Jona J. Sela · Itai A. Bab *Editors*

Principles of Bone Regeneration

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Preface

Bone healing is the process whereby deficiencies and discontinuities in bone tissue are repaired by a regeneration process that rescues the biomechanical properties of the skeleton. Inevitably, this process involves an ultimate net gain in the amount of mineralized matrix at the affected sites. This gain may progress slowly, as in the case of the positive shift of bone remodeling balance induced in the osteoporotic skeleton by bone anabolic agents, or, as an outburst of bone formation and remodeling characteristic of the bone tissue reaction to traumatic insults. The importance of bone healing to medicine and biomedical research is illustrated by the number of publications on the different aspects of the subject, which exceeded 2,000 in 2011 alone.

Either form of bone healing is affected by a multitude of genetic, environmental, mechanical, cellular, and endocrine variables which eventually lead to changes in gene expression that enhance the guided action of osteoblasts (and chondroblasts) to lay down bone that restores, or even improves, the skeletal load bearing capacity and body motion. Needless to say, osteoclasts are also involved in shaping the healed tissue. Recent breakthroughs in understanding the regulatory aspects of bone formation and resorption, at the basic, translational, and clinical arenas, offer new modalities to induce, enhance, and guide repair processes in bone for the benefit of millions of patients with conditions such as osteoporosis, nonunion fractures, critical size defects, orthodontic tooth movement, periodontal bone loss, intraosseous implants, and deformed bones.

An immense number of approaches to treating these conditions are currently under basic, preclinical, and clinical investigations. They range from the development of sophisticated biomaterials for implant surgery, identification of neurotransmitters active in bone and other molecular drug targets, new drugs engineered by cutting edge pharmacological and molecular approaches, and advanced methods for tissue engineering and gene and cell therapies.

Because of the multidisciplinary nature of these efforts, this book addresses the modern aspects of bone healing, with a special attempt to enhance the convergence of the different experimental and clinical approaches designed for the study and treatment of bone healing in its diverse forms and under varying conditions. The information and ideas provided should have value not only for the experimental skeletal biologist and clinician treating bone conditions but also for a general interpretation of healing and regenerative processes in mammals.

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Research Interest and Projects

Gene-expression of bone cells around orthopedic implants. Automated image analysis supported by computerized quantitative morphometry for the study of observations obtained by electron and light microscopy in normal and pathological conditions. Development of novel computerized quantitative histomorphometric methodology to study oral and systemic pathological changes in cancer and wound healing.

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Research Interests and Projects

The bone laboratory is engaged in multidisciplinary research studying the mechanisms involved in skeletal remodeling, metabolic bone diseases, and the integration of endosseous implants. The laboratory studies the effects of different hormone and growth factor derived drugs on bone remodeling, bone mass, and healing of bone injuries. Recently, the laboratory has been engaged in the development of a new scientific field, neuropsychoosteology, which explores the bidirectional interaction between the brain and the skeleton. The methodological approaches employed in the laboratory encompass micro-computed tomography and histomorphometry, cellular and molecular biology, genetics, biochemistry and medicinal chemistry.

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Part I
Physiology of Bone Healing

Chapter 2
Cellular and Molecular Aspects of Bone Repair

Itai A. Bab and Jona J. Sela

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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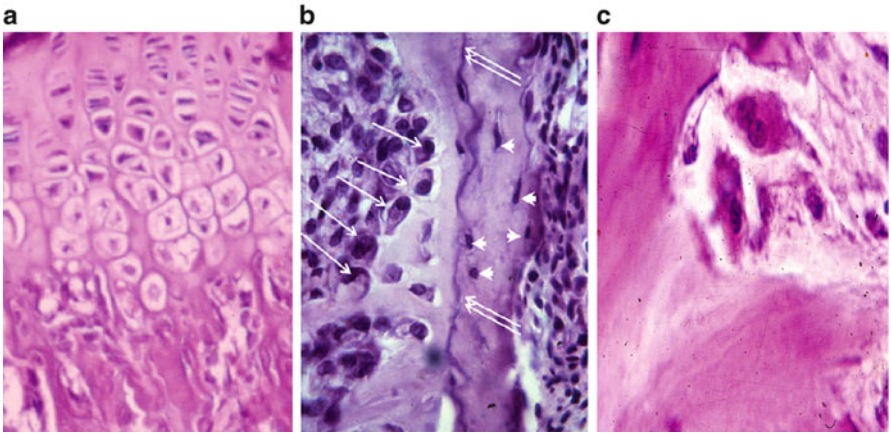

Fig. 2.1 Bone and cartilage cells. (**a**) Chondroblasts. (**b**) Osteoblasts (*arrows*) forming osteoid with Osteoblast–osteocyte transition adjacent to reversal line (*double arrows*) and osteocytes (*arrow heads*). (**c**) Osteoclasts in resorption bay

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

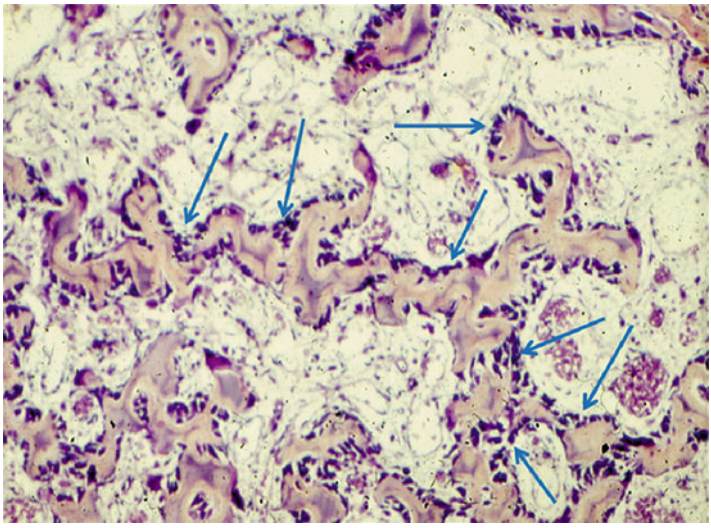

Fig. 2.2 Osteoblasts (*arrows*) aligned along primary bone surfaces

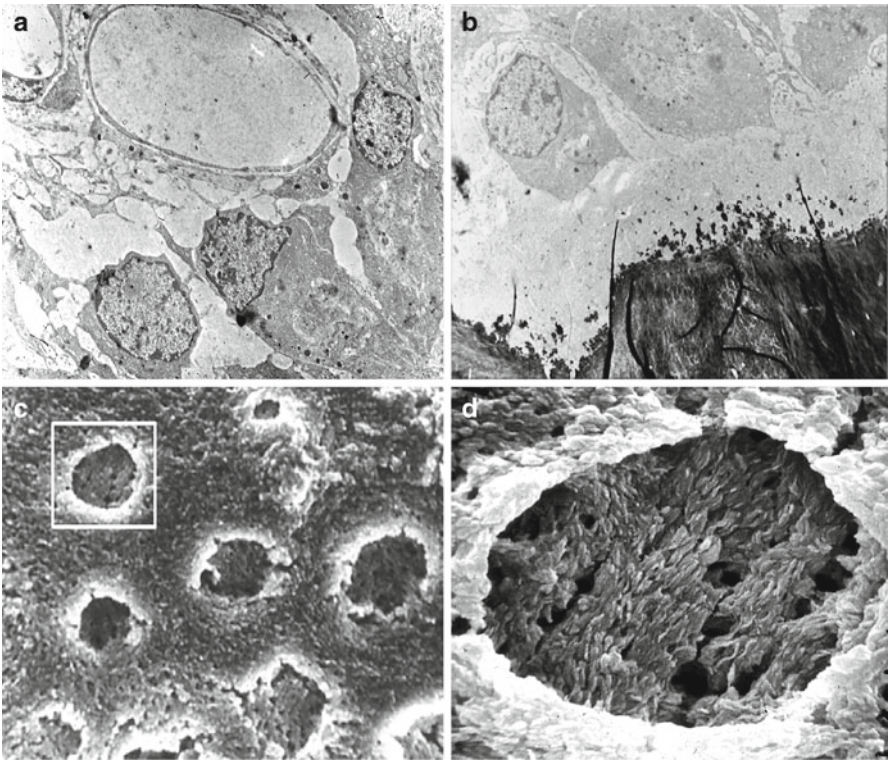

Fig. 2.3 Electron micrographs of different osteoblastic features. (**a**) Osteoblasts adjacent to blood capillary. (**b**) Osteoblasts adjacent to Calcifying front (TEM). (**c**) Osteoblastic lacunae on surface (SEM). (**d**) Higher magnification of the square in C, an osteocytic lacunae (SEM)

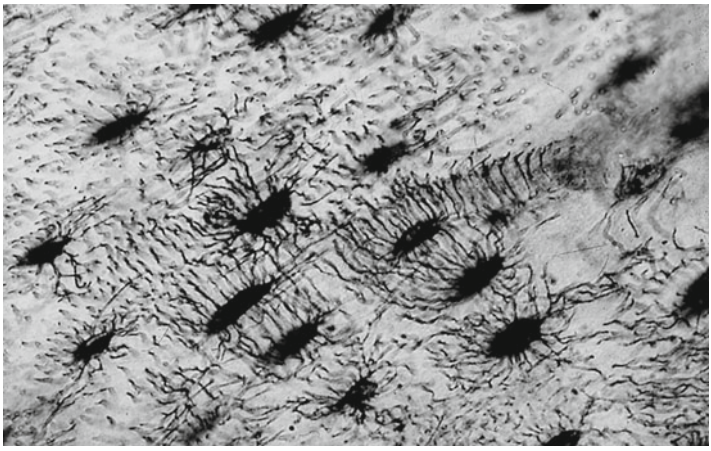

Fig. 2.4 Osteocytes and cellular processes demonstrated by impregnation methodology

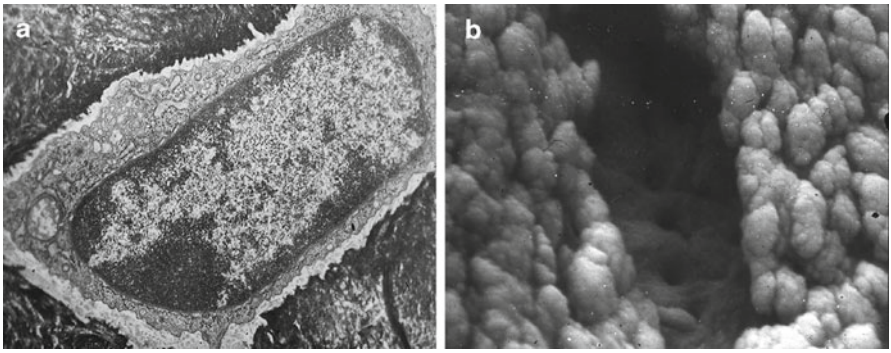

Fig. 2.5 Electron micrographs demonstrating osteocytic features. (**a**) Osteocyte embedded in mineralized matrix (TEM). (**b**) Osteocytic lacuna (SEM)

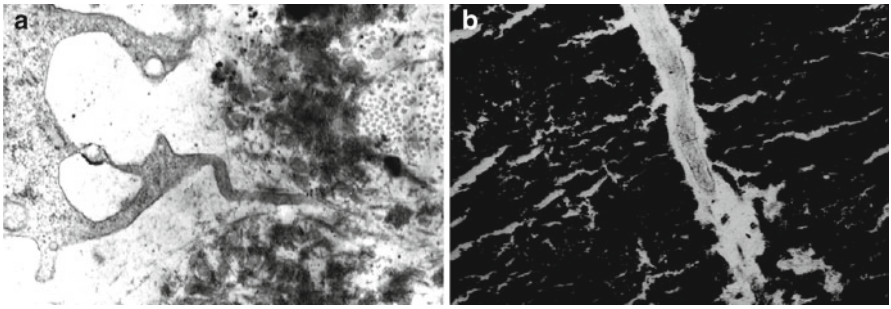

Fig. 2.6 Osteocytic processes (TEM). (**a**) Osteocyte with processes embedded in the mineralized matrix (horizontal). (**b**) Osteocytic process traversing in canaliculus in heavily calcified bone (perpendicular)

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

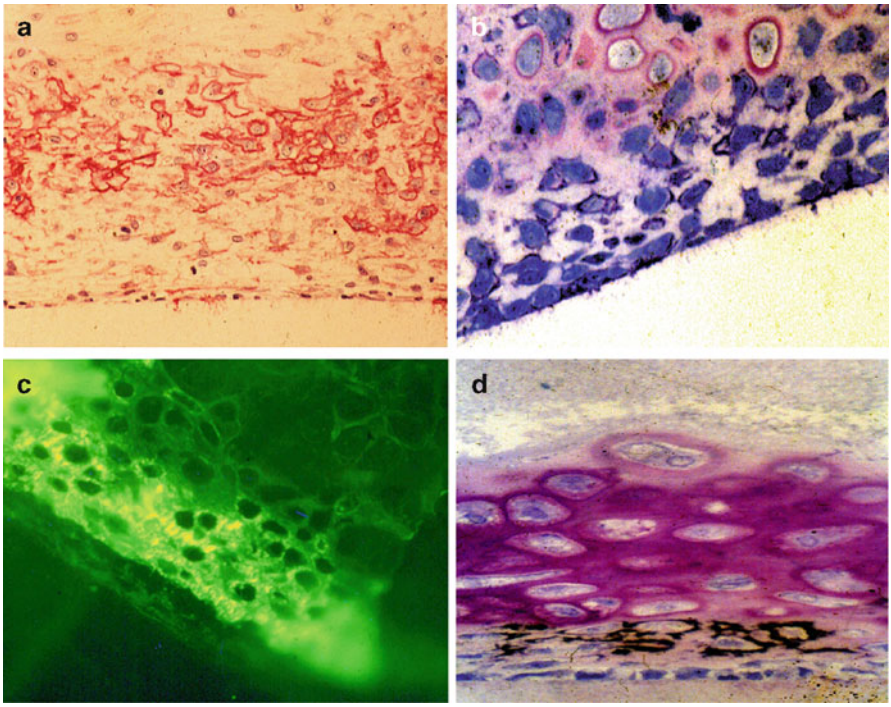

Fig. 2.7 Osteoblasts producing mineralizing matrix in diffusion chamber cultures of MSCs. (**a**) Osteoblast cell membranes stained red with histochemical reaction for alkaline phosphatase. (**b**) Autoradiograph showing distribution of PTH receptors. (**c**) Immunohistochemical staining of collagen 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

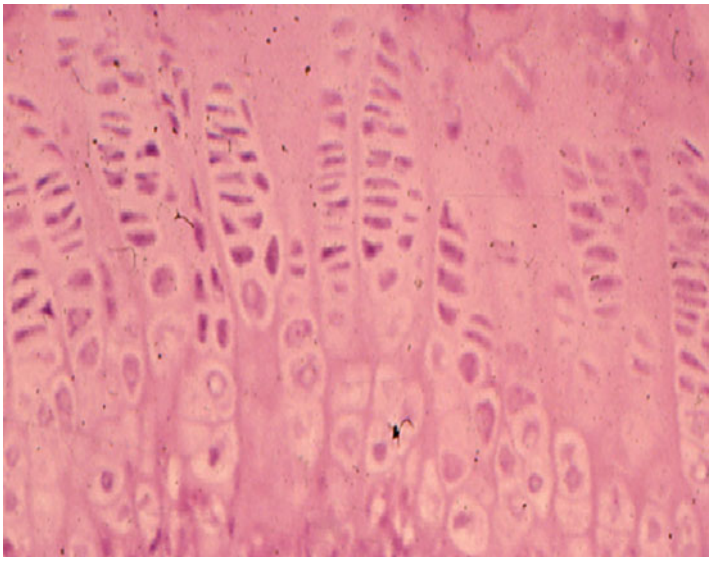

Fig. 2.8 Growth plate cartilage; note, typical palisading chondrocytes. Proliferating cells (*upper*). Hypertrophic differentiated cells (*lower*)

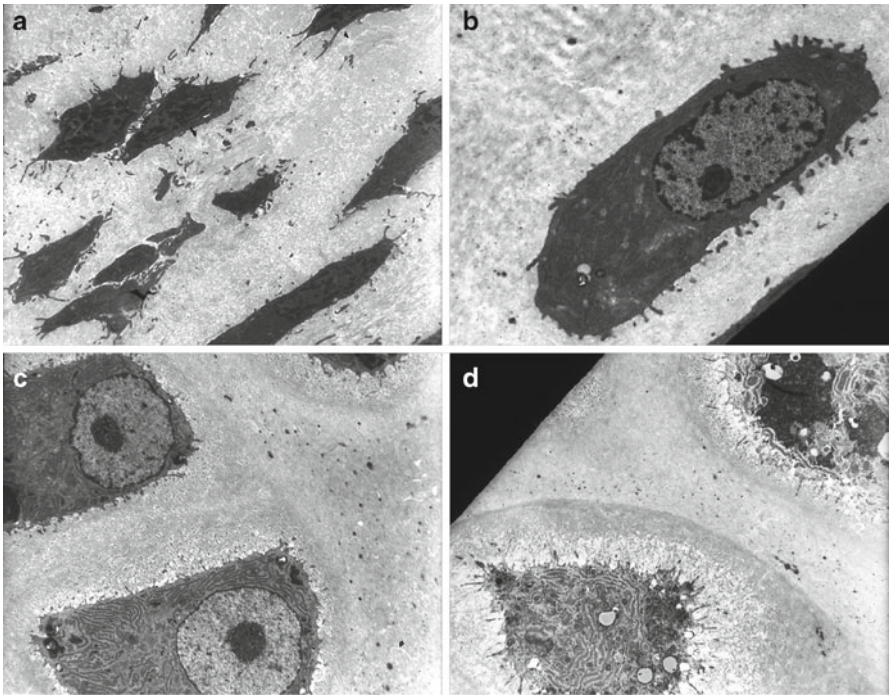

Fig. 2.9 Electron micrographs of growth plate chondroblasts. (**a**) Proliferative zone. (**b**) Prehypertrophic differentiation. (**c**) Hypertrophic chondrocytes. (**d**) Apoptotic chondrocytes

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.

