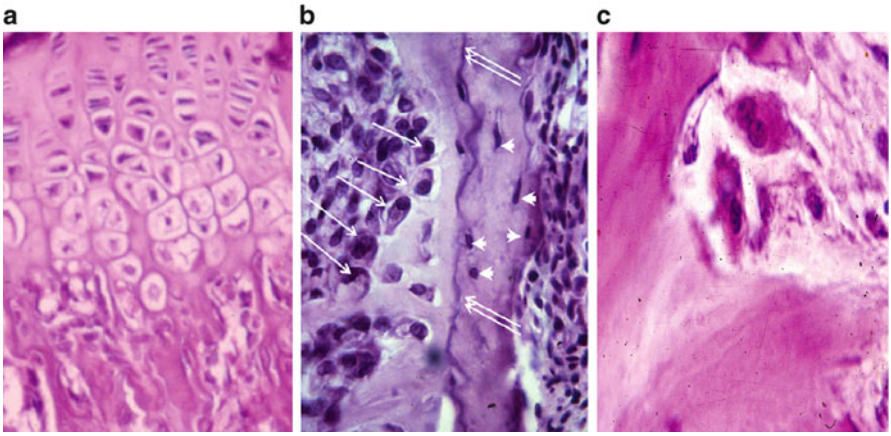

Fig. 2.1 Bone and cartilage cells. (**a**) Chondroblasts. (**b**) Osteoblasts (*arrows*) forming osteoid with Osteoblast–osteocyte transition adjacent to reversal line (*double arrows*) and osteocytes (*arrow heads*). (**c**) Osteoclasts in resorption bay

osseous healing process. Osteoblasts originate from bone marrow mesenchymal stem cells (MSCs) [2]. In healthy bone, a balance of bone formation/resorption is achieved by and large through the coordinated differentiation of these cells from their precursors. The multipotentiality of MSCs is accountable not only for the development of osteoblasts but also to a wide cellular range, including adipocytes, chondrocytes, myoblasts, and fibroblasts. MSC differentiation to the osteoblast versus adipocyte lineage has particular relevance to the maintenance of normal bone homeostasis. Accumulating evidence suggests that a shift in MSC differentiation to favor the adipocyte lineage directly contributes to imbalances in bone formation/resorption that ultimately leads to bone loss [3]. Indeed, conditions associated with bone loss such as osteoporosis and glucocorticoid excess coincides with increased bone marrow adiposity [2, 3]. The balance between adipogenic and osteogenic differentiation is regulated by ligands such as bone morphogenetic proteins (BMPs) and the osteogenic growth peptide (OGP) and receptor/transcription factor PPARγ [4, 5]. However, a multitude of regulators, including neurotransmitters and peptides, hormones, growth factors, and transcription factors are involved in the regulation of the complex and finely tuned process of osteoblast differentiation. During bone healing, the adipogenic–osteoblastogenic balance of stem cell differentiation is completely shifted toward the chondrocyte/osteoblast cell line [4–7]. Cartilage and/or bone matrices serve as provisional bridging of the fracture gap providing mechanically functional components. The coordinated production of these skeletal tissues requires timely recruitment of the progenitor cells at the site and their differentiation into chondroblasts and/or osteoblasts. Disturbances in any one of these events can have a hindering effect on bone repair.

2.1 Osteoblasts

The osteoblasts produce and regulate bone matrix and mineralization during development, remodeling, and regeneration (Fig. 2.2).

Osteoblasts arise from MSCs that develop according to a well-documented course of gene expression, progressing from osteoblastic commitment to proliferation, and final morpho-differentiation. Bone formation and repair by osteoblasts are the basis of healing of skeletal injuries and restorative procedures (Fig. 2.3).

2.2 Osteocytes

The osteocyte is the mature form of the osteoblast.

Osteoblasts and osteocytes [3, 6] produce connections with the existing embedded cells (Fig. 2.4). While becoming engulfed in the matrix, the cells are referred to as osteoid–osteocytes [7].

Mineralization of the matrix completes the osteocytic maturation. The osteocyte embedded in mineralized matrix is the stationary resident responsible for function and metabolism of bone tissue (Fig. 2.5). Osteocytes make up more than 90–95% of all bone cells in the adult skeleton.

Osteoblasts compose less than 5% and osteoclasts less than 1%. Osteocytes are viable for years, even decades, whereas osteoblasts live lifetimes of weeks and osteoclasts of days. The unique feature of osteocytes is the formation of long processes that connect through minute tubules in the bone matrix with one another and

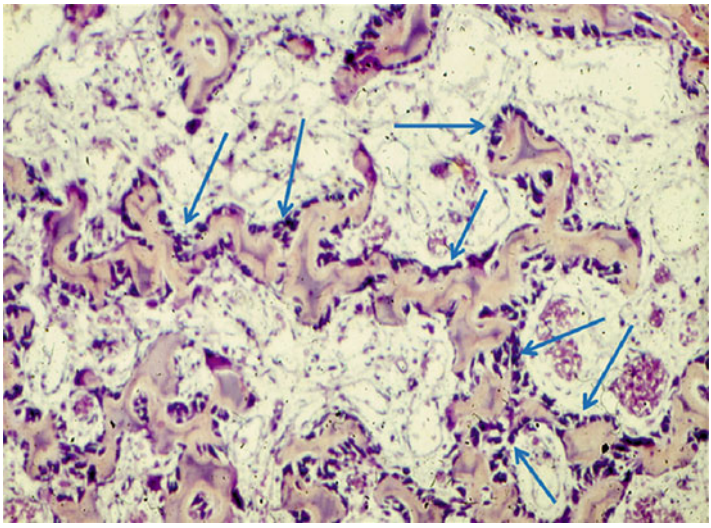

Fig. 2.2 Osteoblasts (*arrows*) aligned along primary bone surfaces

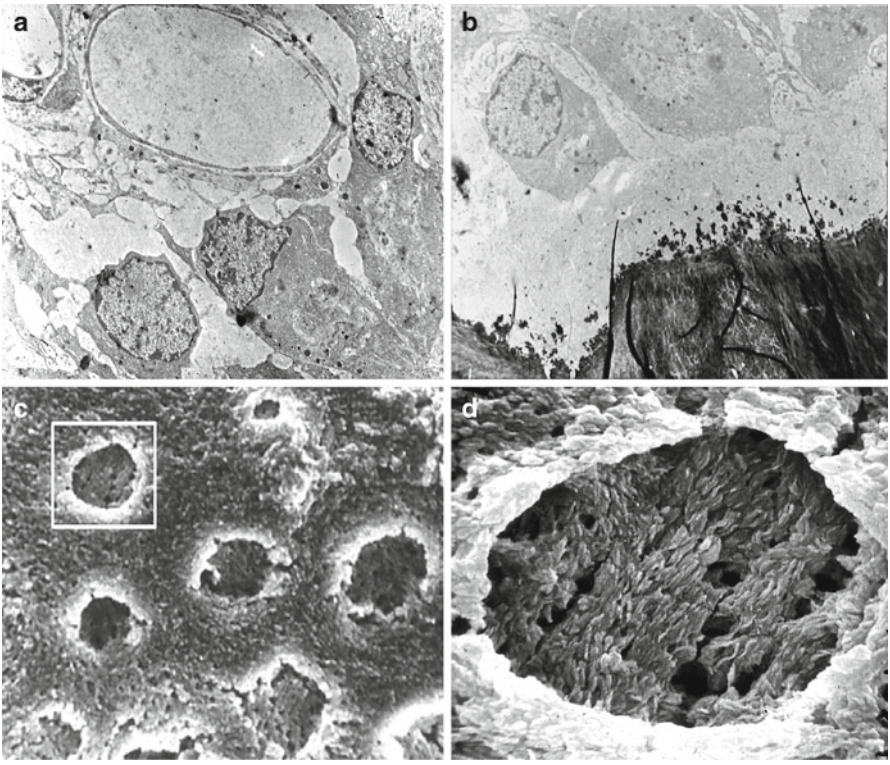

Fig. 2.3 Electron micrographs of different osteoblastic features. (**a**) Osteoblasts adjacent to blood capillary. (**b**) Osteoblasts adjacent to Calcifying front (TEM). (**c**) Osteoblastic lacunae on surface (SEM). (**d**) Higher magnification of the square in C, an osteocytic lacunae (SEM)

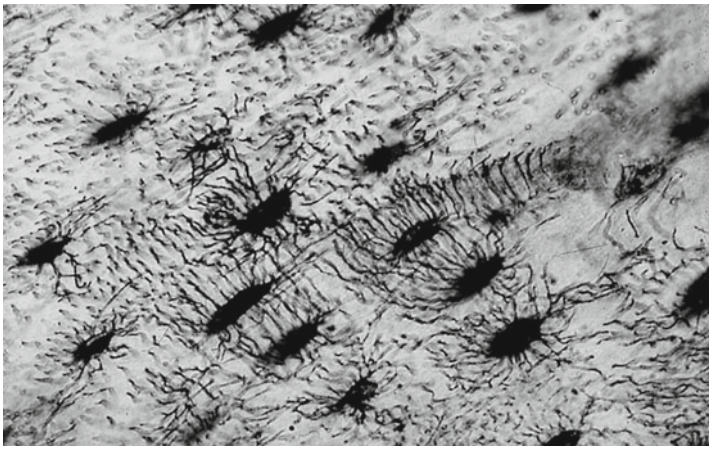

Fig. 2.4 Osteocytes and cellular processes demonstrated by impregnation methodology

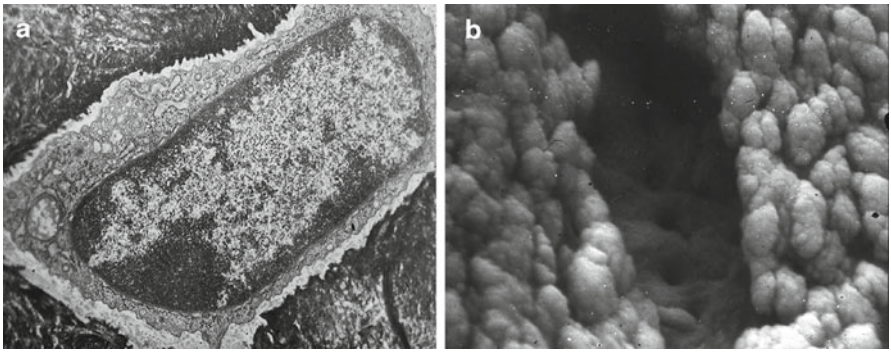

Fig. 2.5 Electron micrographs demonstrating osteocytic features. (**a**) Osteocyte embedded in mineralized matrix (TEM). (**b**) Osteocytic lacuna (SEM)

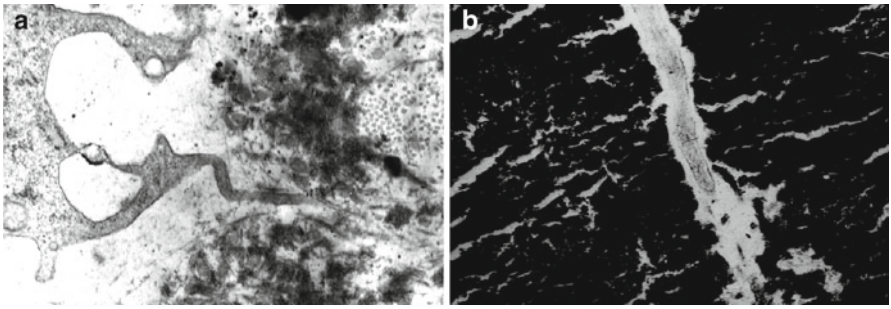

Fig. 2.6 Osteocytic processes (TEM). (**a**) Osteocyte with processes embedded in the mineralized matrix (horizontal). (**b**) Osteocytic process traversing in canaliculus in heavily calcified bone (perpendicular)

with cells on the bone surface. These processes have been shown to extend into the bone marrow [4] (Fig. 2.6).

Osteocytes send signals of both bone resorption and bone formation. It has been proposed that at death phases, osteocytes send signals initiating resorption [5, 8]. Recently, it has been shown that sclerostin, a highly expressed protein in osteocytes, targets osteoblasts to inhibit bone formation [9]. It has been suggested that osteocytes act as orchestrators, directing both osteoclast and osteoblast activity in bone remodeling. A major issue in the understanding of bone regulation concerns the probable sensing of mechanical strains by the osteocyte. It is thought that cells on the bone surface (lining cells, osteoblasts) are subjected to substrate strain, whereas osteocytes "sense" mechanical strain due to fluid flow shear stress. Osteocytes when compared to osteoblasts are more responsive to fluid flow shear stress than to other form of mechanical strain, such as substrate stretching [10]. It has been proposed that osteocytes sense shear stress mainly along their cellular processes and the cell body. Osteocytic deformation in vitro correlates with the extent of shear stress, which in turn is in direct relationship with a biological response manifested in prostaglandin release.

PKD1 and 2 are known to have mechano-sensory functions in the kidney and were shown to be expressed in bone. Deletion of PKD1 function results in animals with a bone defect [11]. In a search for markers highly expressed on osteocytes, the E11/gp38 molecule was found first in MLO-Y4 osteocyte-like cells and also in early embedding osteocytes in bone but not in cells on the bone surface [12, 13]. E11/gp38 is a 40 kDa transmembrane protein thought to play a role in the formation of cellular processes in various cell types. Cells with extensive cellular projections, such as podocytes and type1 alveolar lung cells, etc., express high amounts of E11/gp38. This membrane molecule appears to play a role in dendrite elongation, as MLO-Y4 cells subjected to fluid flow shear stress elongate their processes, and this elongation was blocked by siRNA [13]. Conditional deletion of this gene results in neonatal lethality due to lung defects [14]. In vivo loading induced elevation in both gene and protein expression of E11/gp38, not only near the bone surface but also in deeply embedded bone in response to loading [13]. It was not clear why a molecule proposed to have a role in dendrite formation would be increased in deeply embedded osteocytes-cells thought to have their dendrites stationary and tethered to the walls of their canaliculi [15, 16]. However, dynamic imaging of viable calvarial bone has shown that osteocytes can extend and retract their cell processes [17]. This suggests that E11/gp38 could be involved in the extension of dendrites in osteocytes embedded in bone in response to load. Observations using static data limit our thinking and ability to form more accurate and novel hypotheses, whereas dynamic imaging has opened a whole new area for investigation. Cellular and molecular mechanisms involved in osteoblast formation are of major significance for the progress of curative procedures. Selective expression of master transcriptional regulators is responsible for lineage commitment of MSCs. The myogenic path is regulated by MyoD (myosin dictyostelium); PPARγ (peroxisome proliferator activated receptor gamma) promotes adipogenesis; Sox9 (SRY-sex determining region Y-box 9) and Runx2 (Runt-related transcription factor 2) are responsible for chondrocytic and osteoblast differentiation, respectively [8–11, 18]. Lineage commitment of osteoprogenitors is followed by a proliferative stage, characterized by the production of proteins such as histones, fibronectin, type I collagen c-Fos (antisense oligonucleotide), c-Jun (N-terminal kinases), and p21 (cyclin-dependent kinase inhibitor 1) [12]. Following division, cellular transition expresses genes such as alkaline phosphatase, bone sialoprotein, and type I collagen, producing osteogenic extracellular matrix. Concomitantly the osteoblasts express genes engaged in mineralization of the extracellular matrix such as osteocalcin, osteopontin, and collagenase [13]. Transcription factors including Runx2, Osx (osterix, osteoblast-specific transcription factor), SMADs, TCF/LEF (transcription factor/lymphoid enhancer factor), NFATc1 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1), Twist (twist homolog 1), AP-1 (adaptor-related protein complex 1), and ATF4 (activating transcription factor 4) regulate the program of gene expression and cellular differentiation. Notably, micro-RNAs (miRs) have been identified as regulators of osteoblast gene expression. The mechanistic control of gene expression by cofactors such as acetyltransferases and histone deacetylases (HDACs) has been identified. Numerous transcription factors and epigenetic