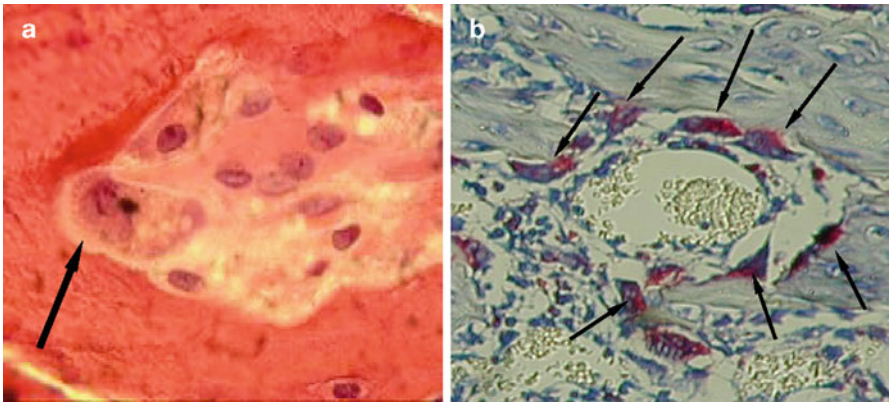

Fig. 2.10 Histological features of osteoclasts. (**a**) A typical appearance of osteoclasts in bone remodelling unit (*arrow*) in H&E Stain. (**b**) Osteoclasts (*arrows*) in tartarate-resistant acid phosphatase staining (*red*)

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

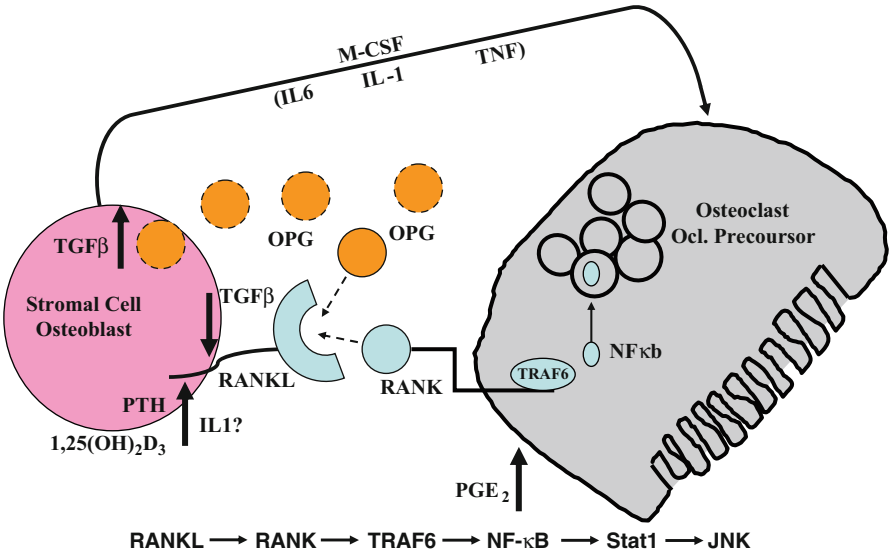

Fig. 2.11 Stromal cell–osteoclast interaction

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.

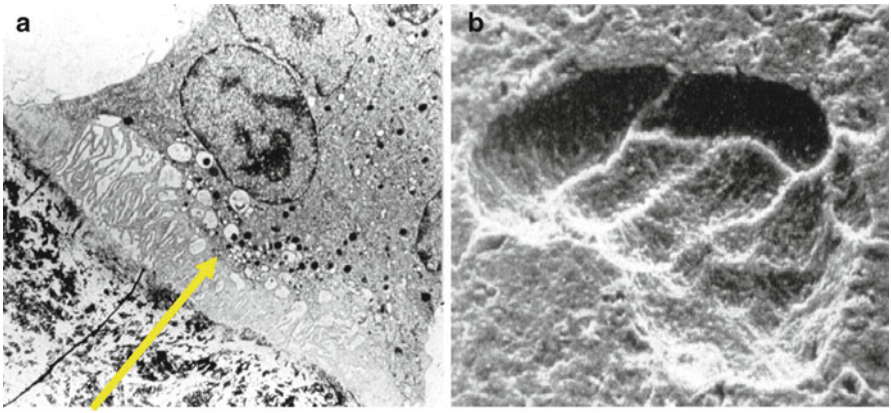

Fig. 2.12 Osteoclastic resorption. (**a**) Transmission electron micrograph of an actively resorbing osteoclast. Note, Ruffled border (*arrow*). (**b**) Howship's Lacunae/*ex vivo* pit formation

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

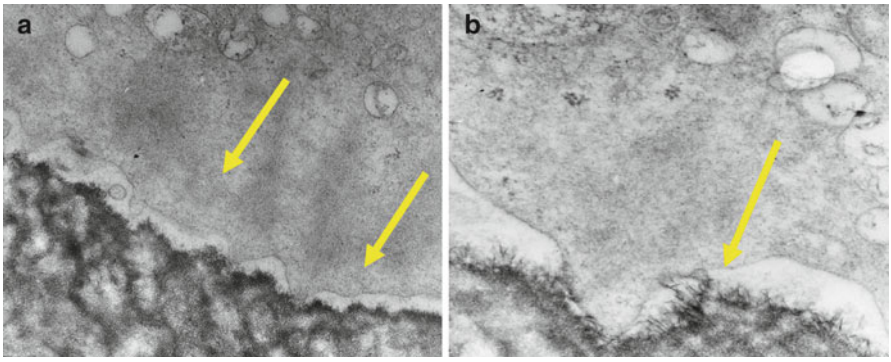

Fig. 2.13 Transmission electron micrographs of seal zone. Note, Podosomes (*arrows*)

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

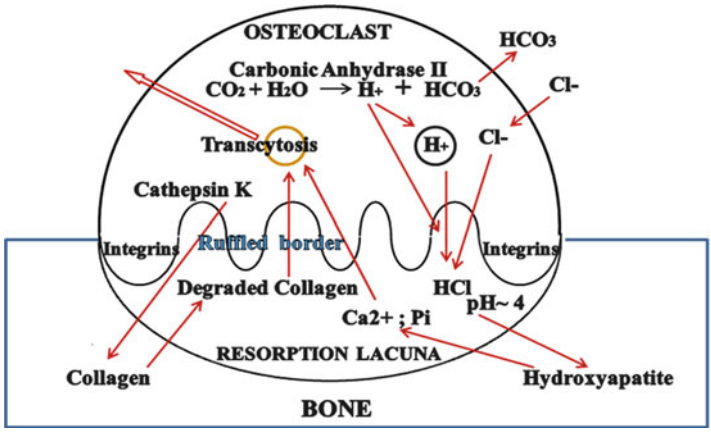

Fig. 2.14 Osteoclastic bone resorption

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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Chapter 3
Primary Mineralization

Jona J. Sela

3.1 Introduction

Primary mineralization in hard tissues is widely documented. Hydroxyapatite crystal (HA) formation in the matrix is regulated by forming cells (chondroblasts, osteoblasts, cementoblasts, and odonoblasts) and commonly initiated within extracellular matrix vesicles (MV). MVs, containing relatively high concentrations of Ca^{2+} and inorganic phosphate (Pi), are an optimal environment for hydroxyapatite crystallization. Alongside this process, a continuous mineralization of the matrix, without MVs, is evident. Concurrently, primary mineralization via MVs has been shown in the early stages of development in cartilage, bone, dentin, and cementum. Furthermore, it is well established that MVs serve as initial loci of calcification in tissues of mesenchymal origin not only in the embryonic stage but also during continuous growth as well as in bone repair and in mineralizing neoplastic conditions [1–25]. Primary mineralization was investigated by ultrastructural, biochemical, and molecular methods.

Proteomic analysis revealed that more than 60% of the total proteins were present in the cellular microvilli in human osteosarcoma cell line (Saos-2). Among all identified MV proteins, cytoskeletal markers of microvilli, including actin, ezrin, radixin, moesin, talin1, and actin-binding proteins such as cofilin1 and transgelin2, were present [26–28]. These findings support the observation that microvilli are the sites of origin of MVs and the finding that actin filament assembly and disassembly are involved in their biogenesis with a metabolically active outer membrane [16, 17, 19, 21, 22]. Vesicular release into the matrix is concomitant with their considerable loading with Ca^{2+} and Pi that produce calcifying foci on the vesicular inner membrane. Subsequent to exocytosis, MVs display high levels of enzymatic activity of alkaline phosphatase (AP), phospholipase-A_2 (PA_2),

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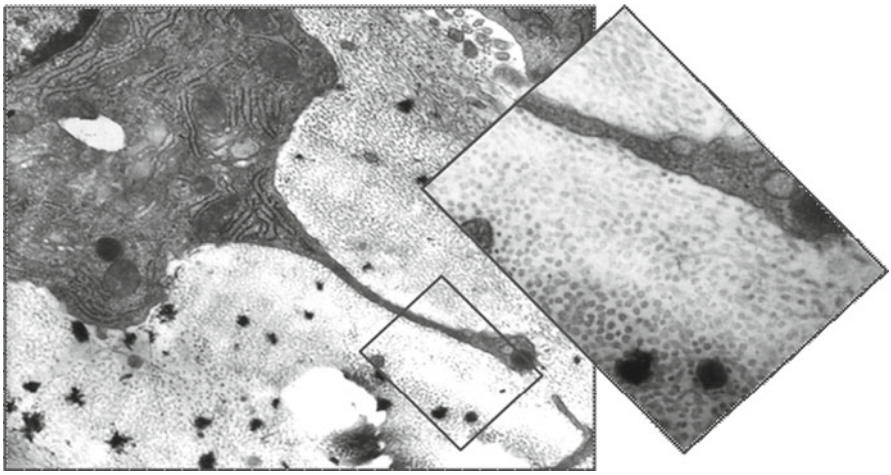

Fig. 3.1 Transmission electron micrograph of osteoblast with its process. *Insert*: Higher magnification. Note, vesicles in process and electron opaque vesicle in the matrix

pyrophosphatase (PP), different ATPases, and elevated contents of phosphatidylserine (PS). AP and annexins participate in the nucleation and formation of HA crystals. Annexins are Ca^{2+} and lipid-binding proteins involved in Ca^{2+} homeostasis in bone cells and MVs; they participate in the formation of calcium ion channel within the MV membrane. AP is associated with Pi regulation by hydrolysis of phosphate compounds. ATP and pyrophosphate, known inhibitors of HA formation, are hydrolyzed by AP, ATPases, and PP. In this respect, antagonistic activities serve in the regulation of the process of mineralization [16, 17, 19, 21, 22, 29, 30]. The process of bone healing is characterized by increased enzymatic activities and PS content on the first and second weeks after injury followed by a decreased activity on the third and fourth weeks. Quantitative-morphometric-ultrastructural studies demonstrated a typical gradient of vesicular distribution from the calcified front, with the ruptured MVs being the closest to the front, and the electron-lucent MVs being farthest away.

The biochemical and the ultrastructural observations clearly demonstrate the vesicular life cycle. Briefly, the vesicle is released from the osteoblast into the extracellular matrix in an electron-lucent form (Fig. 3.1). At this point AP is associated with Pi release in the matrix by hydrolysis of phosphate compounds. Annexins are responsible for influx of ionic calcium and phosphate that form saturated solutions of amorphous calcium-phosphate complexes producing MVs with an electron opaque texture (Fig. 3.2). ATP and pyrophosphate, the principal inhibitors of HA crystallization, are hydrolyzed by AP, ATPases, and PP, allowing intravesicular hydroxyapatite crystals formation (Fig. 3.3). Further crystal growth is accompanied by an increase in PA2 activity, resulting in rupture of the vesicular membranes and release of HA crystals to augment the calcifying fronts (Fig. 3.4) [31–39]. Schematic illustration of the process is summarized in Fig. 3.5.

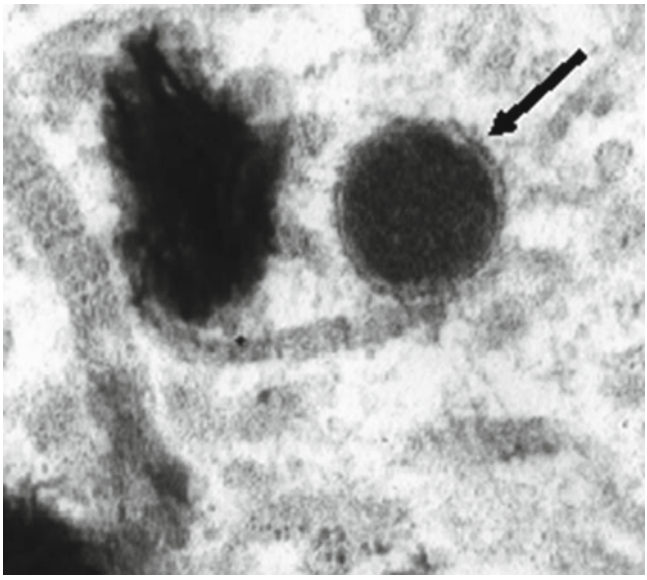

Fig. 3.2 Transmission electron micrograph of an electron opaque vesicle (*arrow*) in the matrix. Note, Collagen with cross banding (*left*) and a small calcospheritic structure constructed of HA crystals

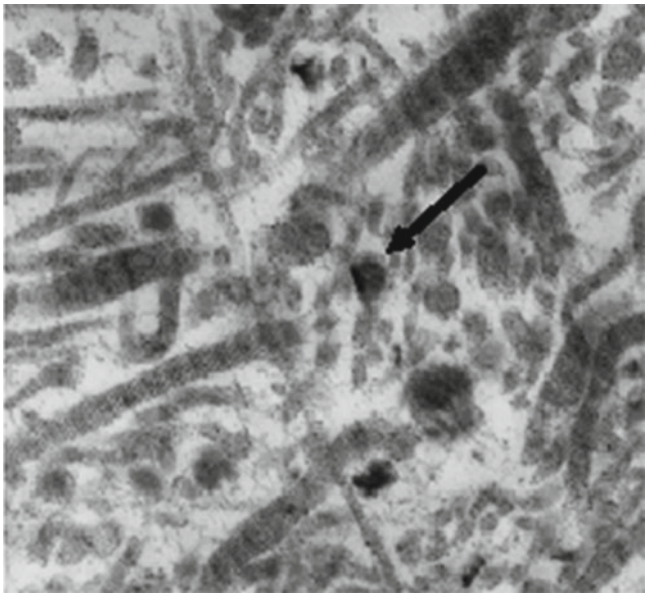

Fig. 3.3 Transmission electron micrograph of crystal containing vesicles (*arrow*) in collagen rich matrix