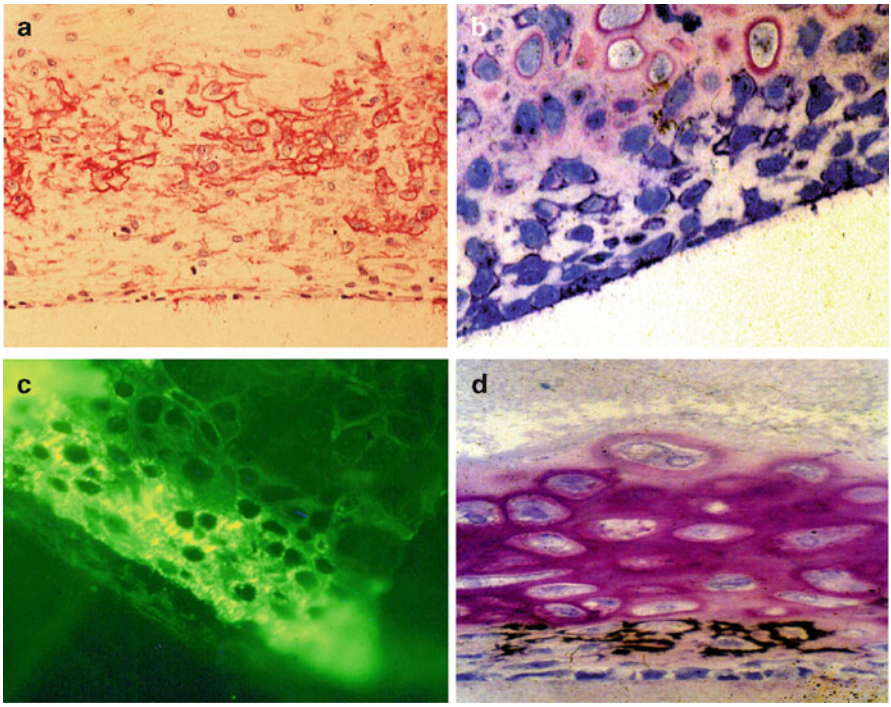

Fig. 2.7 Osteoblasts producing mineralizing matrix in diffusion chamber cultures of MSCs. (**a**) Osteoblast cell membranes stained red with histochemical reaction for alkaline phosphatase. (**b**) Autoradiograph showing distribution of PTH receptors. (**c**) Immunohistochemical staining of collagen type I in osteoblast layer. (**d**) Periosteoblast mineral deposition demonstrated by Von Kossa staining

co-regulators are involved in the genesis of the osteoblast and in the mechanisms that determine the functions as regulators of gene expression (Fig. 2.7, Table 2.1).

Runx2 is often depicted as main regulator of osteoblast-genesis [14] (Table 2.1). It operates during induction, proliferation, and maturation of osteoblasts and controls expression of a range of genes. Haplo-insufficiency of Runx2 causes skeletal abnormalities, delayed ossification of skull-bones, cleidocranial dysostosis, and dental defects. Homozygous mutation of Runx2 is lethal in mice due to a complete lack of mineralized bone [11, 15, 18]. Runx2 expression is poorly correlated with expression of its target genes, indicating that Runx2 activity is regulated by additional factors. In fact, Runx2 is subject to posttranslational regulation by phosphorylation, acetylation, and ubiquitination. In addition to its Runt-class DNA-binding motif, Runx2 protein contains multiple domains that mediate either transcriptional activation or repression through associations with co-activators or co-repressors [16, 17]. These various modes of control enable Runx2 to function as a master

Table 2.1 Sequential marker-gene expression in osteoblast differentiation

Stromal stem cell	Osteoprogenitor	Pre-osteoblast	Osteoblast	Osteocyte
Sca-1	Runx2	Runx2	Runx2	Runx2
Stro-1	Osx	Osx	Osx	Osx
	COL-1	COL-1	COL-1	COL-1
		TNSALP	TNSALP	TNSALP
		PTHRc	PTHRc	PTHRc
			OCN	OCN
				SOST
				DMP1

regulator, integrating diverse signals to activate or repress transcription in a precise spatiotemporal manner and in response to changing physiological needs.

Further co-activators of Runx2 function comprise histone acetyltransferases, p300, CBP, PCAF, MOZ, and MORF [19, 20]. These can add acetyl groups to lysine residues of histone and non-histone target proteins, which modifies protein function by a variety of mechanisms including altered protein–protein interaction and altered protein stability. In the case of nucleosomal histones, acetylation is associated with a more open chromatin structure, recruitment of bromo-domain proteins, and increased transcriptional activity at a locus, while histone deacetylation catalyzed by HDACs is correlated with chromatin condensation and transcriptional repression. The interaction between Runx2 and HDACs is based on the observation that HDAC inhibitors reduce the activities of various Runx2 repression domains [21]. A candidate gene approach confirmed that HDAC6 binds Runx2 and represses its activity. Furthermore, Runx2 is functionally inhibited by HDAC3, HDAC4, HDAC5, and HDAC7 [22–27]. HDAC proteins are known to form large multicomponent repressive complexes composed of cofactors such as NCor, SMRT, and Sin3a, as well as multiple HDACs. It remains poorly understood how these complexes participate in the regulation of Runx2 activity, although it has been shown that Runx2 target gene expression is repressed by HDACs through multiple distinct mechanisms and in response to various osteogenic signals such as BMP2 and PTH. Runx2 was shown to recruit HDAC3 to the BSP promoter, where it represses transcription by deacetylating histones [27]. Runx2 protein is subject to proteolysis in response to Smurf1 (SMAD-specific E3 ubiquitin protein ligase 1) and Schnurri-WWP1 ubiquitin ligases [27–36] (Schnurri, Mammalian Homolog of the *Drosophila* Zinc Finger Adapter Protein Shn). BMP2 protects Runx2 from Smurf1-catalyzed proteolysis by stimulating Runx2 acetylation through a SMAD-dependent mechanism [23]. Runx2 acetylation by p300 is counteracted by HDAC4 and HDAC5, which remove the acetyl groups from Runx2, thus promoting Runx2 ubiquitin-mediated proteolysis (Interestingly, estrogen receptor-related receptor γ, an orphan nuclear receptor whose expression in osteoblasts is stimulated by BMP2, competes with p300 (E1A binding protein p300) for binding to Runx2 and inhibits BMP2-induced osteoblast formation [37]. Runx2 recruits both HDAC6 [21] and HDAC7 [22] to chromatin, which repress Runx2 target gene transcription. However, the mechanism of this repression is still incompletely understood. Inhibitors of deacetylase enzymatic activity facilitate repression by HDAC6 [21], whereas HDAC7 represses Runx2

through a not yet revealed mechanism that does not require its deacetylase domain or catalytic activity [22]. BMP2 activates protein kinase D 1 (PKD1), which phosphorylates HDAC7, leading to a transient export of HDAC7 from the nucleus, and freeing Runx2 from HDAC7's repression [38]. HDACs 4, 5, and 7 can be exported from the nucleus in response to the same set of protein kinases, yet they exhibit different subcellular distributions and respond differently to BMP2 stimulation in osteoblast-like cells [38]. Parathyroid hormone (PTH) regulates skeletal physiology by stimulation of Runx2 interactions with acetyltransferases. PTH is a strong inducer of matrix metallopeptidase, MMP-13 transcription in osteoblasts [39, 40]. Stimulation of these cells with PTH leads to a protein kinase A-dependent binding of p300 to Runx2 on the MMP-13 promoter, resulting in increased histone acetylation and gene transcription [41]. PTH also regulates Runx2 activity through other mechanisms such as phosphorylation [42] and promotes interactions with adaptor-related protein complex 1, AP-1 transcription factors [43, 44]. Finally, PTH decreases Runx2 protein stability by ubiquitin-mediated proteolysis, limiting PTH stimulation of osteoblastic genes [36].

Osterix: (Osx, also known as Sp7) is a Runx2-induced transcription factor expressed in osteogenic cell progenitors, committing them to the osteoblast, rather than chondroblast lineage [45]. Osx-null mice die at birth due to lack of mineralized skeletons. Bones formed by intramembranous ossification are entirely non-mineralized, while endochondral bones exhibit regions of mineralized cartilage, indicating that Osx functions specifically in osteoblasts. Despite its evident importance in bone formation, relatively little is known about regulation of Osx expression, its functional partners, or its direct target genes. Osx expression was believed to be downstream of Runx2, because Runx2 expression is normal in Osx-null mice, while Osx expression is absent in Runx2-knockout mice [45]. This was confirmed through characterization of a Runx2-binding element in the Osx gene promoter [46]. Osterix activation of the Col1A1 (collagen, type 1, alpha 1) promoter is enhanced by binding of NFATc1 to Osx, an interaction that is disrupted by calcineurin [47]. Another function of osterix is as an inhibitor of canonical Wnt signaling by inhibiting DNA binding of transcription factors [48] (Table 2.1).