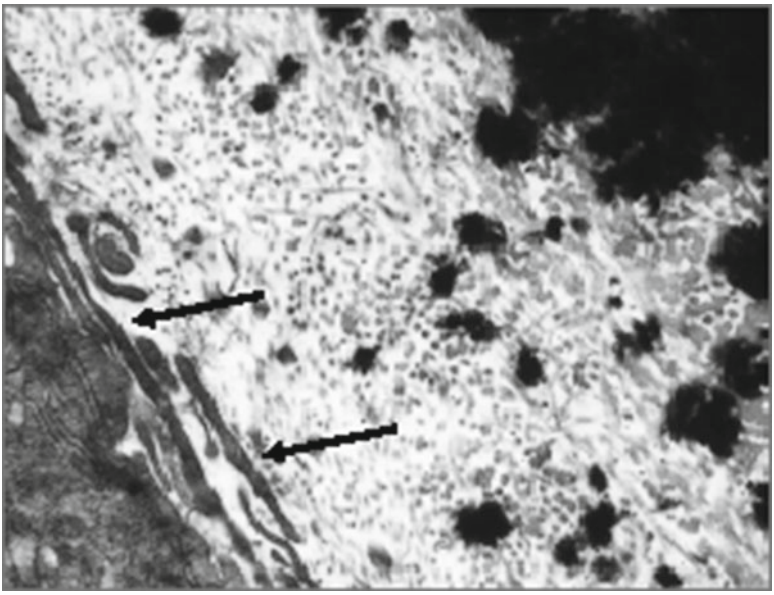

Fig. 3.4 Transmission electron micrograph of osteoblast with flattened processes (*arrows*) separated from the calcifying front (*black*) by collagenous matrix with dispersed calcospheritic structures

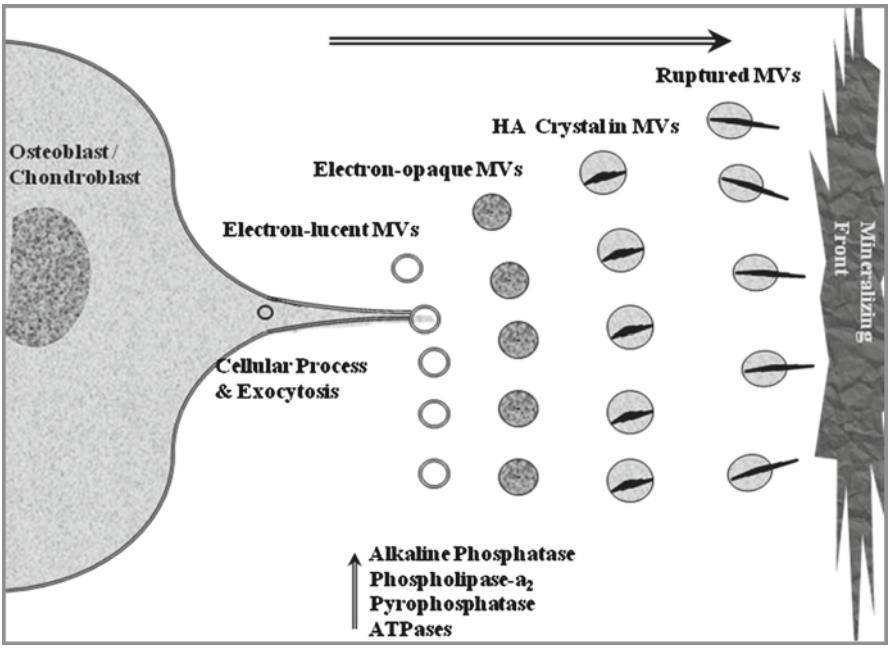

Fig. 3.5 Schematic illustration of MV mineralization process. The vesicle is released from the osteoblast or chondroblast into the extracellular matrix in an electron-lucent form. Increased AP is associated with Pi release in the matrix by hydrolysis of phosphate compounds. Annexins are responsible for influx of ionic calcium and phosphate that form saturated solutions of amorphous calcium-phosphate complexes producing MVs with an electron opaque texture. Intravesicular hydroxyapatite crystals formation and further crystal growth are accompanied by an increase in PA2 activity, resulting in rupture of the vesicular membranes and release of HA crystals that to augment the calcifying front

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Chapter 1
Healing of Bone Fracture: General Concepts

Jona J. Sela and Itai A. Bab

The skeleton is frequently exposed to accidental and iatrogenic insults. Bone, similar to several other tissues, portrays a marked potential for regeneration and repair. Generally, healing proceeds until a complete restoration of the osseous function and anatomy is achieved. Cellular and molecular participants are similar in healing processes of bone and other tissues of mesenchymal origin. Skeletal injury initiates a multifaceted healing process since additional non-osseous tissues are involved. In view of potential complications in the healing process, a methodological approach to expected cellular and molecular therapeutic targets is required. The study of such targets in skeletal morphogenesis reveals that the phases of bone healing display striking similarities to osseous growth and development [1–5].

Classification of the patterns of bone healing is based on a variety of events and factors that influence injury and repair. Currently, the extent of tissue loss is considered to be of critical significance. It is clear that the increase in the amount of bone loss is in direct correlation with the delay in healing. Therefore, the extent of the discontinuity between the fractured edges is accepted to serve as streamline factor for the sorting of the different types of healing. Consequently, the following two major patterns of bone repair are defined:

(a) Healing following close approximation and rigid compression of the fractured edges. This could be considered as healing in primary intention with a minimal replacement of the injured bone by intermediary tissues. The process is concluded

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by a complete union between the fractured edges. Bone healing in this situation is described to occur in both lamellar and trabecular bones in instances of tight proximity of less then 0.1 mm between fractured edges with rigid stabilization. The suggested theory is that this type of healing is mediated by periosteal and endosteal tissues of the intraosseous Haversian system, marrow-derived vessels and mesenchymal cells, osteoblasts, and osteoclasts. Regeneration is characterized by bone remodeling parallel to the streamline of the osteon system. This union is formed by continuous ossification in first intention without cartilaginous or woven bone formation. The osteoclasts, engaged in necrotic bone resorption, are accompanied by the osteoblasts that form lamellar bone. Remodeling of the repaired bone is minimal in this environment consisting of minimal interfragmentary space [4]. *The concept of direct continuous bone regeneration is controversial.* It lacks basic scientific support with histological evidence in the literature. Most researchers would dispute the idea that healing could occur without formation of transient tissues between the fractured edges. It should be noted that a minimal hemorrhage is evident in all cases of trauma, and hence a blood clot, even if minimal, would develop in the fracture area serving as initial matrix for the proliferation of the involved cellular population. However, the theory on direct bone repair serves as a "scientific" justification for various orthopedic procedures. In these instances, the fracture edges are tightly pressed together. Clinical articles report a high rate of successful complete union [4]. It could be pointed out that "green stick" and "stress" fractures would probably heal in a similar manner.

(b) Healing with separated fracture edges involving intermediary tissues. These fractures are characterized by a significant gap formed between the edges with an extent of less than the diameter of the bone. Cases of such discontinuity are proven to heal regularly with artificial fixation. This type of bone healing is probably the most abundant one and is defined as healing in secondary intention (Fig. 1.1).

Clinically, fracture repair is optimized without a tight approximation of the severed edges. The course of healing constitutes several processes along the following possible stages: blood clotting, inflammatory response, granulation tissue formation, macrophage and osteoclast activity, significant bone resorption; formation of cartilaginous callus (endochondral repair) with calcification and young osseous matrix of primary bone. The continuance of the process is characterized by mineralization of the matrix.

It should be pointed out that the newly formed calcifying tissue can serve as a stabilizing but not as a weight-bearing component. Woven bone and cartilage serve as bridging templates. Complete maturation is accomplished by bone remodeling to form biomechanically compatible structures. Osseous regeneration is dependent upon several clinical issues such as location, extent of tissue loss, fracture mobility, infection, and types of reconstructive materials and systemic conditions. In addition, bone regeneration is usually accompanied by restoration of the collaterally damaged tissues, i.e., joints, cartilage, muscles, tendons, ligaments, skin, mucous membranes, bone marrow, periodontal ligament, etc. [3–5].

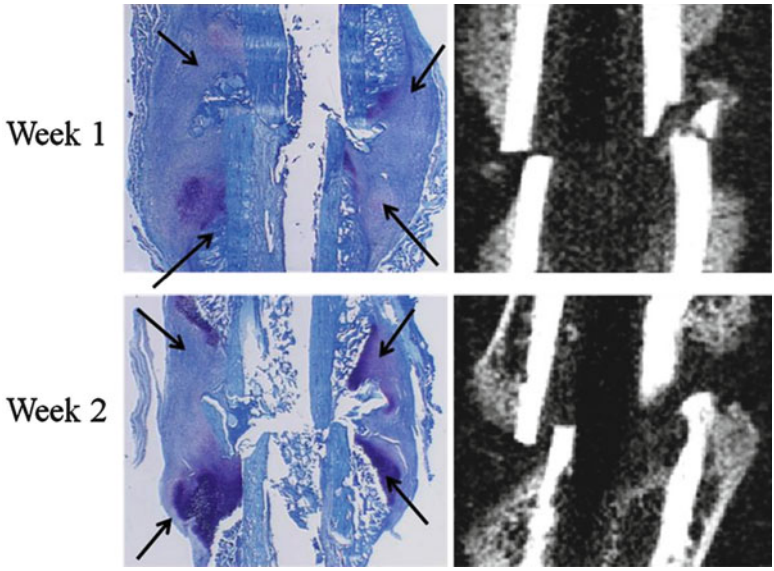

Fig. 1.1 Long bone fractures and callus in first and second weeks of healing. Note, Pairs of histological and μCT representations. *Week 1*: Callus is constructed large cartilaginous component (*arrows*), initially calcified. *Week 2*: Higher calcification (*intense violet*) and reduction of callus size

The natures of the genetic and molecular triggers that initiate and regulate the signaling pathways in the process of cellular activation in bone healing are starting to be disclosed [5–8].

Following trauma, molecules participating in fracture healing comprise pro-inflammatory cytokines, i.e., interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) that are expressed first in the inflammatory phase and later in the remodeling phase. This stage is followed by the involvement of growth and differentiation factors, including transforming growth factor-β superfamily (GDFs, BMPs, TGF-β), platelet-derived growth factor (PGDF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF) that are operative few hours after the fracture time during all the reparative phase [8, 9]. Subsequently, endochondral ossification is characterized by the activities of metalloproteinases, vascular endothelial growth factors (VEGF), and angiopoietin 1 and 2. Molecules antagonist to bone morphogenetic proteins (BMPs) have been identified. Noggin, chordin, sclerostin, follistatin at extracellular setting and BAMBI (BMP and activin membrane-bound inhibitor) were observed during embryogenic development [9–11]. Canonical Wnt signaling pathway has been shown to play a role in fracture repair. This pathway, which activates Lef1/T cell factor (TCF)-dependent transcription, has emerged as a key regulator in embryonic skeletogenesis, positively regulating the osteoblasts. A significant upregulation of β-catenin was found during bone healing process A large molecular array was described to interrelate with each other and with the environment to achieve fracture repair. In this context, regulators of chemotaxis,

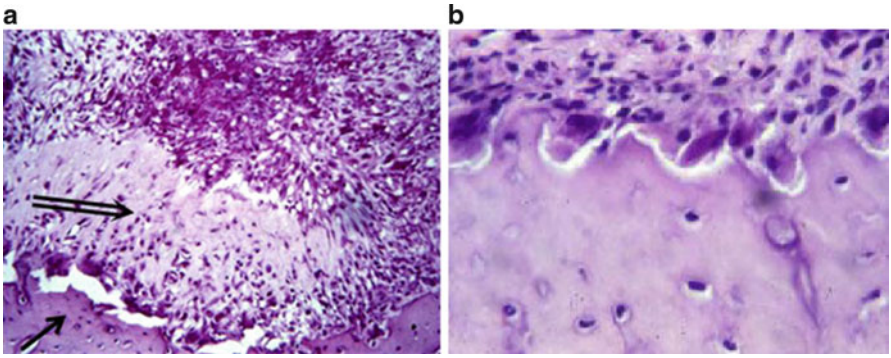

Fig. 1.2 (**a**) Fractured bone (*single arrow*), matrix with osteoblasts (*double arrow*); Note, Granulation tissue (*upper center*). (**b**) Osteoclasts in resorption lacunae (H&E staining)

mitosis, and differentiation such as Wnt, Indian hedgehog, PTHrP genes that respond to hedgehog proteins like Gli 1 and patched (Ptc), platelet-derived GF, matrix metalloproteinases (MMPs), and VEGF a, b, c, and d. Inflammatory cells produce interleukins (IL-1, IL-2, and RANKL). Tumor necrosis factor (TNF x and b) play an essential role [5, 8].

Bone injury is immediately followed by local blood clot formation that serves as a medium that allows cellular migration, proliferation, and capillary budding (Fig. 1.2). Furthermore, the clot was shown to function as a primary source for growth factors [10]. Clot formation is concomitant with the onset of the inflammatory response. At this point, expression of signaling molecules and their proposed functions include IL-1, IL-6, colony-stimulating factors, and TNF-α that play a role in initiating the repair cascade. In addition, TGF-β, PDGF, and BMP-2 expressions increase the initiation of callus formation. Recruitment of mesenchymal stem cells is associated with GDF-8 suggesting its role in controlling cellular proliferation.

It should be emphasized that impaired clotting, due to local or systemic factors, mainly coagulation disorders, anticoagulant drugs and infection, results with a major disruption of healing. The healing process continues with the resorption of the clot and its replacement by granulation tissue. This stage is characterized by an immanent cellular mobilization and vascular in growth from periosteal vessels with extensive neo-angiogenesis mediated by angiopoietins and different VEGFs. A considerable macrophage and osteoclast activity is responsible for the removal and resorption of soft and hard tissue debris by mechanisms mediated by RANKL and MCSF [12–16].

Granulation tissue represents a distinctive pattern of chronic inflammatory reaction, typical to healing in second intention. In bone repair, granulation tissue serves as a transient environment gradually replaced by an ephemeral callus of cartilage and primary bone. Granulation tissue is providing a profuse blood supply and a vehicle for cellular recruitment. At this phase, abundant undifferentiated mesenchymal cells emerge at the site of injury, proliferate, and differentiate, evidently in response to growth factors produced by the injured tissues and from the blood clot. The process

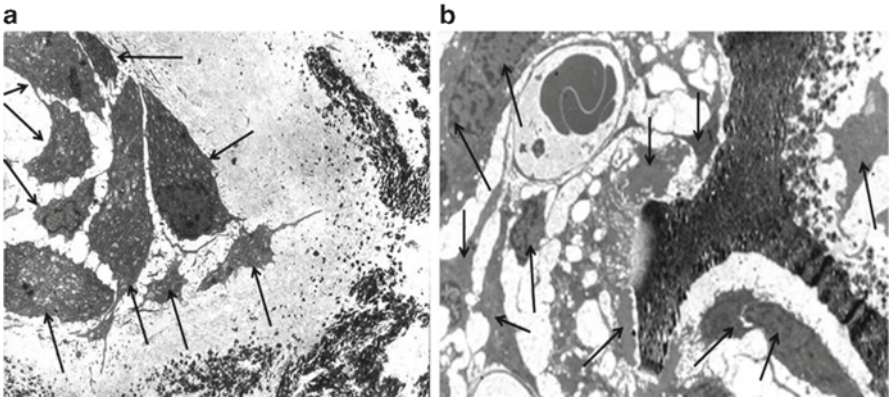

Fig. 1.3 (**a**) Osteoblasts (*arrows*) forming matrix, mineralization (*right*). (**b**) Osteoblasts (*arrows*) surrounding a bone trabecule

described involves both intramembranous and endochondral ossification (Fig. 1.3). Intramembranous ossification involves the formation of bone directly from committed osteoprogenitor cells and undifferentiated mesenchymal cells that reside mainly in the periosteum, in the Haversian tissues, and in the marrow resulting in hard callus formation [1, 4].

In endochondral ossification, chondrogenesis is assumed to be triggered by local ischemia, namely low oxygen tension, and regulated by factors such as IGF-I, PTHrP, IHH, and HIF-Iα, and mesenchymal cells differentiate into chondrocytes, producing cartilaginous matrix, which then undergoes calcification and eventually is replaced by bone. The formation of primary bone is followed by extensive remodeling until the damaged skeletal elements regain the original shape and size. As stated, these processes resemble embryonic bone formation, suggesting that fracture repair is a reiteration of normal bone development [1–5]. Regarding the molecular events, increased levels of TGF-β2, TGF-β3, and GDF-5 are associated with stem cell mobilization, chondrogenesis, endochondral and woven bone ossification. BMP-3, -4, -7, and -8 promote recruitment of cells of the osteoblastic lineage. BMP-5 and -6 rise in association with cell proliferation in intramembranous ossification. TNF-α RANKL and MCSF rise in association with mineralized cartilage resorption, apoptosis of hypertrophic chondrocytes, and matrix proteolysis. Bone remodeling coupled with osteoblast activity is associated with IL-1 and IL-6 rise, whereas RANKL and MCSF display diminished levels. Establishment of marrow is marked by diminished expression of members of the TGF-β superfamily [17]. β-Catenin signaling has been shown to play a role in fracture repair. The β-catenin signaling pathway, which activates TCF-dependent transcription, has emerged as a key regulator in embryonic skeletogenesis, positively regulating osteoblasts. A significant upregulation of β-catenin was found during bone healing process. β-Catenin functions differently at different stages of fracture repair. In early stages, precise regulation of β-catenin is required for pluripotent mesenchymal cells to differentiate to either osteoblasts or chondrocytes. Once these undifferentiated cells have become committed

to the osteoblast lineage, β-catenin positively regulates osteoblasts. This is a different function for β-catenin than that has previously been reported during development. Activation of β-catenin by lithium treatment has potential to improve fracture healing, but only when used in later phases of repair after mesenchymal cells have become committed to the osteoblast lineage [18]. It is noteworthy that matrices formed during bone repair bear high similarities to those produced during embryonic limb development. Extracellular matrices are formed in healing fractures. Structural proteins, type I collagen in bone, and types II and X collagen in cartilage callus. Type III collagen is the major collagen of the fibrous matrix that forms along the periosteal surface. Type I collagen is secreted in large amounts as trabecular woven bone develops. Type V collagen is found in both fibrous tissue and bone. This type is particularly associated with blood vessels. Type II collagen is the last of the major collagens to be synthesized. Its synthesis is dependent on the mechanical conditions under which the fractures are healing particularly in instances of a large area of cartilaginous callus. Type II collagen formation is typical to mechanically unstable fractures. Type IX collagen is present throughout the large areas of cartilage. Type X is present only in calcified regions [19].

Transcription core binding factor 1 (cbfa 1) stimulates osteoblast differentiation. Additionally, bone morphogenetic proteins (BMP2, BMP3 osteogenin, BMP4, BMP7, osteogenetic protein, OP1) play a major role in bone repair [20].

Fibroblast GF2. Both fibroblast growth factors-1 (acidic FGF) and -2 (basic FGF) increase the proliferation of osteoblasts and chondrocytes in vitro and FGF-2 stimulates angiogenesis and bone formation in vivo. The application of FGF-1 or FGF-2 to normally healing fractures of the rabbit tibia did not have a significant effect on the rate of healing. Smads 1–8 serve in intracellular signaling for transforming growth factor beta (TGF-β). TGF-β was shown to stimulate bone and cartilage formation in calvarial and long bones. The effect of exogenous TGF-β2 on normally healing fractures was investigated to see if healing can be accelerated. TGF-β2 did not stimulate fracture healing under either stable or unstable mechanical conditions during the initial healing phase. Osteogenic growth peptide (OGP) was characterized in regenerating marrow. OGP-induced stimulation of bone formation in vivo suggests a role for this peptide in mediating systemic osteogenic response [15].

1.1 Distinctive Patterns of Bone Repair

1.1.1 Bone Healing Following Trauma or Marrow Injury Devoid of Fracture

This type of healing occurs in cases of bone wounds, marrow ablation, and socket healing after tooth extraction, and it is mediated by woven bone formation. Bone healing in these instances is characterized by the following stages: formation of a blood clot which is substituted by granulation tissue. The granulation tissue is replaced by

woven bone. This transient tissue is remodeled to a complete restoration of the tissues in the region of the injury. In instances of bone wound healing, a full recovery is expected in accordance with the individual location of the repair. Tooth socket healing is completed after restoration of the continuity of the maxillary anatomical and histological features. In the case of bone marrow injury or ablation, reconstruction of the marrow marks the completion healing.

1.1.2 Critical Size Defect

This is defined as an extensive bone loss that prevents spontaneous healing. The gap in these cases is clinically determined to be twice the diameter of the injured bone. Due to the inability of the osseous tissue to regenerate, this condition results with repair by soft tissue callus with subsequent pseudoarthrosis usually referring to a spontaneous fractures which progress to nonunion. Experimental nonunion models in different laboratory animals have been reported with emphasis on the importance of the critical size bone defect in testing bone-regenerating materials [21–24].

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Part II
Systemic Factors in Bone Healing

Chapter 4
Anabolic Agents in Bone Repair

Itai A. Bab

Systemically administered bone anabolic agents that stimulate bone and cartilage formation in fracture healing have attracted much attention. These anabolic agents include parathyroid hormone (PTH), osteogenic growth peptide (OGP), statins, and vitamin D (Vit D).

PTH is an 84-amino acid polypeptide hormone secreted by the parathyroid glands. Its main function is to maintain extracellular calcium at normal levels by upregulating renal calcium and phosphate reabsorption, and bone resorption and release of calcium from the skeleton. It has been repeatedly demonstrated that all the known biological activities of PTH reside within the 1–34 N-terminal fragment. In experimental models of osteoporosis, intermittent treatment with PTH leads to the rescue of bone mass and mechanical strength consequent to increased osteoblastic activity. These anabolic effects are in contrast to the catabolic actions induced by continuous exposure to PTH. Teriparatide is a synthetic polypeptide hormone that consists of the 1–34 amino acid fragment of human PTH [rhPTH (1–34)]. It is used clinically to treat osteoporosis. Daily subcutaneous injections of teriparatide in osteoporotic patients stimulate cancellous bone formation, increase bone mineral density, and reduce the risk of fractures. Also, recombinant PTH (1–84) is used in the treatment of osteoporosis. Over the past several years, there has been an increasing interest in potential technologies for enhancing fracture healing. Part of this interest is derived from the growing age of the population and the recognition that increased age carries an increased risk of complications after fracture. Although use of locally implanted or injected growth factors has received the most attention, systemic treatments for the enhancement of bone repair, especially for situations in which bone repair may be diminished or delayed, are now under investigation. Since the approval of

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teriparatide as an anabolic treatment for osteoporosis, there has been an increasing interest in other potential clinical uses for this compound, as well as other bone anabolic agents, in musculoskeletal conditions. Recently, a growing body of evidence has supported the conclusion that PTH (1–34) will also be an effective anabolic therapy for the enhancement of bone repair after fracture. Indeed, ongoing research has demonstrated the potential of PTH (1–34) in the management of bone repair in a number of bone healing models. In naive rodents, intermittent PTH (1–34) administration at doses of 30–200 μg/kg/day, initiated after fracture, stimulates the structural and biomechanical properties of the callus and enhances fracture healing. However, as PTH (1–34) is approved for anti-osteoporotic therapy, and because of the increased fracture risk in osteoporosis, it is also important to see if fractures occurring during ongoing PTH (1–34) treatment show a similar response. Indeed, PTH (1–34) pretreatment in rats does improve fracture healing unless supported by continuous treatment after fracture. Studies in cynomolgus monkeys, a species more closely related to humans, also showed acceleration of mid-femoral fracture healing by low-dose intermittent PTH (1–34) treatment. The above studies demonstrate the feasibility of using PTH, either the full length peptide or 1–34 amino terminal part, for the enhancement of regular fracture repair. Importantly, PTH has been shown to be effective also in experiment models for situations known to restrain bone healing such as aging, gonadal hormone deficiency, and malnutrition. Literature search could not reveal studies on the effect of PTH in animal models of critical size fracture nonunion. Clinical trial in humans showed that teriparatide shortened the time of healing of distal radial fractures in postmenopausal women by 2 weeks. Although this result means that PTH (1–34) may be effective in human, it has to be substantiated in trials involving more patients and additional skeletal sites [1–5].

OGP is a 14-amino acid chain identical to the C-terminal region of histone H4 (H4). It is produced by cells of the stromal lineage such as fibroblasts and osteoblasts and present in the blood circulation at micromolar concentrations. OGP and some of its naturally occurring and synthetic analogues have an established role as bone anabolic agents and hematopoietic stimulators. The discovery of OGP in the early 1990s followed the observation of enhanced systemic bone formation associated with post-ablation bone marrow regeneration [6]. A highly reproducible and controllable model system of experimental myelopoiesis occurs during regeneration of bone marrow after mechanical, chemical, or radioablation. Following such injuries, bone marrow regeneration is preceded by an intermediate phase of osteogenesis in which the affected medullary cavity is transiently filled with primary trabecular bone [7–10]. The intramedullary bone is resorbed and replaced by normal bone marrow. This osteogenic phase is accompanied by a systemic increase in bone formation [11]. OGP was initially isolated from primary bone during post-ablation healing bone marrow [12]. It regulates the local osteogenic response and transferred to the blood circulation and stimulates bone formation systemically [11–16]. OGP also promotes osteoblastic differentiation of bone marrow stromal stem cells through the stimulation of heme oxygenase-1 levels. It has been shown in vitro and in vivo that OGP levels are regulated by an autocrine/paracrine circuit as well as by binding

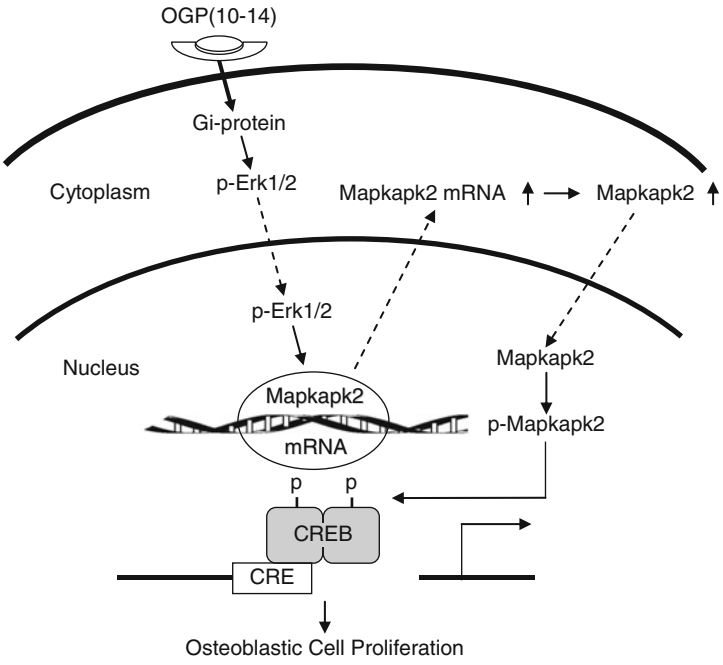

Fig. 4.1 OGP signaling. Following dissociation of the OGP–OGPBP complexes, the OGP is proteolytically processed in the extracellular milieu generating the active OGP. The formation of a putative OGP–OGP receptor complex leads to the activation of the intracellular Gi protein-CREB signaling cascade

to and dissociation from α2M. In rodents, exogenously administered OGP and OGP [12–17] stimulate osteoblastic activity and trabecular bone formation, thus preventing or reversing bone loss. The enhanced fracture healing was accompanied by elevated expression of genes involved in osteogenesis such as type II transforming growth factor-beta (TGF-β2), type I collagen, and the receptor for basic fibroblast growth factor [18, 19]. These studies suggest a role for OGP in the physiologic regulation of osteoblastic activity and bone mass and demonstrate its therapeutic potential as a bone anabolic agent for the systemic and local stimulation of bone formation. It has been demonstrated in osteoblasts that this pentapeptide binds to and activates a Gi protein-coupled receptor, which targets a mitogenic Erk1/2-Mapkapk2-CREB signaling pathway (Fig. 4.1) [20–26].

The biosynthesis of OGP is presented in Fig. 4.2. OGP is a H4 gene product. A pre-OGP is translated from H4 mRNA by a mechanism known as alternative translational initiation. The pre-OGP is proteolytically processed to become OGP. In the circulation, most of the OGP is present as a complex with α2-macroglobulin (α2M) thus being protected from proteolysis. Upon dissociation from α2M, the free OGP is proteolytically activated into its biologically active form, OGP [27–38].

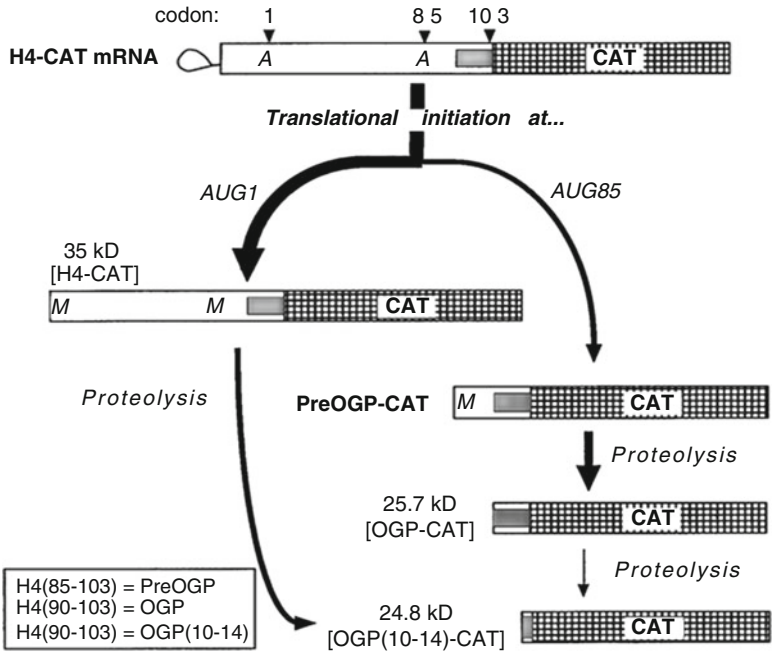

Fig. 4.2 Biosynthesis of OGP via alternative translational initiation at AUG85 of histone H4 mRNA. Pre-OGP [H4 (85–103)] is produced by alternative translational initiation at AUG85, and then proteolytically cleaved to yield OGP. OGP is slowly converted to OGP [i.e., H4 (99–103)], which, in addition, can be produced from full length H4 by proteolytic cleavage. The chloramphenicol acetyl transferase (CAT) derivatives are illustrated to directly reflect the actual data. A and M represent AUG codon and methionine residue, respectively. The correspondence of OGP-related sequences to H4 carboxyl terminal-derived peptides is presented in the *box* at the *lower left corner*

The increase in serum OGP after bone marrow ablation or acute blood loss is closely correlated with the enhancement of bone formation associated with these manipulations (Figs. 4.3 and 4.4). The OGP-induced increase in trabecular bone volume in these instances occurs consequent to an increase in trabecular thickness and connectivity. It has been demonstrated in rabbits and rats that systemically administered OGP stimulates fracture healing by enhancing the cartilage-to-bone transition in the fracture callus [39, 40].