ATF4 (*activating transcriptionfactor 4*): RSK2 is a ribosomal serine/threonine kinase mutated in Coffin–Lowry Syndrome, a disorder that includes various skeletal abnormalities. The positive role of ATF4 on osteoblast formation was recognized with the findings that it is a substrate for the RSK2 kinase and ATF4-deficiency decreased bone formation [49], while forced accumulation of ATF4 induced osteoblastic gene expression in non-osseous cells [50]. ATF4 forms a complex with Runx2 at the osteocalcin promoter to increase osteocalcin *transcription* [51]. The transcriptional activity of this complex is furthered by PTH signaling and by associations with C-EBP (CAAT-enhancer binding protein) and the TFIIAγ (General Transcription Factor IIA-Gamma) [52–54]. (CCAAT is the abbreviation for cytidine–cytidine–adenosine–adenosine–thymidine.) Interestingly, ATF4 in osteoblasts was recently found to regulate energy metabolism through decreased insulin production and insulin responsiveness via altered osteocalcin and leptin endocrine signaling pathways [55, 56].

2.3 SMADs (A Combination of Two Abbreviations, SMA and MAD)

SMAD proteins are homologs of both *Caenorhabditis elegans* protein SMA and the drosophila protein, mothers against DPP = DecaPentaPlegic MAD. The BMP and TGFβ families of growth factors have long been recognized as vital regulators of skeletal physiology. TGFβ or BMP signaling leads to phosphorylation and nuclear translocation of receptor-activated SMADs (rSMADs). These interact directly with the DNA and associate with other transcription factors to regulate gene transcription. rSMADs direct mesenchymal cells into the osteoblast lineage through induction of Runx2 expression[57]. They also interact with the Runx2 protein to synergistically regulate transcription [57–61]. The SMAD-interaction domain in Runx2 has been identified and is continuous with the nuclear matrix targeting sequence, which is necessary for Runx2 function [60–62]. SMADs are inactivated by Smurf-directed ubiquitination, resulting in their proteolytic degradation. An interesting feedback loop between BMP/SMAD/Runx2 signaling is indicated by recent studies which showed that BMPs act through Runx2 to induce expression of SMAD6, an inhibitory SMAD protein that represses BMP signaling [63]. SMAD6 stimulates Runx2 ubiquitination and degradation by Smurf1 [64]. This process would be a potential mechanism to prevent excess BMP/Runx2-mediated osteogenesis (Table 2.1).

NFATc1/Calcineurin: NFATc1 (nuclear factor of activated T-cells) is a transcription factor that plays a central role in osteoclast formation and in T-cell development [65]. In unstimulated cells, NFATc1 is highly phosphorylated and localized to the cytoplasm. Intracellular calcium signaling activates the phosphatase calcineurin, which dephosphorylates NFATc1, permitting its nuclear import and NFATc1-mediated gene expression. Given the importance of NFATc1 in osteoclastogenesis, it would be expected that administration of calcineurin inhibitors would suppress resorption and increase bone mass; however, calcineurin inhibitors actually result in ostepenia. Koga et al. resolved this paradox by showing that in addition to inhibiting osteoclasts, calcineurin inhibitors blocked osteoblast maturation and mineralization by preventing a previously unknown synergy between NFATc1 and osterix in osteoblasts [47]. In a subsequent study, Choo et al. showed that overexpressed constitutively active (nuclear) NFATc1 inhibited MC3T3 E1 osteoblast differentiation in vitro and reduced expression of osteocalcin as a result of inhibited TCF/LEF transcriptional activity, which was due to sustained recruitment of HDAC3 and decreased histone acetylation at the proximal osteocalcin promoter [26].

Twist: Twist is a basic helix-loop-helix transcription factor that regulates differentiation of multiple cell types. Heterozygosity for Twist-1 in mice or humans results in premature fusion of the skull sutures, suggesting that Twist antagonizes osteoblast formation [66–70]. One mechanism through which Twist-1 acts to impair osteoblastogenesis is by binding to the Runx2 DNA-binding domain and inhibiting its ability to bind DNA [69]. Twist also inhibits BMP/SMAD responsive transcription by forming a complex with Smad4 and HDAC1 [70].

AP-1: The AP-1 class of transcription factors is composed of heterodimers of Fos-related factors (c-Fos, Fra1, Fra2, and FosB) and Jun proteins (c-Jun, JunB, and JunD). Multiple Fos and Jun proteins are highly expressed in proliferating osteoprogenitors. Their expression decreases during differentiation such that Fra2 and JunD are the primary AP-1 components present in mature osteoblasts [71]. Targeted deletion and transgenic overexpression strategies have been used to examine the role of individual Fos and Jun proteins in mice. Deletion of c-Fos had little effect on bone formation [72], while its overexpression led to osteosarcomas [73]. Fra1 and ΔFosB (an alternative splice variant of FosB) overexpressing mice exhibit enhanced osteoblast formation [74, 75], while deletion of Fra1 or JunB reduced bone mass. Recent work by Chang et al. demonstrates that inhibition of NF-κB signaling specifically in differentiated osteoblasts promotes bone formation through increased Fra1 expression [76]. These observations indicate that AP-1 proteins promote bone formation. In contrast, deletion of JunD increased bone mass, apparently by increasing expression of Fra1, Fra2, and c-Jun, suggesting that JunD represses expression of other AP-1 proteins in osteoblasts [77]. A number of direct targets of AP-1 in osteoblasts have been identified, and include the osteocalcin, collagenase-3 (MMP13), bone sialoprotein, and alkaline phosphatase promoters [78]. At these promoters, AP-1 physically and functionally interacts with other transcription factors such as the vitamin D receptor and Runx2 to regulate gene expression. Yet another layer of complexity to AP-1 signaling involves alternative protein isoforms. As mentioned above, ΔFosB, which is a splice variant of FosB that lacks the amino-terminus, promotes osteoblast formation through incompletely understood mechanisms. Translational initiation of the ΔFosB mRNA from an internal methionine can produce a further truncated protein, known as Δ2ΔFosB, which lacks any known transcriptional activation domains, yet enhances osteoblast formation by increasing BMP/SMAD signaling [78].

Tcf7/Lef1 TranscriptionFactors: Tcf7 proteins and Lef1 are high mobility group proteins best known as nuclear effectors of canonical Wnt signaling. Activation of the canonical Wnt signal transduction pathway stabilizes β-catenin, which translocates to the nucleus and associates with Tcf/Lef1 transcription factors, displacing HDACs and other co-repressors while recruiting additional co-activators to stimulate gene expression [79–83]. Tcf7 (also known as Tcf1), Tcf7L2 (Tcf4), and Lef1 are expressed in osseous cells [84–87]. Although Tcfs are functionally redundant in some instances, emerging evidence demonstrates distinct roles for Tcf7/Lef1 factors in osteoblasts. Expression of a mutated and constitutively activated version of the Tcf7/Lef1 co-activator, β-catenin, in osteoblasts using the (2.3)ColIA promoter stimulated osteoprotegerin (OPG) expression, leading to decreased osteoclastogenesis and bone resorption, but had little effect on osteoblast formation [84]. Conversely, Tcf7 knockout mice showed decreased OPG expression, enhanced osteoclast activity, and increased resorption [84]. Lef1 also contributes to osteoblast function. Lef1+/− female mice exhibited reduced osteoblast activity resulting in decreased bone mass [88], while homozygous Lef1−/− mice show reduced body size and die by 2 weeks of age [86]. Lef1 expression decreases during osteoblast differentiation and overexpression of Lef1 inhibited differentiation and expression of late osteoblast markers, indicating that Lef1 inhibits late stages of osteoblastogenesis

[85, 89]. Subsequent work by Hoeppner et al. identified an alternative variant of Lef1, Lef1ΔN, which lacks the N-terminal β-catenin binding domain [90]. Lef1ΔN expression increases during differentiation and in response to BMP signaling and Runx2, and leads to accelerated osteoblast formation. Likewise, although Runx2 expression is directly enhanced through canonical Wnt signaling through TCF-7 [91], Runx2's transcriptional activity is repressed by binding to Tcf/Lef transcription factors in osteoblasts, providing a novel means for feedback between Wnts signaling and Runx2 [85]. Wnt signaling is believed to act downstream of BMP signaling in the differentiation of pre-osteoblastic cells, as induction of osteoblasts by Wnt3a or activated β-catenin is independent of BMP signaling, whereas attenuated Wnt signaling impairs BMP2-induced expression [92–94]. Wnt-responsive transcription in osteoblasts is also antagonized by FGF signaling, through decreased expression of frizzleds and TCF/LEFs [95] (TCF/LEF—T-cell factor/lymphoid enhancer factor). Together, these studies demonstrate functional complexity within the TCF/LEF family and illustrate some of the opportunities for regulatory crosstalk to integrate diverse signals and modulate gene expression in osteoblasts.