Statins are 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors. They are anti-lipidemic, thus lowering cholesterol and reduce the incidence of cardiovascular disease. High doses of orally administered simvastatin have previously been shown to improve fracture healing in a mouse femur fracture model. In vitro and in vivo evidence could suggest that there are anabolic effects of statins in bone metabolism. Although evidence in patients with osteoporosis is conflicting, several studies have shown that the use of statins is associated with increases in bone mass density and reduction in fracture risk. The conflicting data may be due to different routes of administration, types of statins employed, and low doses used. In laboratory animals,

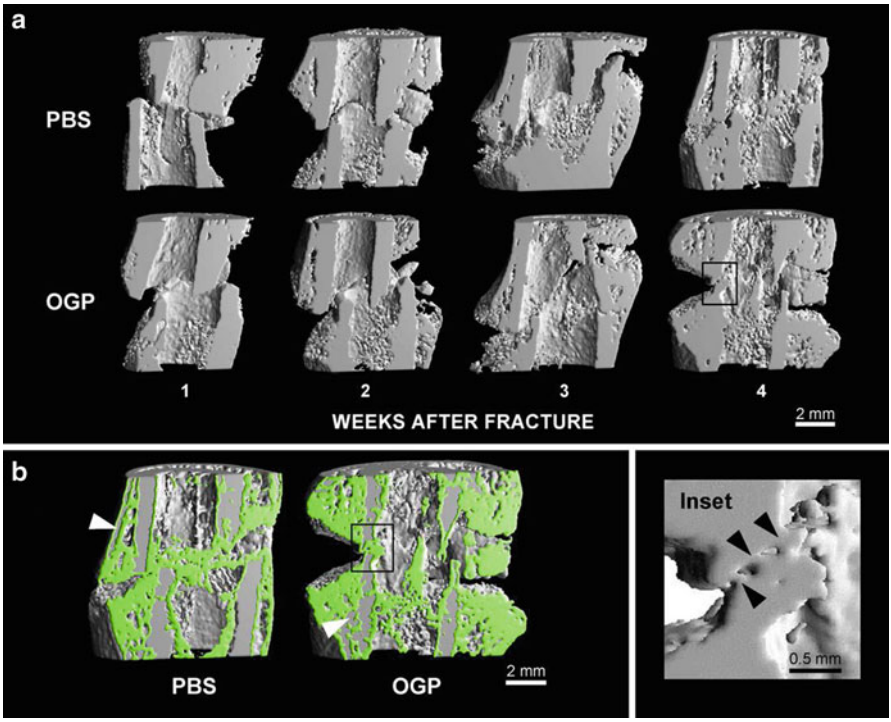

Fig. 4.3 Three-dimensional μCT images of fractured femora. (**a**) Effect of healing time and treatment on osseous components. *Inset*, high magnification of framed zone (4-week OGP). Note partially remodeled cortical union (*arrowheads*). (**b**) Overlay label of newly formed bone in specimens from 4-week OGP and control animals

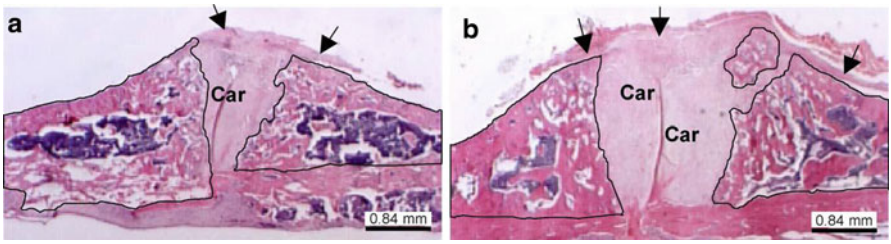

Fig. 4.4 Four-week fracture callus. (**a**) OGP-treated animal; (**b**) control animal. *Car*, cartilaginous callus; *contoured areas*, osseous callus; *arrows*, fibrous tissue. Hematoxylin and eosin

a positive effect on biomechanical parameters of fracture healing by simvastatin treatment was demonstrated following direct application at the fracture area. Statins have been shown to stimulate BMP2 transcription and bone formation. This raises the possibility that they could be useful for enhancing fracture repair. Observational studies in patients treated with oral statins for lipid lowering have been controversial.

The likely reason for their inconsistent effects is that the statin concentration reaching the periphery was too low after oral administration to produce a reproducible biologic effect. Taken together, there is sufficient evidence to suggest further clinical trials to establish the effect of statins on fracture healing [41–45].

Vitamin D. Adequate dietary intake of Vit D and calcium is essential to building and maintaining healthy bones. Animal studies have shown increased mechanical strength of the callous and other beneficial effects with Vit D treatment after a fracture. Given the few potentially harmful side effects, calcium and Vit D supplementation have long been advocated in an effort to augment bone healing. In the only trial reported in the literature on the role of Vit D and calcium supplementation was performed in treating osteoporotic women aged 78 years who had experienced an acute fracture of the proximal humerus. The primary outcome was the difference in bone mass density at the fracture site between the normal and osteoporotic. There was a significant difference in callous formation 6 weeks after fracture in the treatment group, but this was not sustained at 12 weeks. The clinical relevance of this study is questionable, even if applied to the study population, and it certainly cannot be generalized to other populations [46–48].

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Chapter 5
Bone Repair in Diabetes

Gail Amir

Abstract Diabetes is associated with an increased risk of fracture of bone and impaired fracture healing. Wound complications are more serious and the rate of postoperative complications is higher than that in non diabetic subjects. The etiology and pathogenesis of impaired bone repair in human subjects and animal models of type I and type II diabetes are reviewed. In addition the adequacy of bone repair around orthopedic and dental implants is addressed and a role for hyperglycosylation in impaired bone repair in diabetic subjects is discussed.

5.1 Delayed Fracture Healing in Diabetes Mellitus

In general, osseous fracture is repaired to an extent that morphologically and functionally the original bone is restored. A sequential cascade of events occurs which can be divided into four overlapping histological stages. The inflammatory stage begins with hematoma formation at the site of the fracture. The hematoma is invaded by neutrophils and macrophages that digest and remove debris and then by fibroblasts that form a collagenous framework. In the second stage (soft callus formation) progenitors are recruited to the fracture site where they proliferate and differentiate into chondrocytes and osteoblasts. Chondrocytes produce cartilage giving rise to the soft fibrocartilagenous callus. The cartilage mineralizes and then undergoes apoptosis and is removed by osteoclasts. Hard callus formation occurs by osteoblastic proliferation giving rise to bony replacement of the fibrocartilagenous callus. The bony callus then undergoes remodeling by cycles of resorption and bone formation until the original architecture of the bone is restored [1]. At the cellular level, inflammatory cells, vascular cells, osteochondral progenitors, and osteoclasts

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are the key players in the repair process. At the molecular level, fracture repair is driven by three main classes of factors: pro-inflammatory cytokines and growth factors, pro-osteogenic factors, and angiogenic factors [2].

Diabetes has been shown to cause osteopenia, particularly in patients with type 1 diabetes, and is associated with an increased risk of fracture of the hip, proximal humerus and foot, and significantly impaired fracture healing in humans and in animal models of type 1 and type 2 diabetes [3–7]. The literature on the effects of DM on fracture healing in man pertains almost exclusively to healing of lower limb and ankle fractures. Delayed union is seen in displaced fractures of the lower extremity in type 1 and type 2 diabetic patients [6], and diabetes is a risk factor for nonunion of ankle fractures [8]. Fractures in diabetic patients treated surgically have a higher incidence of prolonged union time and serious wound complications compared to nondiabetic patients [9], and the overall rate of postoperative complications of fracture is higher [10]. Insights into the mechanisms of diabetes-induced delayed fracture healing come mainly from the study of animal models. Delayed and impaired healing of fractures has been observed in several models of type I diabetes. In 1965, Wray first demonstrated that the tensile strength of fracture callus in diabetic rats is significantly reduced compared to that in nondiabetic rats [7]. More recent studies confirm delayed recovery of structural strength [11] and decreased mechanical strength of fractures in diabetic rodent models [12]. Histological studies of animal models of DM have demonstrated evidence of delayed fracture callus formation characterized by reduced early cellular proliferation, delayed chondrogenesis [12–14], and reduced vascular response, corresponding with impaired mechanical tensile strength of the bone [12, 14–16]. The etiology and pathogenesis of poor fracture healing in diabetic patients are largely unknown and are probably multifactorial. Diabetic patients have an increased susceptibility to infection, and they frequently have comorbidities that impair fracture healing such as macro- and microangiopathy and neuropathy [8]. Diabetic neuropathy may contribute to impaired blood flow as well as to inappropriate weight bearing before adequate union is achieved [9]. In view of the complex cascade of events contributing to fracture repair and the multifaceted interactions of DM and bone metabolism, it is difficult to assess the significance of the contribution of any single dysfunction to the impairment of fracture healing.

A wealth of literature has accumulated implicating various etiologic factors in the disturbances of callus formation in diabetic subjects. These include hypoinsulinemia, hyperglycemia, increased oxidative stress, increased cell death, and inappropriate levels of growth factors at the site of the fracture.

Insulin has an anabolic effect on bone [17]. Hypoinsulinemia has been implicated in delayed fracture healing. Systemic insulin treatment reverses impaired bone healing in diabetic animals [18, 19]. Local delivery of insulin to a fracture site ameliorates fracture repair in diabetic rats without affecting blood glucose levels, indicating a direct effect of insulin on fracture healing [20]. Some studies suggest that many of the defects of fracture healing in diabetic models are a direct result of hyperglycemia. Impaired collagen synthesis by osteoblasts and chondroblasts correlates with the degree of hyperglycemia in diabetic models and is thought to play a major role in the impairment of callus formation. Blood glucose control in

this model results in improved cellular proliferation and fracture healing and in normalized tensile strength [14, 15]. On the molecular level, high glucose levels modulate osteoblast gene expression resulting in bone loss and impaired osteoclast differentiation and function in vitro [21, 22]. This may be due to increased formation of advanced glycation endproducts (AGEs) induced by systemic hyperglycemia, possibly mediated through the AGE cell surface receptor, RAGE, on osteoblasts [23]. Oxidative stress is due to overproduction of reactive oxygen species (ROS). Increased oxidative stress is induced by a variety of mechanisms in diabetes, and it has been proposed as one of the major mechanisms of the hyperglycemia-induced trigger of diabetic complications [24]. Oxidative stress has been shown to inhibit osteoblast differentiation, to induce apoptosis of osteoblasts [25–27], to accelerate destruction of calcified tissue by osteoclasts, and is associated with a low turnover osteopenia. These changes can be reversed by administering antioxidants [28, 29]. The WNT pathway is involved in glucose homeostasis, and one of its functions is the regulation of differentiation of mesenchymal stem cells to osteoblasts or adipose cells, mediated by ß-catenin. It has been proposed that oxidative stress antagonizes the anabolic skeletal effects of Wnt/ß-catenin by diverting the limited pool of ß-catenin to FOXO-mediated transcription in osteoblasts [30, 31]. The FOXOs serve as a defense mechanism against oxidative damage by inducing cell cycle arrest and quiescence. Treatment with an antioxidant or with insulin suppresses oxidative stress and is associated with reversal of diabetes-associated osteopenia [32]. These findings suggest a role for oxidative stress in diabetic bone disease and may lead to the development of pharmacotherapeutic strategies to deal with impaired bone formation in diabetes [32, 33].

Impaired cartilage formation during fracture healing has been described in diabetic animals. They develop smaller fracture calluses than do nondiabetic animals during the period of transition from cartilage to bone, associated with increased cartilage resorption due to apoptosis of chondrocytes and increased osteoclastogenesis [34]. This contributes to impaired fracture healing by decreasing the scaffold for endochondral new bone formation [35]. The increased apoptotic rate of chondrocytes and increased osteoclastogenesis are normalized by insulin therapy, indicating that the adverse effects on fracture healing are directly related to the diabetic condition. Release of growth factors and cytokines at the fracture site is critical for organization and maturation of the early callus. Studies on diabetic rats have demonstrated decreased levels of platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), insulin-like growth factor I (IGF-I), vascular endothelial growth factor (VEGF), in early fracture callus associated with decreased cellular proliferation and impairment of collagen and extracellular matrix production, chondrocyte proliferation/differentiation and new blood vessel formation [13, 15, 36]. Local application of recombinant PDGF reverses these effects leading to enhanced fracture healing [37]. Low levels of expression of collagen type II, type X, and osteopontin have also been observed in diabetic rat callus [38]. Other studies implicate both the inflammatory stage and the soft callus stage. Inadequate callus formation and enhanced removal of cartilage from the callus in diabetic rats may be explained on a molecular level by the elevated levels of mRNA expression for aggrecanases that degrade cartilage (ADAMTS-4 and ADAMTS-5) and for cytokines that induce osteoclastogenesis

(TNF-α, M-CSF, RANKL, VEGF-A) [34, 35]. The impaired callus formation and the increased levels of cytokines are both reversed by insulin therapy. The final bone forming and remodeling stage of callus formation may also be impaired in diabetic subjects. Marrow ablation in diabetic mice is followed by reduced bone formation associated with reduced expression of critical transcription factors Runx2 and Dlx5 that regulate osteoblastic differentiation [39]. RANKL, its receptor RANK, and osteoprotegerin (OPG) provide the cellular and molecular basis for osteoblast–osteoclast cross talk which is crucial during bone remodeling. Interaction between RANK and RANKL on osteoclast precursors induces osteoclast differentiation leading to bone resorption. OPG is a decoy receptor of RANKL that antagonizes osteoclast differentiation. An imbalance between RANKL and OPG occurs at the fracture site in diabetic rats and may contribute to the delayed fracture repair in the diabetic condition [40].

Patients with diabetes have a higher complication rate after fracture. In addition to malunion, delayed union, and nonunion, these patients are also at risk for impaired wound healing, infection, and Charcot arthropathy. The latter is commoner in patients who were initially undiagnosed and had a delay in immobilization and in patients treated nonsurgically for displaced fractures [41]. There are many options for nonoperative and operative treatment of fractures in diabetic subjects. Correct soft tissue management and stable, rigid fixation with prolonged immobilization and prolonged restricted weight bearing are required in order to minimize problems and restore full function, particularly in patients with vasculopathy or neuropathy [42]. Regardless of whether insulin has a direct effect on fracture healing or whether its primary effect is to reverse hyperglycemia, the importance of managing serum glucose levels during fracture healing has been stressed by many investigators [14, 15, 34]. A better understanding of the cellular and molecular mechanisms of delayed fracture healing is expected to provide the basis for a new generation of drugs in the future to help promote fracture healing in diabetic subjects.

5.2 Delayed Bone Healing Around Orthopedic and Dental Implants in Diabetic Subjects

Diabetic patients undergoing arthroplasty have a higher rate of postoperative complications, particularly wound, medical, and orthopedic complications. They also have a higher revision rate and lower postoperative function scores than nondiabetic patients [43]. In addition, the long-term clinical results are worse than those of a control population due to the associated systemic complications of diabetic patients. Nevertheless, the long-term probability of implant survival is no different from that of nondiabetic subjects [44–46].

Assessment of the degree of osteointegration around metallic dental implants in experimental models of diabetes has given rise to conflicting reports. In many experimental models of type I diabetes, there is a reduced bone formation and reduced level of bone-implant contact. These changes are reversed after institution of insulin

therapy, and they do not develop in insulin-controlled diabetic rats [47–50]. In other studies of insulin-dependant rats impaired osteointegration has been noted, but this was associated with increased bone response around the implant [51, 52]. The results of studies on osteointegration of implants in type II diabetes models are also contradictory. There are some reports of impaired bone-implant contact [53] while others have not found significant differences in osteointegration or trabecular bone volume around implants [54]. Diabetes is not a contraindication to dental implant placement [55]. Nevertheless, a higher failure rate of dental implants is seen in type I diabetic subjects compared to nondiabetic subjects. Most of these occur in the first year of functional loading, suggesting microvascular complications as a possible etiologic factor [48]. Increased liability to infection is another factor. Diabetic subjects have a higher incidence of bacterial-induced mucositis (soft tissue inflammation) and peri-implantitis (associated with bone loss) [56]. Good control of serum glucose levels has been shown to improve implant survival. Type II diabetes does not impact on dental implant survival or complication rate [57].

5.3 The Role of Hyperglycosylation

It has been proposed that chemical modification of proteins in diabetes alters the structure and function of tissue proteins giving rise to AGEs. AGEs are slowly and irreversibly formed on proteins exposed to carbonyl and substrate stress, especially in conditions of prolonged hyperglycemia, hyperlipidemia, and/or oxidative stress. The main targets for AGE formation are long-lived proteins such as collagen. Accumulation of AGE has been implicated in the development of long-term complications in diabetic subjects such as reduced elasticity and increased permeability of blood vessels [58]. AGE-mediated collagen cross linking can cause loss of flexibility and elasticity and increased brittleness of tissues like bone [59]. Long-term exposure to AGE-modified proteins has been shown to inhibit proliferation, differentiation, and mineralization of osteoblastic cultures while increasing apoptosis of osteoblasts and ROS production [60–62]. In addition, osteoclastogenesis is enhanced by upregulation of RANKL [63]. These changes are mediated via specific receptors for AGE on osteoblasts (RAGE) [23, 64, 65]. This mechanism is thought to play an important role in the reduced strength, increased fracture risk, and impaired bone healing in diabetes and may in the future prove to be a useful drug target [60, 66].

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Chapter 6
Cannabinoids in Bone Repair

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Abbreviations

2-AG	2-Arachidonoylglycerol
β2AR	β2-Adrenergic receptors
BMP	Bone morphogenetic protein
DAGL	Diacylglycerol lipase
FAAH	Fatty acid amide hydrolase
IL-6	Interleukin 6
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
OGP	Osteogenic growth peptide
OPG	Osteoprotegerin
OVX	Ovariectomy
PTH	Parathyroid hormone
RANKL	Receptor activator of NFκB ligand
SNP	Single nucleotide polymorphism
THC	Δ9-Tetrahydrocannabinol
TRPV1	Transient receptor potential vanilloid type 1 receptor

The Marijuana plant, *Cannabis sativa*, has been cultivated throughout history for medical and recreational use. Its psychoactive properties are exploited generally for drug abuse. However, it is well established that in addition to its effect on the nervous system, it is involved in the functioning of other organs in the body.

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The psychoactive component of marijuana and hashish, Δ9-tetrahydrocannabinol (THC), acts on two distinct receptors that are distributed throughout the body, only one of which mediates the psychotropic effects. These receptors respond to endogenous ligands, termed endocannabinoids, with THC just mimicking the activity of these physiological activators. The endocannabinoids are produced and degraded by specific enzymes. Together, the receptors, ligands, and enzymes comprise the endocannabinoid system. It is well established that THC produces numerous beneficial effects, including analgesia, appetite stimulation, nausea reduction, and reduction of intraocular pressure. THC also affects fertility, short-term memory, tumor growth, and motor coordination. Recently, there has been a rapidly growing interest in the role of cannabinoids in the regulation of skeletal remodeling and bone mass, addressed in basic, translational, and clinical research [1–13]. Studies published in the past decade propose an important role for the endocannabinoid system in the regulation of skeletal remodeling. The primarily neural CB1 cannabinoid receptor has been identified in sympathetic terminals innervating the skeleton. However, its function in controlling bone turnover is only partially understood. The predominantly peripheral CB2 receptor is expressed in bone cells. Its mechanism of action in bone cells, corroborated by human genetic considerations, has been reported in detail. Important genetic risk factors for low bone mass are attributed to polymorphism in CNR2, the gene encoding CB2. In this chapter, these considerations are extrapolated to address the potential role of the endocannabinoid system in bone wound healing. Several key components of the endocannabinoid system have been identified in bone. The main physiologic involvement of CB2 is associated with the maintenance of the balance of bone remodeling (Fig. 6.1). CB2 agonists are possible candidates for a combined anti-resorptive and anabolic therapy for osteoporosis. These considerations open an important therapeutic avenue in the treatment of impaired bone remodeling, bone healing, and bone implant acceptance, and control of bone mass and biomechanical function.

6.1 Cannabinoid Receptors and Ligands

CB1 and CB2 are G protein-coupled receptors [14], which share 44% of the overall identity (68% identity for the transmembrane domains). CB1 is perhaps the most abundantly expressed G protein-coupled receptor in the central nervous system. It is also present in peripheral neurons and gonads and to some extent in several other peripheral tissues. CB2 is expressed in the skeleton, immune system, cirrhotic liver, arteriosclerotic plaques, inflamed gastrointestinal mucosa, and glial and inflammatory cells in pathological brain conditions [15, 16]. That CB1 and CB2 are not functionally identical is demonstrated by the presence of cannabinoid agonists and antagonist with distinct binding specificities to either receptor [14–25]. Both receptors signal via the Gi/o subclass of G proteins, inhibiting stimulated adenylyl cyclase activity. Further downstream, the CBs induce the activation of p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase, AP-1, the

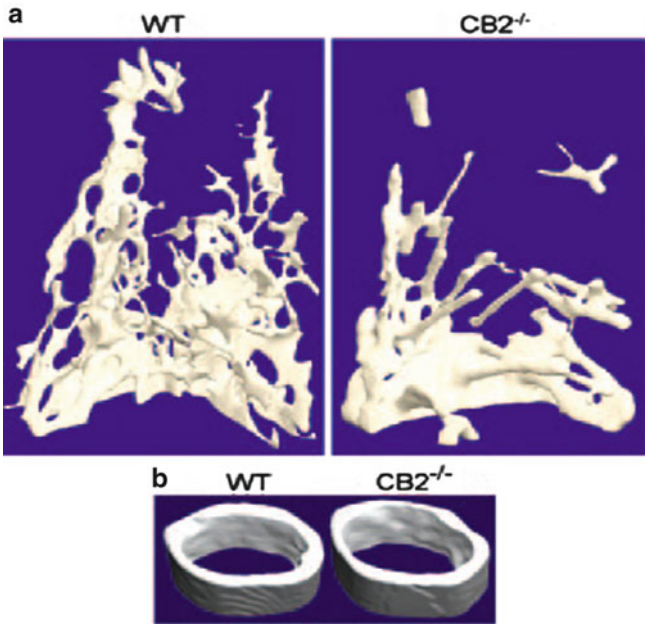

Fig. 6.1 Sever osteoporosis in femoral bones of aging CB2-deficient mice compared to wild-type (WT) femoral bones. Microcomputed tomographic images of trabecular bone in distal metaphysic (**a**) and mid-diaphysis (**b**)

neural form of focal adhesion kinase, protein kinase B, and K^+ and Ca^{2+} transients. Recently, it has been shown that the mitogenic action of CB2 in osteoblasts is mediated through a Gi-protein–Erk1/2–Mapkapk2–CREB–cyclin D1 axis (Fig. 6.2).

It has been proposed that GPR55 and TRPV1 may be also involved in endocannabinoid triggering of these event [26–34].

The main CB1 and CB2 endogenous ligands are *N*-arachidonoylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG). Anandamide is present in a variety of tissues such as the brain, kidney, liver, spleen, testis, uterus, and blood in picomol/g concentrations, with the highest levels reported in the central nervous system. The low anandamide concentrations have been attributed to low substrate levels and/or the short anandamide half-life in vivo. Anandamide is biosynthesized through *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD)-dependent and -independent pathways. The main anandamide degrading enzyme is fatty acid amide hydrolase (FAAH) [35–38]. In general, the tissue distribution of 2-AG is similar to that of anandamide. However, its concentration is three orders of magnitude higher (ng/g range). 2-AG production has been demonstrated in the central nervous system, platelets, and macrophages, especially in response to stimulation by inflammatory agents such as lipopolysaccharide. It is generated from arachidonic acid-enriched membrane phospholipids, such as inositol phospholipids, through the combined actions of phospholipase C and diacylglycerol lipases (DAGLα and DAGLβ). It is metabolized by a monoacylglycerol lipase (MAGL) [39–43].

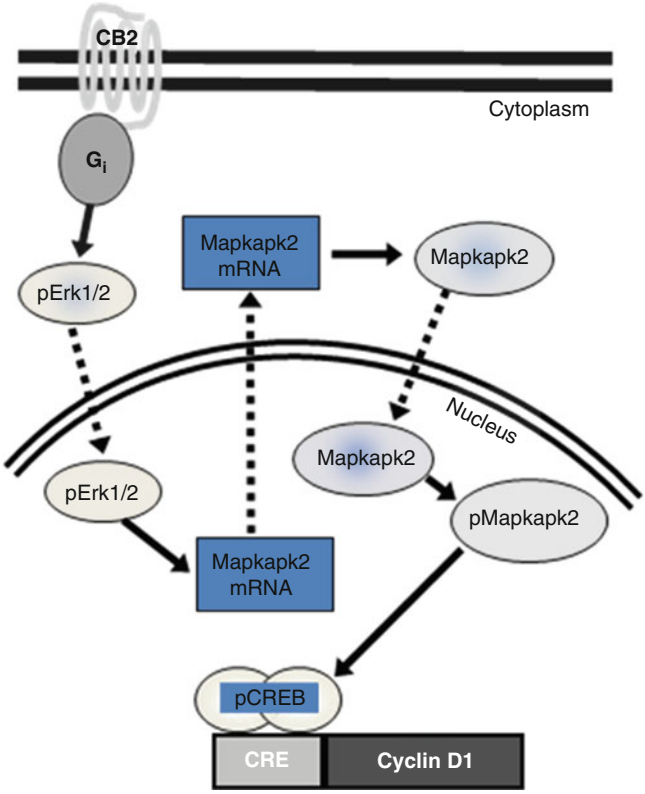

Fig. 6.2 Model of CB2 mitogenic signaling in osteoblasts

6.2 The Skeletal Endocannabinoid System

The major key components of the endocannabinoid system have been identified in bone (Fig. 6.3). Anandamide and 2-AG are present in this tissue at levels similar to those found in the brain. Because the blood endocannabinoid levels are several orders of magnitude lower than those found in bone, it is very likely that anandamide and 2-AG are synthesized locally in the skeleton. Indeed, both ligands are produced by osteoblasts and osteoclasts in culture. In addition, DAGLα and β are expressed in osteoblasts, osteocytes, and bone-lining cells (Fig. 6.4).

NAPE-PLD and FAAH are also expressed in bone cells. Although both 2-AG and anandamide are perceived as nonselective agonists of CB1 and CB2, findings in bone and bone cell cultures suggest differential effects of these ligands. While 2-AG activates CB1 in the sympathetic nerve terminals following a single or chronic administration to mice, thus stimulating bone formation, it has no effect on osteoblasts and may even act as an inverse agonist in these cells [16]. Like the CB2 selective

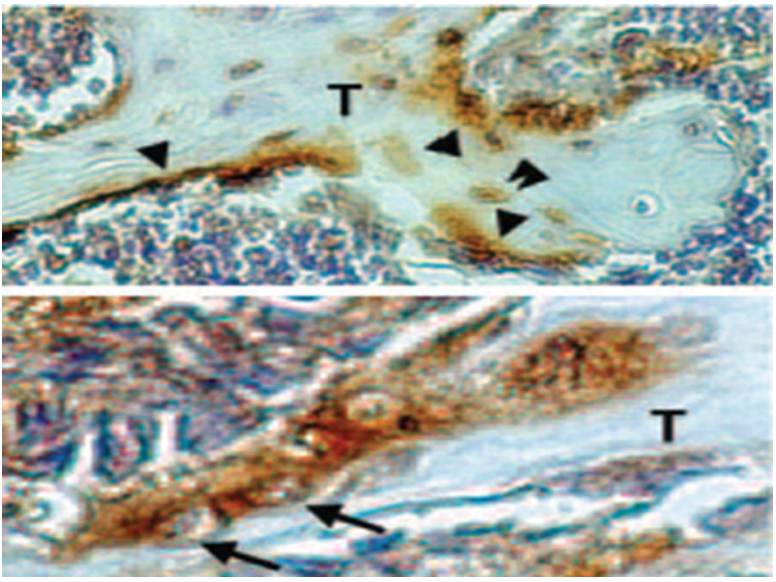

Fig. 6.3 Expression of CB2 in mouse osteoblasts, osteocytes, and osteoclast. T, trabecule; *arrows*, osteoclasts; *arrowheads*, osteoblasts; *double arrowheads*, osteocytes. Immunohistochemical staining using anti-CB2 antibodies

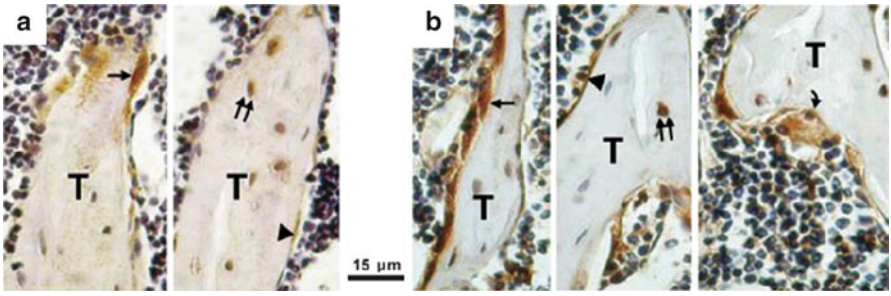

Fig. 6.4 Expression diacylglycerol lipase (DAGL) in bone cell. (**a**) DAGLα; (**b**) DAGLβ. T, bone trabecule; *arrows*, osteoblasts; *double arrows*, osteocytes; *arrow heads*, lining cells; *bent arrow*, osteoclasts

agonist, anandamide stimulates in vitro osteoblast proliferation. In addition, the number of osteoclasts in culture is increased by a direct challenge with anandamide or through the action of the FAAH inhibitor URB597 that leads to increased anandamide levels endogenously [15]. It remains to be seen whether these actions of anandamide are mediated by CB1, CB2, GPR55, and/or TRPV1.

6.3 Effects on Bone Cell Differentiation and Activity

Activation of CB2 has different effects in early osteoblast progenitors and in more mature osteoblastic cells. In the early precursors, represented by bone marrow-derived, partially differentiated osteoblastic cells that show limited CB2 expression, the specific CB2 agonist HU-308 [44] (Fig. 6.5), but not the specific CB1 agonist noladin ether [45], triggers a mitogenic effect and consequent expansion of the preosteoblastic pool. Ex vivo osteoblastic colony (CFU-Ob) formation by bone marrow stromal cb2−/− cells is markedly diminished, whereas CFU-Ob formation by wild-type cells is stimulated by HU-308. In mature osteoblastic cells, represented by the MC3T3 E1 cell line, the same ligand stimulates osteoblast-differentiated functions such as alkaline phosphatase activity and matrix mineralization [13, 45]. Hence, CB2 signaling is involved in several regulatory, pro-osteogenic processes along the osteoblast lineage.

In mouse bone marrow-derived osteoclastogenic cultures, CB2 activation inhibits osteoclast formation by restraining mitogenesis at the monocytic stage, before incubation with RANKL. It also suppresses osteoclast formation by repressing RANKL expression in osteoblasts and osteoblast progenitors [40]. Likewise, it has been recently shown that the cannabinoid receptor agonist ajulemic acid also suppresses osteoclastogenesis [42]. By contrast, another study reported the stimulation of osteoclast formation and bone resorption by cannabinoid receptor agonists and their inhibition by antagonists [13, 44]. These allegedly paradoxical effects may be species and/or agonist dependent, as in human osteoclasts and other cells anandamide

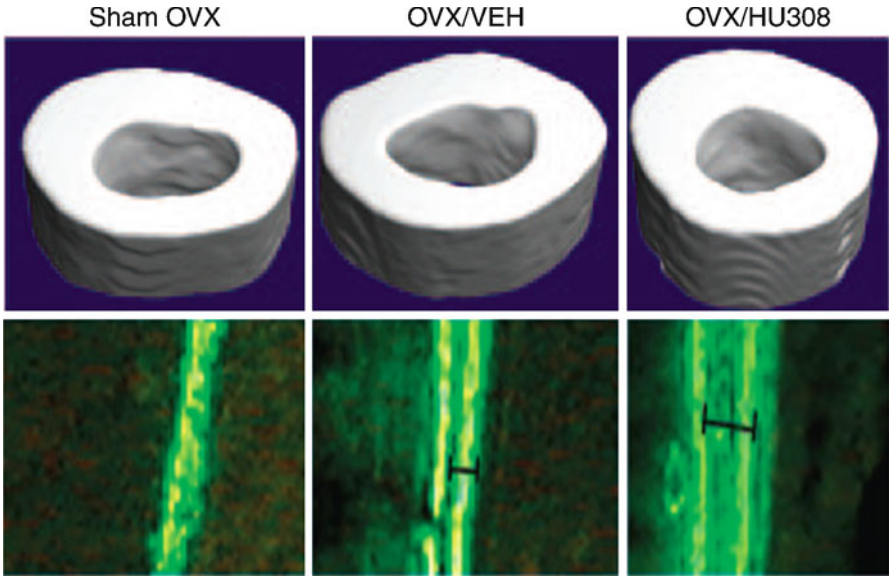

Fig. 6.5 Increased endosteal bone formation in OVX mice treated with the CB2 agonist HU-308

has been shown to activate also TRPV1. TRPV1 activation in the human osteoclasts and osteoclast precursors enhances osteoclast formation and activity and may modify the effect of selective CB2 agonists. In addition to CB2, low levels of CB1 mRNA have been reported in bone cell cultures [13, 44].

6.4 Skeletal Phenotypes of Cannabinoid Receptor-Deficient Mice

Cannabinoid receptor mutant mice have been used to assess the physiologic role of CB1 and CB2 in the control of bone mass. CB1-deficient mice have a low bone mass phenotype accompanied by increased osteoclast counts and decreased bone formation rate (Fig. 6.6). Our recent findings suggest that CB1 controls osteoblast function by negatively regulating norepinephrine (NE) release from sympathetic nerve terminals in the immediate vicinity of these cells.

NE suppresses bone formation by binding to osteoblastic β2AR [40]; this suppression is alleviated by activation of sympathetic CB1 (Fig. 6.7). That Cb1 agonists may be used to speed up bone wound healing is a corollary of the enhancement in fracture healing repeatedly reported in patients after traumatic brain injury (TBI) [45].