ZFP, *Zinc FingerProteins*: Two major families of zinc finger transcription factors are the Kruppel-like factors (KLFs) and specificity proteins (Sps). Members of both groups participate in regulation of gene expression in osteoblasts through interactions with other transcription factors at target gene promoters. Zfp521, a KLF protein, is expressed in osteoblast precursors, osteoblasts, and osteocytes, as well as chondrocytes [96]. Its expression increases during osteoblast differentiation and in response to PTHrP, while BMP2 decreases ZFP521 levels. ZFP521 binds to Runx2 and antagonizes Runx2 gene transactivation, and overexpression of ZFP521 in in vitro osteoblast cultures impairs their differentiation. These observations indicate an inhibitory role for Zfp521 in osteoblasts. Unexpectedly, mice overexpressing ZFP521 in osteoblasts, under control of the OG2 osteocalcin promoter element, exhibit increased bone mass, even though isolated calvarial osteoblasts from these mice show impaired osteoblastic differentiation. The authors speculated that this difference may stem from the OG2 promoter not being expressed until relatively late in osteoblastogenesis. The Sp family of transcription factors is ubiquitously expressed (with the exception of Osx, which is also known as Sp7), and is involved with both basal and induced gene expression. Sp1 cooperates with ETS transcription factors at the Runx2 P1 promoter to stimulate transcription of Runx2 [97]. In osteoblasts, Sp1 and Sp3 cooperate with TGFβ-responsive SMADs to induce the β5 integrin promoter [98]. Similarly, Sp1 cooperates with Runx2 to mediate PTH-induction of the matrix gla protein promoter, while Sp3 is an inhibitor of this promoter [99].

2.4 Regulation of Osteoblast Gene Expression by MicroRNAs

Progress in the understanding of the regulation of osteogenesis involves the role of miRs. Short noncoding RNAs, range from 18 to 25 nucleotides, which regulate gene expression by binding to the 3′-UTR of mRNAs for specific target genes and

inhibiting gene expression by either promoting degradation of the target mRNAs or inhibiting their translation [100]. Many of these miRs inhibit osteogenesis through repression of osteoblastic genes. In an important study, Li et al. identified a novel mechanism through which BMP-2 promotes osteoblastogenesis [101]. By RNA expression profiling, they identified a set of 22 miRs whose expression was reduced by BMP2 stimulation of C2C12 mesenchymal cells. These miRs are predicted to inhibit a range of pro-osteogenic factors; hence, reduced levels of these miRs should enhance expression of osteogenic genes. Osteoblastic proliferation is inhibited by miR-125b, which inhibits the ErbB2 receptor tyrosine kinase [101]. Two miRs that inhibit expression of Dlx5 (distal-less homeobox 5) in pre-osteoblasts have been identified [102]. MiR-29a and miR-29c are expressed in response to canonical Wnt signaling and inhibit expression of the extracellular matrix protein Osteonectin, which is important in numerous processes in skeletal physiology [103]. Not all miRs are functional inhibitors of osteoblastogenesis. TGFβ signaling inhibits osteogenesis, and MiR-210 acts as a positive regulator of osteoblastic differentiation by inhibiting expression of ACVR1B (activin receptor 1B) for TGFβ [104].

2.5 Chondroblast

The process of fracture healing exhibits often a high similarity to endochondral ossification. This has been confirmed by numerous histological, cellular, and molecular studies. Consequently, a description of the cells involved in this process and their regulation are briefly reviewed. Since the body of information on the cellular and molecular processes in growth plate cartilage (Fig. 2.8) is substantially greater, this process is described below as a paradigm highly relevant to fracture healing.

In endochondral ossification, progenitor cells present in the resting zone serve as a reservoir for the proliferative cellular zone. Further maturation is characterized by termination of cell division and further differentiation into prehypertrophic and later to hypertrophic chondrocytes [105–108] (Fig. 2.9).

Both proliferating and hypertrophic chondrocytes secrete extracellular matrices that typically contain collagen type II and type X, respectively. The extracellular matrix in the hypertrophic cell zone mineralizes. Following resorption by chondroclasts/osteoclasts, the cartilage is replaced by trabecullar bone [106, 108–110].

Although critically affected by growth hormone, the regulation of endochondral ossification has been attributed primarily to mechanisms intrinsic to the cartilage [105, 111]. The cartilage maturation and eventual resorption and replacement are associated with structural changes such as reduced heights of the proliferative and hypertrophic cell zones, as well as reduced hypertrophic cell size and column density [112]. It has been suggested that this decline occurs since the progenitor cells have a definitive proliferative capacity that is gradually exhausted [105, 112, 113]. The cartilaginous intrinsic paracrine factors that regulate chondrocyte proliferation, differentiation, and senescence are insulin-like growth factors I (IGF-I), Indian

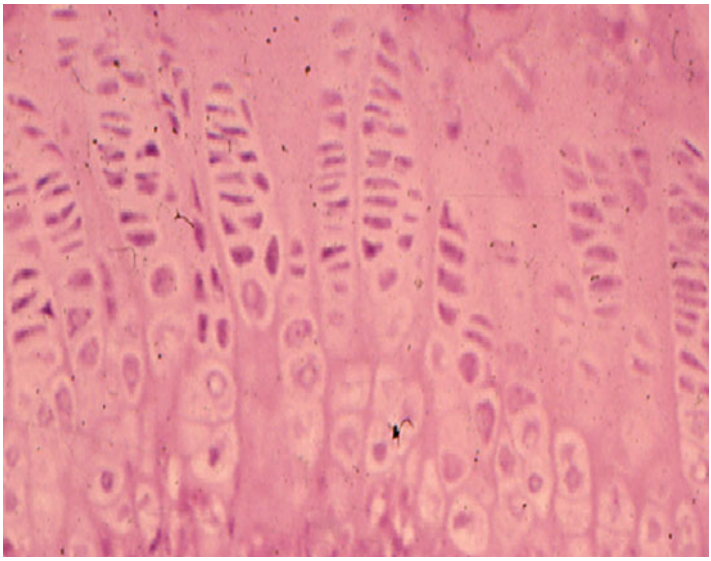

Fig. 2.8 Growth plate cartilage; note, typical palisading chondrocytes. Proliferating cells (*upper*). Hypertrophic differentiated cells (*lower*)

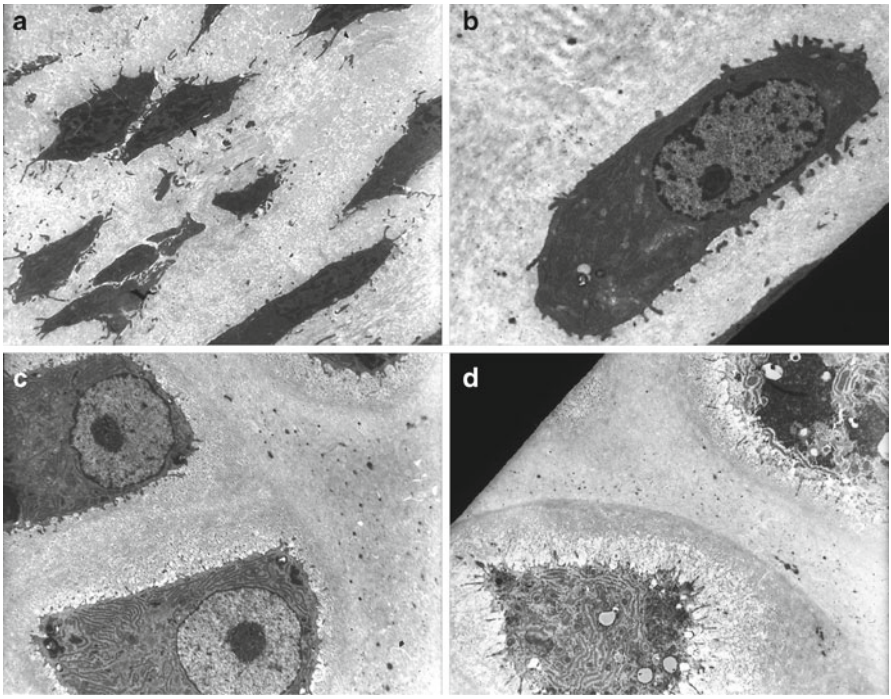

Fig. 2.9 Electron micrographs of growth plate chondroblasts. (**a**) Proliferative zone. (**b**) Prehypertrophic differentiation. (**c**) Hypertrophic chondrocytes. (**d**) Apoptotic chondrocytes

hedgehog (Ihh), PTH-related protein (PTHrP), fibroblast growth factors (FGF), transforming growth factor β (TGFβ), and BMPs.

IGF-I: The main cartilage intrinsic regulator is IGF-I, which is expressed in proliferating chondrocytes and to a lesser extent in hypertrophic chondrocytes [114]. It should be noted that exogenously administered IGF-I can markedly improve linear skeletal growth, supporting a suggested role for circulating IGF-I [105, 115].

IGF-Binding Proteins (*IGFBPs*): The cellular availability of IGF-I is also regulated by the IGFBPs, a family of proteins with high affinity especially for IGF-I. IGFBP-2, -3, -4, and -5 are present in all layers of the osteogenic cartilage, with IGFBP-3, -4, and -5 expressions being reduced in hypertrophic chondrocytes. IGFBPs are regulated by IGF-I and to a lesser extent by IGF-II [116].

Ihh: Indian hedgehog is a member of the family of hedgehog (HH) proteins that includes also sonic HH (SHH) and desert HH (DHH). The HH signal is received and transduced via a specific receptor complex composed of patched (PTCH) and smoothened (SMOH) transmembrane proteins [117]. Ihh is expressed in the prehypertrophic cells that have just stopped proliferating [118–121]. Its main action in the osteogenic cartilage is through the regulation of PTH-related peptide (PTHrP) [118–120]. Ihh appears to be both necessary and sufficient for PTHrP expression [122]. It inhibits hypertrophic chondrocyte differentiation, and thereby delays the mineralization of the cartilage matrix and its resorption. Ihh regulates cartilage development through PTHrP-independent pathways as well. Ihh stimulates differentiation of periarticular to columnar chondrocytes thereby regulating column length independently of PTHrP [119, 121, 123].

PTHrP: This is an auto/paracrine factor [118] that binds to and activates the PTH/PTHrP receptor, which is also activated by PTH [124], a G protein-coupled receptor. In osteogenic cartilage, PTHrP mRNA is expressed by perichondrial cells and proliferating chondrocytes in the periarticular region [122]. The PTH/PTHrP receptor is expressed in proliferating/prehypertrophic chondrocytes. Its activation delays maturation thus ensuring a supply of proliferating chondrocytes, which is essential for skeletal growth and repair [125]. Cells at a distance from the source of PTHrP withdraw from the cell cycle and begin terminal differentiation. PTHrP appears to promote chondrocyte proliferation and delays differentiation by several mechanisms [121, 125]. It inhibits production of p57, an inhibitor of cyclin-dependent protein kinases. It also regulates phosphorylation of the transcription factor Sox9, a master transcription factor in chondrogenesis. Sox9 phosphorylation increases its transcriptional efficiency and decreases terminal differentiation. PTHrP also decreases the production of Runx2 [125, 126], a transcription factor essential for osteoblast-specific gene expression and for bone formation. Recent experiments have shown that Runx2 is expressed in the prehypertrophic and hypertrophic zones of embryonic mouse cartilages and plays a role in chondrocyte maturation. [122, 125, 126].

The Ihh-PTHrP circuit is regulated by IGF-I. Lack of IGF-I alters this circuit, dissociating the regulation of Ihh and PTHrP, which results in downregulation of Ihh expression and upregulation of PTHrP expression [127]. In the growth plate,

IGF-I deficiency results in an elevated, abnormally distributed, PTHrP expression in proliferative and hypertrophic chondrocytes. This would be expected to delay the rate of differentiation of chondrocytes, and delay mineralization, similar to overexpression of PTHrP in transgenic mice [127].

FGF: The FGFs comprise a family of secreted proteins that form a trimolecular complex by binding to one of four high affinity FGF receptors (FGFRs) and heparan sulfate proteoglycans [128–132]. All FGFRs are expressed in the osteogenic cartilage. FGFR3 has gained more attention than the other FGFRs. It is a master inhibitor of chondrocyte proliferation and growth [118, 132, 133]. Mutations in FGFR3 lead to short stature syndromes [118, 132, 133]. This effect of FGFR3 signaling involves direct action on chondrocytes as well as suppression of Ihh expression [120]. The FGFRs are activated by FGF-1,-2,-7,-17,-18,-19, and -22 [132].

Wnt Proteins (Wingless-type MMTV Integration Site Family) (MMTV abbreviation of Mouse Mammary Tumor Virus): Wnt proteins are powerful secreted signaling factors that regulate a number of developmental processes [123, 134]. The vertebrate Wnt family currently comprises 20 members. Wnt proteins act by binding to Frizzled and low-density lipoprotein receptor-related protein cell surface receptors. Upon Wnt binding, Frizzled receptors transduce signals via the β-catenin-LEF/TCF pathway, Ca2-calmodulin–PKC pathway, or JNK-dependent pathway [134]. Depending on the developmental stage, disruption of the canonical β-catenin pathway either blocks chondrocyte hypertrophy and endochondral ossification (early stages) or stimulates hypertrophy and ossification (later stages) [134]. The Wnt family member mainly implicated in growth regulation is Wnt9a, acting as a temporal and spatial positive regulator of Ihh [123].

TGFβ: TGFβ-related proteins form a large family of secreted molecules including, among others, TGFβs, activins, and BMPs [106, 109, 135]. These molecules form either homodimers or heterodimers, and exert their activity through type I and type II serine/threonine kinase receptors. ALK5/TGFβRI and TGFβRII are expressed in proliferating and hypertrophic chondrocytes and in the perichondrium [106, 109, 135]. TGFβ is secreted by chondrocytes and stimulates PTHrP production in perichondrial cells [109]. Disrupting the TGFβ signaling pathway (SMAD 3) lead to progressive cartilage abnormalities, including premature hypertrophy of growth plate chondrocytes and disorganization of the growth plate columns resulting in decreased longitudinal growth [109, 136].

BMPs: In the osteogenic cartilage, most of the BMP expression is found in the perichondrium (BMP-2, -3, -4, -5, and -7). In addition, BMP-2 and -6 are present in hypertrophic chondrocytes and BMP-7 in proliferative chondrocytes [118]. The BMPs are positive modulators of chondrocyte proliferation and negatively regulators of chondrocyte terminal differentiation [118, 137]. BMP-6 accelerates calcified matrix deposition, thus being involved in the cartilage-to-bone transition [138]. The type I BMP receptors exhibit characteristic expression patterns in the cartilage. BMPR1A is highly expressed in the perichondrium and in proliferating and hypertrophic chondrocytes. BMPR1B is found throughout the cartilage

[137, 139]. The type II BMP receptor is also expressed throughout the cartilage. BMPR1A has been implicated in the differentiation of proliferating chondrocytes toward hypertrophic chondrocytes [140]. BMPs interact with the IHH/PTHrP pathway by promoting Ihh expression by prehypertrophic chondrocytes and can therefore increase the proliferation of chondrocytes [124, 137]. IHH controls BMP levels, operating in a positive feedback loop [137].

Hypoxia: The osteogenic cartilage is largely avascular, resulting in low O_2 tension. There is an O_2 gradient with lowest levels of O_2 in chondrocytes of the core hypertrophic zone [140, 141]. The hypoxic signals are transmitted to the cells by prolyl hydroxylases (PHDs), which are O_2 sensor proteins found in chondrocytes [141]. When activated, PHDs enhance hydroxylation of specific prolyl and asparagyl residues of the transcription protein, hypoxia-inducible factor 1 (HIF-1). HIF-1 is composed of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed whereas HIF-1α protein is highly unstable, and its accumulation is regulated by the von Hippel–Lindau (VHL) protein, an E3-ubiquitin ligase. Under normoxic condition, this ligase targets HIF-1α to the proteasomal degradation. Conversely, in hypoxic conditions, HIF-1α is not recognized by VHL. It translocates to the nucleus and forms a complex with HIF1-β, which binds to a HIF response element present in HIF target genes. HIF-1α negatively regulates chondrocytes proliferation and promotes their survival [118, 141].