In a mouse model for TBI, we have demonstrated a critical regulatory role for CB1 and 2-AG in the stimulation of bone formation (Fig. 6.8). Therefore, peripherally selective specific CB1 agonists that do not cross the blood–brain barrier, and therefore do have any central adverse effects, could serve for the stimulation of

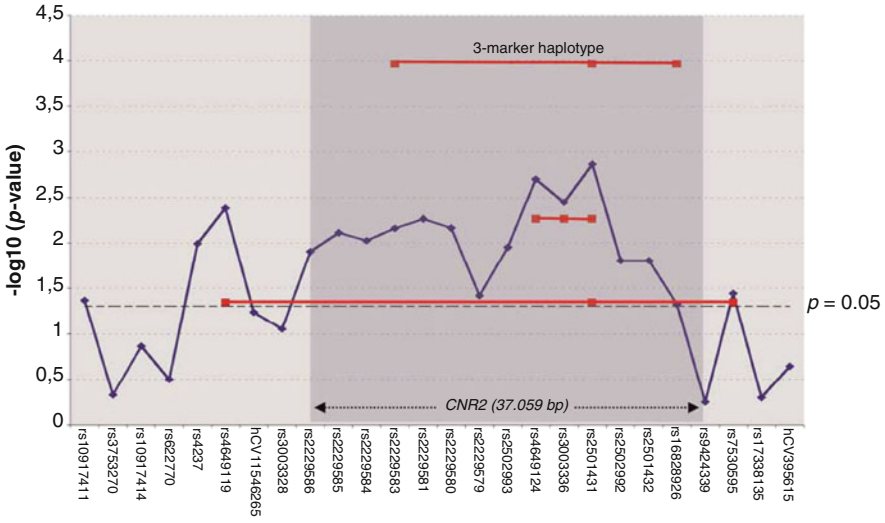

Fig. 6.6 Association of single nucleotide polymorphisms in the CNR2 gene with human osteoporosis

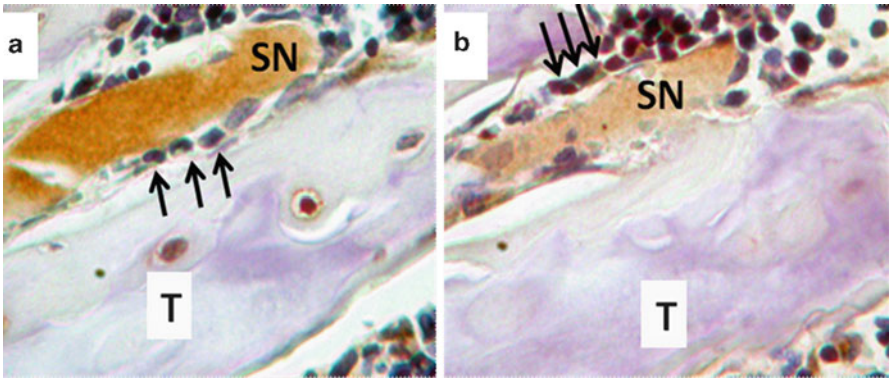

Fig. 6.7 CB1 expression in skeletal sympathetic nerve (SN) terminals. Serial sections stained with anti-tyrosine hydroxylases (**a**) and anti-CB1 (**b**) antibodies. T, trabecule; *arrows*, osteoblasts

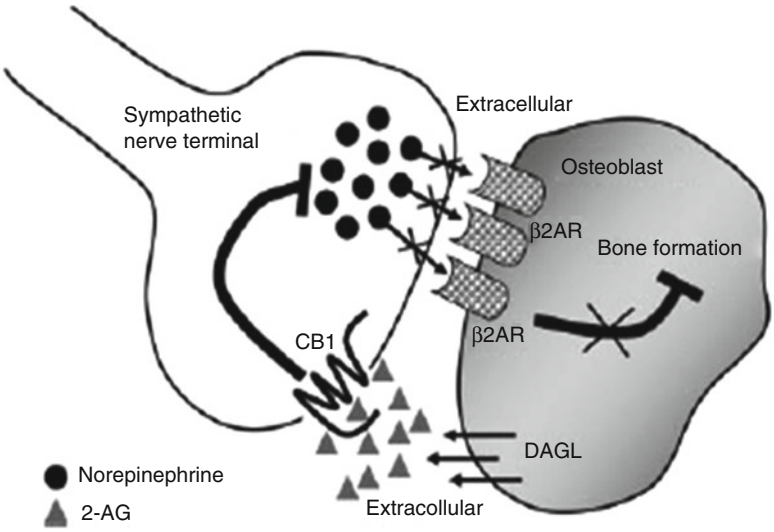

Fig. 6.8 Regulation of skeletal norepinephrine levels by osteoblast-derived 2AG

fracture repair. Alternatively, MAGL inhibitors could be used to stimulate CB1 by increasing the endogenous 2-AG levels. Animals with a CNR2-mutated gene have also a typical skeletal phenotype (Fig. 6.6). During their first 2–3 months of life, CNR2−/− mice accrue a normal peak trabecular bone mass, but later display a markedly enhanced age-related bone loss; their trabecular bone volume density at 1 year

of age is approximately half compared to wild-type controls. Reminiscent of human postmenopausal osteoporosis, the CNR2−/− mice have a high bone turnover with increases in both bone resorption and formation which are at a net negative balance [38]. Importantly, low bone mass is the only spontaneous phenotype so far reported in these mice. Hence, because healthy CB2 mutant mice are otherwise normal, it appears that the main physiologic involvement of CB2 is associated with maintaining bone remodeling at balance. Polymorphisms in the human CNR1 and CNR2 loci were studied to assess the cannabinoid receptors as targets for the risk assessment and treatment of osteoporosis. The CNR1 locus is located on chromosome 5q15 and encompasses a single coding exon that is preceded by several noncoding 5′ exons, indicating a complex transcriptional regulation of this gene by different promoters. The CNR2 locus is located on chromosome 1p36. This genomic region and its mouse ortholog on chromosome 4 have been linked to BMD and osteoporosis in several independent association analyses. However, these analyses did not consider CNR2 as a potential candidate gene. Like CNR1, the CNR2 gene also consists of a single coding exon, which is preceded by a noncoding upstream exon. Thus far, two genetic association studies have been reported dealing with the relationship between polymorphisms in CNR genes and osteoporosis. The first study was carried out in a French Caucasian sample comparing postmenopausal osteoporotic women with a low bone mineral density (BMD) and age-matched healthy controls. Analysis of four single nucleotide polymorphisms (SNPs) spanning nearly 20 kb around the CB1 coding exon revealed no significant association with the osteoporosis phenotype, suggesting that the CNR1 locus does not have a major role in this sample. In the CNR2 gene, a total of 26 SNPs were analyzed, spanning approximately 300 kb around the CNR2 locus. Several of these SNPs showed a significant association with the disease phenotype, suggesting that CNR2 polymorphisms are important genetic risk factors for osteoporosis. The most significant *p*-values for allele and genotype associations were observed with SNPs located within the CB2 coding region ($p = 0.0014$ and $p = 0.00073$, respectively). Furthermore, when BMD at the lumbar spine was analyzed as a quantitative trait, highly significant differences were found in BMD between individuals carrying different SNPs in the CB2 coding region. Indeed, sequencing the CB2 coding exon in all patients and controls identified two missense variants, Gln63Arg and His316Tyr, with the Arg63 variant being more common in the osteoporotic patients than in the healthy controls. Taken together, these findings suggest that a common variant of the CB2 receptor contributes to the etiology of osteoporosis in humans. The second is a prospective study, which analyzed several candidate quantitative trait loci in BMD, including CNR2, in a cohort of 1,110 women and 1,128 Japanese men, 40–79 years of age [46]. For the *CNR2* locus, a single SNP (rs2501431, A→G) was assessed, which had shown the strongest association in the previously published French sample. BMD, measured by peripheral quantitative computed tomography or dual-energy X-ray absorptiometry, was consistently lower in women with the AA genotype compared to the AG and GG genotypes. Together, these studies strongly suggest that *CNR2* is the susceptibility gene for low BMD and osteoporosis on chromosome 1p36 [47–53].

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Part III
Bridging of Skeletal Defects and Implants

Chapter 7
Mesenchymal Stem Cells for Bone Gene Therapy

Gadi Pelled, Olga Mizrahi, Nadav Kimelman-Bleich, and Dan Gazit

Bone tissue has regenerative capabilities enabling the self-repair and regeneration of fractures and other forms of damage. However, in extreme situations where the extent of bone loss or damage due to trauma, surgery, or metabolic diseases such as osteoporosis is too large, full regeneration will not occur. In these situations, the extent of bone loss, or the conditions leading to its loss, is beyond the capabilities of the bone self-repair mechanism. Large bone defects that do not spontaneously heal are thus called nonunion defects, and they present a major problem for orthopedic surgeons. Currently, nonunion fractures are treated by metallic implants that tend to fail in the long run. In addition, extensive bone formation is required in spine surgery when a fusion of two vertebrae is considered in the treatment for intervertebral disc degeneration leading to back pain. It is clear, therefore, that for these clinical conditions a biological approach that will be able to enhance bone formation is required. Most of the biological approaches undertaken to overcome the loss of large bone segments involve the administration of either cells with osteogenic potential or of osteogenic growth factors. Both approaches are aimed at enhancing the bone formation process by introducing one of its components: i.e., bone forming cells and osteogenic growth factors that promote the proliferation and differentiation of osteoprogenitors into mature osteoblasts that are responsible for the lay-down of new bone tissue. Gene therapy was applied to this setting as a means to deliver recombinant DNA sequences encoding for the osteogenic growth factors. However, in delivering the desired gene in vivo, two strategies may be taken:

1. The direct delivery of the construct via viral or nonviral vectors or
2. The delivery of the desired gene product via cells that were genetically engineered to express the desired gene, ex vivo. The latter approach is termed Cell-Based Gene Therapy, and it will be the main focus of this chapter.

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7.1 Stem Cells

Stem cells are a distinct population of cells that can give rise to different tissues. Two main features characterize all types of stem cells: self-renewal and the ability to give rise to differentiating daughter cells. Stem cells can be further divided into two major groups. The first group constitutes the embryonic stem (ES) cells, which together with the totipotent zygote present a cell population able to develop into a multitude of cell types and tissues [1]. The second group constitutes adult stem cells, which reside in adult tissues and give rise to differentiated, tissue-specialized cells. These adult stem cells are responsible for the regenerative capacities of tissues in the body. Generally, adult stem cells present a more limited range of differentiation compared with ES cells. In studies done by Jiang et al., it was reported that multipotent adult progenitor cells (MAPCs) purified from adult bone marrow can differentiate at the single cell level, to cells with visceral mesoderm, neuroectoderm, and endoderm characteristics in vitro [2]. Adult stem cells are preferable for therapeutic purposes since they are considered safer for transplantation with lesser proliferation capacity and tumorogenecity than ES cells. In addition, adult stem cells are more easily directed to specific lineages than ES cells which can give rise to a wide range of tissues following local transplantation [3]. Skeletal tissues such as bone, cartilage, tendon, and ligament that are the focus of orthopedic medicine vary in their ability to self-regenerate. While bone tissue is considered to have high regeneration capacity and ligament tissue a somewhat lesser degree, cartilage tissue is considered to have a very low self-repair ability [4, 5]. All these tissues are believed to have originated from similar common stem cells termed mesenchymal stem cells (MSCs). MSCs are stem cells residing in a variety of adult mesenchymal tissues. The MSCs and their self-repair ability correlate with their capability in recruitment of adult MSCs from their local environment [6, 7].

MSCs were readily isolated from the bone marrow and expanded in culture [8]. They were shown to differentiate into various mesenchymal lineages including bone, cartilage, adipose, muscle, and tendon [9]. Their accessibility and ease to manipulate in vitro have made them natural candidates for orthopedic gene therapy studies and the focus for the development of therapeutic approaches in orthopedic therapy. However, bone marrow-derived MSCs are not the only stem cells found to differentiate to various skeletal tissues. Stem cells from other tissues, such as muscle and adipose tissue, were also found to have similar properties [10, 11].

7.2 Bone Marrow-Derived MSCs for Bone Gene Therapy

Since MSCs and osteoprogenitors are relatively easily isolated from bone marrow and cultured in vitro, it is conceivable to use them as vehicles for the delivery of therapeutic genes in vivo, a strategy known as stem cell-based gene therapy [12]. Most gene therapy studies directed to bone healing attempt to induce bone formation in a model of bone nonunion fractures or as a means to achieve spinal fusion.

Indeed, some studies have used primary MSCs and cell lines for the expression and delivery of osteogenic genes inducing bone formation [13–17]. These studies implemented various types of MSCs including cell lines such as C3H10T1/2 and primary marrow-derived stem cells for the delivery of bone morphogenetic protein-2 (BMP-2). The delivery of growth factors of the bone morphogenetic protein family is often used in these studies, since these factors promote osteogenic differentiation and bone formation [18, 19]. In particular, BMP-2 was commonly used because it is a highly osteoinductive agent, well studied and known to induce bone in vivo in ectopic and orthotropic sites [18, 20–30]. Other members of the BMP family, such as BMP-4 and -9, were also used for stem cell-mediated gene therapy [31–36]. The hypothesis of these studies was that healing of bone defects could be achieved by long-term production of osteoinductive agents in the vicinity of bone defects, inducing new bone formation and defect repair.

Bone marrow-derived MSCs are good candidates for gene therapy directed to bone regeneration, not only because of their accessibility, but also because they form the source stem cells for osteoprogenitors and osteoblasts, the bone forming cells, in the bone environment [6]. Osteogenic differentiation begins with the commitment of the undifferentiated MSC to the osteogenic lineage, giving rise to committed osteoprogenitor cells that gradually differentiate into mature osteoblasts [6]. It was postulated that using genetically engineered MSCs for bone cell-mediated gene therapy may have a particular advantage [12]. When these cells are engineered to express osteogenic growth factors such as BMP-2, upon transplantation in vivo, the expressed transgene exerts its effect not only on host mesenchymal tissue (paracrine effect) but also on the engineered MSCs (autocrine effect). Thus, the engrafted, engineered MSCs differentiate and contribute to the bone formation process and in parallel recruit and induce osteogenic differentiation in other host stem cells (Fig. 7.1). It was hypothesized that these dual autocrine and paracrine effects may promote bone formation to a larger extent than any other cell type merely exerting a paracrine effect. Using murine C3H10T1/2 MSC line that were engineered to express BMP-2, the authors were able to demonstrate their increased osteogenic potential over non-MSCs engineered CHO cell lines that also expressed BMP-2 [12]. Engineered MSCs were able to heal murine nonunion radial defects to a greater extent than non-osteogenic CHO cells, despite the fact that these cells secreted more BMP-2 protein than the engineered MSCs.

Using MSCs as vehicles for gene delivery has an additional benefit over direct in vivo delivery of proteins or genes. Engineered MSCs can potentially engraft into the damaged tissue in vivo and express the therapeutic genes for long periods, whereas local, one time administration of genes or protein will have a limited time effect. BMP family members are known for their ability to induce bone formation in vivo and repair bone defects when applied locally in the injury sites [37–39]. In order to compare the efficiency of stem cell-mediated gene therapy with BMP-2 protein delivery, Moutsatsos et al. have analyzed the extent of bone tissue produced by engineered MSCs (C3H10T1/2) expressing BMP-2 compared with local administration of a high dose of BMP-2 in a murine radial nonunion defect [40].

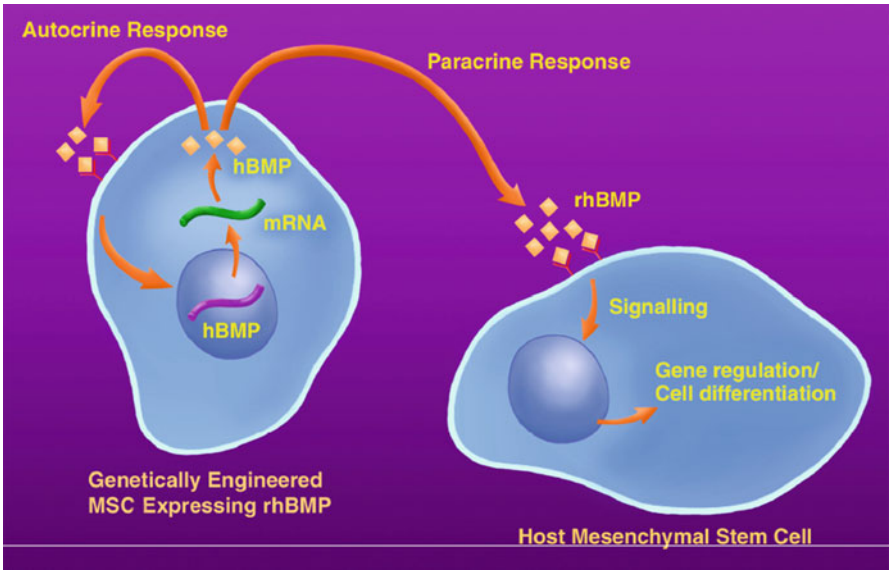

Fig. 7.1 Genetically engineered MSCs exert both autocrine and paracrine responses leading to a synergistic effect of osteogenesis in vivo

The authors have found that the engineered MSCs produced significantly more bone tissue then was produced following local administration of BMP-2 protein.