Apoptosis: Apoptosis of hypertrophic chondrocyte, which occurs at the cartilage vascular interface, is central to endochondral ossification and elongation. Changes in mitochondrial function initiated by early apoptotic events and modulated by the Bcl-2 (B-cell leukemia/lymphoma 2) family of proteins regulate calcium accumulation and release [107, 109]. Calcium released from hypertrophic chondrocytes generates matrix calcification nucleated by matrix vesicles, the remnants of apoptotic chondrocytes. Apoptosis triggered events lead to activation of pretenses on the cell surface and within the matrix, and the destruction of the cartilage matrix. Apoptosis initiated activation and release of growth factors regulates the homeostatic maintenance of growth plate width, stimulation of blood vessel invasion, stimulation of osteoblast recruitment, and the formation of blood vessels and osteoid [142]. Chondrocyte apoptosis is regulated by signals triggered by local factors such as FGF-2 that leads to increased apoptosis or PTHrP that upregulates Bcl-2 expression as part of its mechanism to control the rate of chondrocyte turnover [107].

2.6 Osteoclast

Bone fracture is followed by a unique healing process, which initially shares certain features with healing processes in other connective tissues. The injury may involve consequent to the location, cortical bone, periosteum, bone marrow and additional soft tissues. The trauma sets off an inflammatory response characterized by a series of molecular and cellular events concurrent with substantial MSC recruitment.

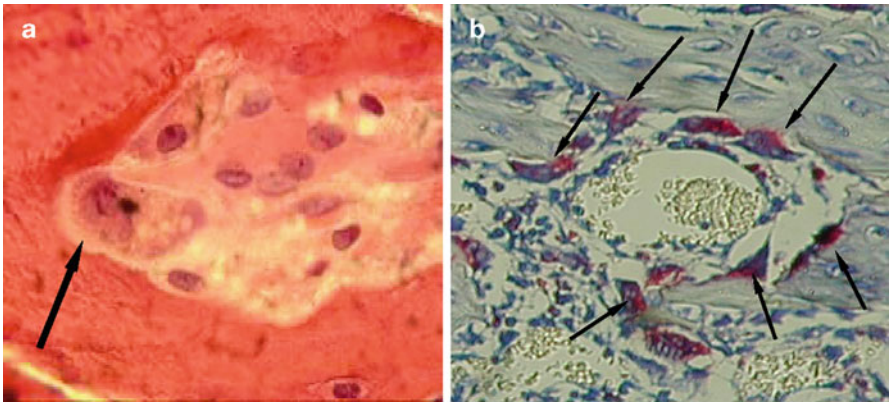

Fig. 2.10 Histological features of osteoclasts. (**a**) A typical appearance of osteoclasts in bone remodelling unit (*arrow*) in H&E Stain. (**b**) Osteoclasts (*arrows*) in tartarate-resistant acid phosphatase staining (*red*)

This is followed by the emergence of a large number of osteoclasts, primarily responsible for an extensive cartilage and bone resorption, in which the mineralized constituent and the organic matrix are disintegrated (Fig. 2.10). Concomitantly, endothelial cells initiate angiogenesis and progenitor cells differentiate into chondroblasts and osteoblasts that form a bridging callus at the fracture gap. Further resorption and ossification brings about restoration of the original bone. Callus remodeling concludes with the regeneration of a mechanically competent osseous structure.

Origin and Genesis of Osteoclasts. The osteoclast is a multinuclear phagocyte derived from bone marrow HSCs. These cells serve as a common origin to all blood cells as well as other members of the immune system. Multinucleation is ascribed to the fusion of precursor monocytes. The osteoclast constitutes an essential linkage between the immune and the osseous systems. Evidently, a variety of cytokines, their receptors, and downstream signaling pathways are operative in both systems. Cells of the osteoblastic lineage, as well as immune cells, express factors that induce osteoclast formation. Among those, macrophage colony stimulating factor (M-CSF), receptor–activator of NFκB ligand (RANKL), and tumor necrosis factor (TNF) are counted. These factors induce mononuclear cells to fuse and form multinucleated osteoclasts with bone-resorbing capability. Such factors are minimally expressed in intact bone. However, they are markedly increased following bone fracture [143, 144]. These factors, in particular M-CSF and RANKL, are essential for osteoclastogenesis. Their expression is markedly increased before the onset of calcified cartilage removal. Fracture healing in mice deficient of these cytokines is prolonged due to blockade of the transition of calcified cartilage to bone. Such deficiencies could be involved in the etiology of a subclass of nonunion fractures, which demonstrate the persistence of calcified cartilaginous callus [145]. Cellular multinucleation is the key feature distinguishing osteoclasts from their precursors. Dendritic cell-specific

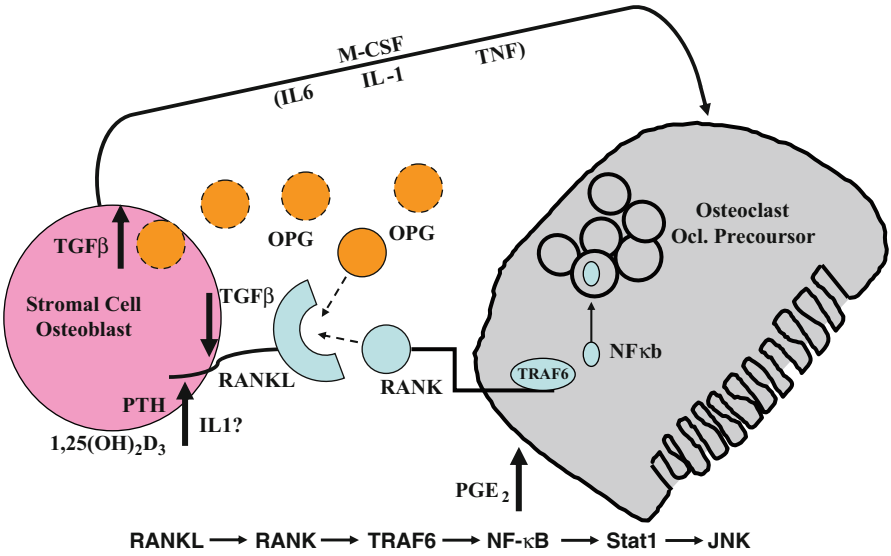

Fig. 2.11 Stromal cell–osteoclast interaction

transmembrane protein (DC-STAMP) was found to be critical for fusion of the mononuclear precursors to form multinucleated osteoclasts. DC-STAMP-deficient cells fail to fuse, yet exhibit normal features of an osteoclast with actin ring and ruffled border formation [146]. The current hypothesis regarding the developmental stages from the firstly identifiable osteoclast precursor to the mature active resorbing cells is illustrated in Fig. 2.11.

The M-CSF-RANKL system has been in the focus of osteoclast research for more than a decade. In the presence of M-CSF RANKL activated its receptor, RANK, leading monocytes/macrophages into the osteoclastic pathway [147]. RANKL is mostly a membrane anchored protein of the osteoblast lineage. Hence, a cell–cell interaction is required for its action. However, this may not be the whole scenario, as soluble RANKL is produced by T cells and is osteoclastogenic, together with M-CSF, ex vivo cultures. The divergence from the macrophage/dendritic cell toward the osteoclast is shown in Fig. 2.10b. An important modifier of the RANKL–RANK interaction is system OPG, produced by several cells and tissues including osteoblasts and stromal cells. Like RANK, OPG belongs to the TNF receptor family and acts as a soluble decoy receptor, competing with RANK on the binding to RANKL, thus inhibiting osteoclastogenesis [148, 149].

Migration and Targeting. Conceptually, bone resorption should involve the recruitment of osteoclasts and/or their precursors to the site of degradation of the mineralized matrix. Indeed, several matrix proteins such as type I collagen peptides, a2HS glycoprotein, osteocalcin, and stromal cell-derived factor-1 demonstrate monocyte chemoattraction. Whether they function in this capacity remains to be investigated.

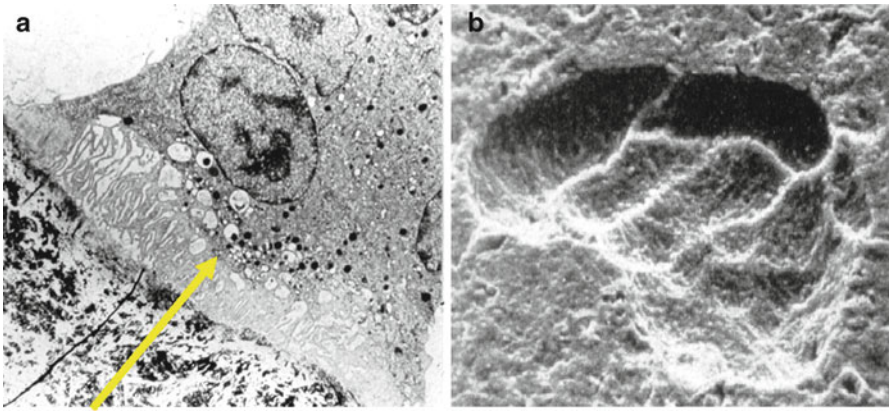

Fig. 2.12 Osteoclastic resorption. (**a**) Transmission electron micrograph of an actively resorbing osteoclast. Note, Ruffled border (*arrow*). (**b**) Howship's Lacunae/*ex vivo* pit formation

Another class of chemotactic signals could originate in osteocytes. The association between micro cracks to bone remodeling raised the suggestion that dying osteocytes at the crack site may signal to the attraction of osteoclast precursors [150]. Also, intact osteocytes may inhibit resorption; when the osteocyte originating osteoclast restraining signals are alleviated, the osteoclast precursors could migrate toward the fracture site [151]. Matrix metalloproteinases (MMPs) were found to be critical for the migration of the precursor cells. MMP14 in particular carves the path for osteoclastic cell migration through the degradation of non-mineralized matrices. In addition, MMP9 could probably release chemo-attractants like vascular endothelial growth factor (VEGF) [152].

2.6.1 Structure and Function

The osteoclast is a large (~300 mm) cell with up to eight nuclei. The reason for these features is unclear. The osteoclast has two major opposite plasma membrane domains, the functional secretory domain (FSD) that faces the mineralized matrix and the basolateral domain (BLD), usually in a close proximity to a blood vessel [147, 153]. At the FSD the cytoskeleton reorganizes and assumes polarization of F-actin to a circular structure, the "actin ring." The plasma membrane beneath the actin ring forms a tight attachment with the mineralized matrix. The attachment mediated by avb3 integrin through the recognition of bone protein sequences such as osteopontin and sialoprotein. The primary adhesion structures of osteoclasts are dot-like, actin-rich structures known as podosomes. This attachment outlines the sealed zone, which is the space between the mineralized matrix and a highly convoluted, ruffled, resorbing part of the osteoclast cell membrane (Fig. 2.12). Hydrogen ions and matrix degrading enzymes are secreted into the sealed zone through the ruffled

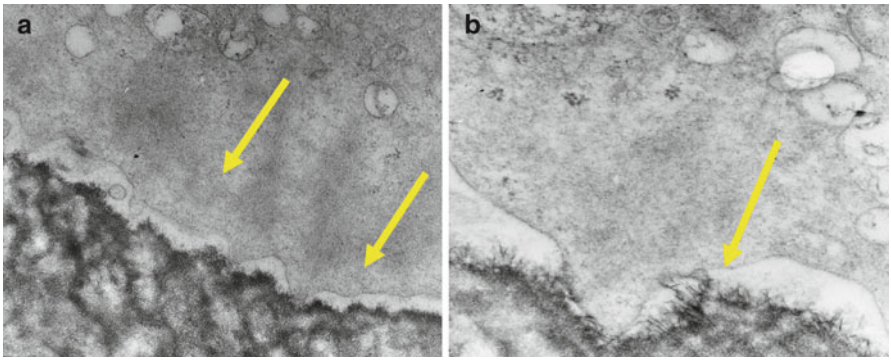

Fig. 2.13 Transmission electron micrographs of seal zone. Note, Podosomes (*arrows*)

membrane. Mineral dissolution and organic matrix degradation are followed by removal of the products from the resorption lacuna. This step involves transcytosis and secretion into the circulation at the BLD [154].

Mineral Dissolution and Organic Component Degradation. Osteoclast attachment to bone with isolation of a sealed space and formation of a ruffled border (Fig. 2.13) creates a secluded compartment at the resorption site. Acidified conditions of pH ~4.5 develop at this location by the generation of hydrochloric acid (HCl) that dissolved the bone mineral. The HCl is formed by the mobilization of hydrogen (H^+) and chlorine (Cl^-) ions from inside the osteoclast across the ruffled membrane. The HCl is mobilized by fusion of acidic vesicles with the ruffled border coupled to an electrogenic proton pump (H^+-ATPase) coupled with a Cl^- channel. The functional separation of the ruffled border from the rest of the cell membrane by the sealing zone enables concentration of the HCl. To enable a constant release of HCl into the resorption area, protons are continuously produced by the activity of carbonic anhydrase II, an enzyme that is highly expressed in osteoclasts and facilitates the hydration of CO_2, resulting in the production of protons and HCO_3^-. The latter is substituted to chloride by the chloride-bicarbonate exchanger located in the basolateral membrane. The osteoclast is characterized also by a high number of mitochondria required to produce energy for the resorption process. The organic matrix is degraded probably by more than one enzyme. It seems, however, that cathepsin K is the main bone matrix breakdown enzyme [155] (Fig. 2.14).

Osteoclastic Bone Resorption. Carbonic anhydrase II catalyzes the hydration of CO_2 resulting in the supply of protons that accumulate in the resorption area by proton pump and through vesicular transport (Fig. 2.14). The HCO_3^- produced together with the proton is exchanged for chloride ions that are transferred through chloride channels into the resorption area. The HCl dissolves the hydroxyapatite and cathepsin K exocytosed from the cell degrades the collagen. The ions and collagen degradation products are endocytosed by the ruffled membrane, the vesicles

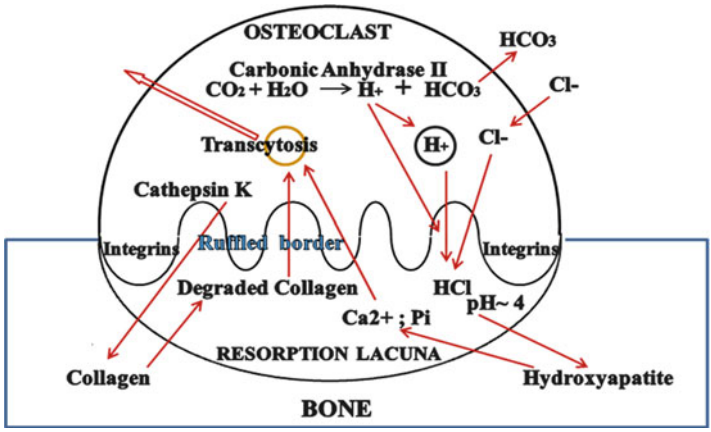

Fig. 2.14 Osteoclastic bone resorption

are fused to the membrane opposite to the ruffled membranes, and the resorption products are disposed.

Disposal of Resorption Products. Efficient resorption requires an instantaneous removal of the ions and the collagen fragments produced. The FSD is the area where degradation products are targeted [156]. They are endocytosed into the osteoclast. The endocytic vesicles, derived from the ruffled border, fuse with the FSD, and the degradation products are released into the extracellular fluid, mainly the blood stream, at the BLD.

Mineralized Tissue Resorption in Bone Healing. Osteoclasts have a key role in the cartilage-to-bone transition of fracture healing and in the consequent remodeling and maturation of the bony callus toward regeneration of the cortical bone. It has been shown that inhibition of bone resorption during fracture healing, by agents such as bisphosphonates, leads to enlarged callus and delays its replacement by bone. The biomechanical properties of the consequential bony callus are diminished [157]. Increased resorption, as in the case of partially stabilized and fractures and bone injuries in aged individuals, is associated with diminished trabecular bone parameters and callus strength [158]. Although the clinical significance of these findings has not been fully elaborated, special care, such as rigid fixation, and a close follow-up should be implemented in elderly patients and those receiving anti-resorptive medication.

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