MSCs or osteoprogenitor cell-mediated gene therapy holds yet another advantage over protein delivery or even other types of gene delivery. When analyzing the healing process in bone defects following transplantation of MSCs engineered to express rhBMP-2, an interesting pattern is observed. Engineered MSCs have produced bone in an organized manner by augmenting new bone on top of the defect edges, forming continuous regeneration between the original defect edges and the newly formed bone [12, 40, 41]. In comparison, BMP-2 protein delivery or the implantation of non-MSCs CHO cells expressing BMP-2 resulted in the formation of diffused bone foci with no continuity to the original bone [12]. This phenomenon can be attributed to the ability of MSCs to localize and orient themselves to particular sites in the defect area following transplantation. It was found that MSCs localized mainly surrounding the defect edges rather than being randomly distributed in the defect site [12]. Apparently, being stem cells, MSCs can respond to local factors and developmental signals that direct and guide their orientation in the transplantation site and affect the healing process in a manner similar to the process that takes place during bone formation in developmental stages. Liechty et al. demonstrated that human MSCs possessed these characteristics by showing that these cells were able to engraft in various fetal mesenchymal tissues following systemic administration in utero in sheep [9]. Moreover, human MSCs were able to localize into the osteoprogenitor layers of calvaria bone when transplanted subcutaneously adjacent to the calvaria in SCID mice [42].

Human bone marrow-derived MSCs are expected to have the same regenerative benefits described above for murine MSCs. However, if stem cells engraft and respond to local signals, what possible advantage does genetic engineering have? This question is stressed in the case of human MSCs which were previously found to regenerate bone in vivo upon local transplantation to bone defect, even without any genetic engineering [43]. On the other hand, additional studies have shown that human MSCs cannot form bone when engineered to express the LacZ gene or nonengineered at all, compared with human MSCs engineered to express BMP-2 [41]. It was found that human MSCs infected with adenoviral vector encoding for human BMP-2 were able to differentiate to osteogenic cells both in vitro and in vivo forming cartilage and bone tissues, and healing nonunion defects created in CD nude mice [29, 41]. Human MSCs infected with adenoviral vector encoding the LacZ reporter gene were not able to form bone or cartilage in vivo. The type of matrix used to carry for human cells when delivered in vivo could explain this discrepancy. It was found that the ability of human MSCs to form bone in vivo is dependent upon osteoinductive matrices such as hydroxyapatite/TCP that are non-biodegradable [44]. Consequently, genetic engineering of human MSCs may elicit the osteogenic potential of MSCs regardless of the carrier type with the use of biodegradable carriers [41, 45]. One can safely assume that in large bone defects, nonengineered human MSCs will not be sufficient in order to induce repair compared to genetically engineered cells. The above-mentioned studies demonstrate the unique features that MSCs possess, granting them additional advantage for the use in bone gene therapy and gene delivery. These stem cells can serve as "smart" vehicles that in addition to expressing the transgene in specific areas of the damaged tissue can also actively participate in the neo-tissue formation process.

7.3 Cell Sources for Stem Cell-Mediated Bone Gene Therapy

Adult MSCs that reside in the bone marrow are the natural stem cells for bone forming cells, since this is the natural reservoir from which cells are recruited for bone repair. However, this does not exclude the use of other sources of stem cells for bone gene therapy. The most prominent cells studied in this regard are fat and muscle stem cells [11, 46–52]. Muscle-derived stem cells can be conveniently isolated from a muscle biopsy and expanded in vitro. Muscle tissue contains stem cells with the ability to differentiate into osteoblasts under the influence of a proper osteogenic factor such as BMP-2 [10]. These cells though originating from murine muscle tissue, following engineering to express BMP-2 or BMP-4, were able to differentiate into osteoblasts and osteocytes and could heal critical nonunion bone defects in the calvaria [50, 53]. Likewise, engineered cells from human skeletal muscle were shown to have in vitro and in vivo osteogenic potential when engineered to express rhBMP-2 [49]. Since a muscle cell is one of the possible differentiation pathways of MSCs, these results are not surprising.

Fat tissue-derived stem cells can also respond to BMP signaling by converting from the adipogenic differentiation pathway toward the osteogenic one [47, 52, 54]. These stem cells could be obtained from routine liposuction procedures and cultured in vitro. Osteogenic differentiation of adipose-derived stem cells was induced by the over-expression of BMP-2 and was further used for the healing of critical sized femoral defects and induce spinal fusion [48, 51, 55]. MAPCs that were co-purified with MSCs from adult marrow differentiated not only into mesenchymal cells but also into cells with mesoderm, neuroectoderm, and endoderm characteristics in vitro. In vivo, MAPCs engrafted and differentiated to the hematopoietic lineage and to the epithelium of liver, lung, and gut. However, these cells were not integrated in skeletal tissues, an issue which might be attributed to the low turnover characteristic of these tissues [2]. Nevertheless, as MAPCs proliferate at a high rate, they may also be considered as another source for stem cell-mediated therapy. ES cells originating from the inner cell mass of an embryo in the blastocyst stage have a wide differentiation potential both in vitro and in vivo. These cells can give rise to a variety of cell types including neural cells, cardiomyocytes, vascular cells, and hematopoietic cells [56]. They were also found to differentiate into osteogenic cells in vitro [57, 58]. However, there is still no data on osteogenic differentiation in vivo. In addition, the method of obtaining human ES cells introduces some major ethical issues [59, 60]. Moreover, it was found that MAPCs obtained from the bone marrow possess a pluripotent range of differentiation that includes all cell types found in the embryo [61]. For these reasons, one can expect that ES cells will not be the main target cells for gene therapy applications in orthopedics in the future, whereas the more easily obtained, manipulated, and less controversial MSCs will be a more realistic choice.

7.4 Angiogenesis and Bone Gene Therapy

Most gene therapy strategies to facilitate bone regeneration, as was discussed above, focus on the delivery and expression of osteoinductive genes, such as members of the BMP family. Such growth factors promote osteogenic differentiation of MSCs, osteoprogenitors, and osteoblasts. This approach is aimed at initiating and promoting the primary process that is responsible for osteogenesis. A different, novel approach was suggested that targets initiating secondary processes supporting new bone formation by promoting angiogenesis [32, 40, 62]. Angiogenesis was found to be closely correlated to enchondral bone formation during development [40, 63]. It was found that vascular endothelial growth factor (VEGF), a known inducer of angiogenesis, couples the transition from cartilage to bone in developing bones. Moreover, it was found that applying TNP-470, an angiogenesis inhibitor, could markedly reduce BMP-2-induced ectopic bone formation in muscle tissue. Other studies have found a correlation between angiogenesis and GDF-5, a member of the BMP family, and SMAD5, a BMP signal molecule [64–66]. Moutsatsos et al. reported an important finding linking angiogenesis and together with new bone formation induced by BMP-2 secreting MSCs [40]. Increased blood vessel formation was observed,

coupled with new bone and cartilage created in ectopic muscle tissue transplanted with engineered MSCs. A CAM assay further indicated that BMP-2 protein induced angiogenesis and may, in part, mediate the angiogenesis observed in transplants of genetically engineered MSCs in vivo [40]. These studies clearly indicate the important supporting effect that angiogenesis and its mediator, VEGF, have on bone formation.

The use of angiogenic growth factors for bone formation was demonstrated by Peng et al. In this study, the authors implemented a combination of BMP-4 and VEGF genes both infected into muscle-derived stem cells. It was found that VEGF alone expressed in muscle stem cells did not elicit any bone response; however, when expressed together with BMP-4, a synergistic effect of VEGF and BMP-4 was observed. Timing and ratio between VEGF and BMP-4 expression were found to be most crucial in this study [67]. Once again, the importance of angiogenesis in new bone formation was demonstrated when the soluble VEGF antagonist Flt1 was able to inhibit new bone formation elicited by BMP-4. These authors have also shown similar, but not identical, effects on accelerated bone formation when they combined BMP-2 and VEGF-expressing muscle-derived stem cells [67]. These studies represent a new approach, which combines two growth factors that promote key role processes in bone formation.

7.5 Gene Therapy for Systemic Bone Diseases

The most common pathology in bone that has been addressed by gene therapy studies was nonunion bone defects [12–16, 32, 40, 41, 45, 49, 50, 67]. As discussed above, adult stem cell-based gene therapy has successfully addressed this problem in animal models by using MSCs of bone marrow, adipose tissue or muscle origin, genetically engineered to express osteogenic growth factors, primarily members of the BMP family, which were transplanted in the fracture site. Several studies have aimed to develop gene therapy for systemic and metabolic bone diseases. These diseases present more complex pathologies since they require systemic rather than local repair and also the possible involvement of different genes.

Osteoporosis is a disease resulting in bone loss and osteopenia. Though the results of bone loss are the same, two types of osteoporosis are commonly recognized: Type I or postmenopausal osteoporosis and Type II or senile osteoporosis [68, 69]. Type I is related to increased osteoclastogenesis resulting in over resorption of bone due to estrogen depletion, whereas Type II is related to decreased osteogenesis due to bone marrow MSCs senescence, that is reflected by decreased number, proliferation, and osteogenic activity [70–74]. Although osteoclastogenesis is increased in Type I osteoporosis, there is ample evidence of decreased osteogenesis as well [75]. It is therefore a rational approach to attempt to increase bone mass in osteoporosis by increasing osteogenesis. Indeed, Turgeman et al. have shown that systemic administration of BMP-2 protein to osteoporotic mice of both Type I and Type II osteoporosis models has resulted in increased osteogenic potential of bone marrow MSCs leading to restoration of bone mass [70]. Moreover, the same group has shown that

human bone marrow MSCs obtained from osteoporotic patients had increased osteogenic activity and proliferation following infection with adenoviral vector encoding for BMP-2 [41]. The engineered cells were able to form bone in vivo and regenerate nonunion defects in CD nude mice. These studies indicated the potential use of bone marrow MSCs engineered to express osteogenic growth factor as BMP-2 for the treatment of osteoporosis. Since bone marrow MSCs are affected in both Type I and Type II osteoporosis, it is conceivable to target these cells for gene therapy applications.

An opposite approach directed at blocking osteoclastogenesis was suggested by Goater et al. to prevent the loosening of prosthetic implants due to bone resorption. The authors engineered synovial fibroblasts to express osteoprotegrin (OPG), a RANKL receptor antagonist that counteracts the osteoclast differentiation action of RANKL [76]. Engineered fibroblasts were able to inhibit osteoclastogenesis induced by debris in mouse calvaria. This approach can be easily duplicated and applied to the bone marrow in osteoporosis using MSCs as vehicles for OPG expression. However, in this approach MSCs would serve merely as vehicles for OPG delivery and would not have an anabolic influence on bone formation. Another interesting approach directed mainly toward age-related bone loss due to Type I osteoporosis was suggested by Yudoh et al. The authors' approach was directed toward the pathological mechanism of senescence affecting bone marrow osteoblasts, which subsequently leads to low bone mass. In order to overcome this, the authors transduced human osteoblasts and osteoblastic cell lines that display senescence phenotype with the telomerase reverse transcriptase (hTERT) gene [77]. The forced expression of hTERT resulted in increased telomerase activity in these cells, and consequently elevated replication capacity and delayed senescence were observed. It was the authors' suggestion to further use this approach for cell-based gene therapy for osteoporosis. Osteopetrosis is a genetic disease that results in the opposite phenotype of osteoporosis. Excessive bone is formed, eliminating the bone marrow from the bone compartment and eventually resulting in death due to lack of sufficient hematopoiesis [78]. Osteopetrosis is caused by a decrease in osteoclastogenesis due to a genetic mutation of essential growth factors important for osteoclast development such as CSF-1. The op/op mouse carries a genetic defect in CSF-1 and serves as a model for osteopetrosis. Abboud et al. have suggested the over-expression of soluble forms of CSF-1, specifically in osteoblasts, as a potential model of gene therapy for osteopetrosis [79]. To corroborate the notion that expression of CSF-1 by osteoblasts can restore the osteopetrotic phenotype, the authors have created a transgenic op/op mouse that harbors the CSF-1 cDNA under the control of the osteoblastic-specific osteocalcin promoter. The authors report that within 5 weeks postnatal, the osteopetrotic phenotype was completely reversed to the wild-type phenotype. Bone marrow derived could be potentially transduced to express the CSF-1 gene, returned to the bone marrow and promote osteoclastogenesis.

Osteogenesis imperfecta is a genetic disease that affects the quality of the bone formed in the body. Due to a mutation in one of the subtypes of procollagen chain genes, the resulting assembly and production of mature collagen fibers are impaired [78]. In order to overcome this genetic mutation; the delivery of the correct form of

collagen pro-chain gene must be achieved. The *oim* mouse model, which harbors a defect in Proα2(I)-chain gene, presents a phenotype resembling osteogenesis imperfecta. Niyibizi et al. have shown evidence for a potential therapeutic use of stem cell-based gene therapy in osteogenesis imperfecta. They have over-expressed the correct Proα2(I) cDNA in *oim* mice bone marrow-derived MSCs using an adenovirus. The authors reported that the corrected gene was expressed in vitro, and the cells were able to form a stable Type I collagen fiber composed of Proα1(I) and Proα2(I) in the correct ratio of 2:1 [80]. The studies mentioned above demonstrated that MSCs play a crucial role in the pathophysiology of systemic and metabolic bone diseases. However, even in cases where the pathophysiology is not directly connected to MSCs, they can still serve as powerful candidates for cell-mediated gene therapy.

7.6 Genetic Engineering of MSCs

The relative ease of MSC isolation from different skeletal tissues and expansion in vitro has made them readily available for genetic manipulation with various vectors. The most common vectors that have been used were adenoviral vectors [29, 41, 49, 50, 80]. Retroviral vectors have also been used for transducing MSCs and osteoprogenitors but with relatively poor results [15]. Modifications of retroviral infection techniques were suggested to improve the transduction rate of MSCs with these vectors. Kuhlcke et al. showed positive results when tissue culture vessels were preloaded with retroviral vectors by low-speed (1,000 g) centrifugation [81]. Various cell types cultured in these conditions were efficiently transduced into T cells with up to 85% efficiency. Recently, the use of a Lentiviral vector encoding for the BMP-2 gene has been shown to be effective in expressing the transgene in rat MSCs leading to osteogenesis in vitro and in vivo [82, 83]. MSCs were also effectively transduced with other vectors such as vesicular stomatitis virus (VSV) [84–86]. Human bone marrow-derived MSCs were found to be highly susceptible to VSV infection achieving high rates of transduction with more than 81% efficiency [84]. A safety aspect was also encountered as transduction with VSV did not alter the proliferation and differentiation potential of bone marrow-derived MSCs.

Achieving high transgene expression is a desired goal in some cases in gene therapy. An interesting and novel approach was undertaken by Peng et al. for enhancing the secretion of BMP-4 transgene from transduced bone marrow MSCs, ex vivo [87]. The authors used an MFG-based retroviral vector pseudotyped with a VSV-G envelope. In order to increase BMP-4 secretion, the authors created hybrid constructs encoding for BMP-4 peptide linked to a BMP-2 propeptide sequence. Replacement of the BMP-4 propeptide region with that of BMP-2 has resulted in increased secretion of BMP-4 from MSCs engineered to express this hybrid.

Achieving high expression of a transgene is not always the ultimate goal of stem cell-based gene therapy, especially in orthopedics. Limiting the expression in terms of intensity and duration is necessary in certain cases. One way to ensure

limited expression of the transgene is the use of tissue-specific promoters. Bone- and osteoblast-specific promoters will ensure expression in the bone marrow zone where active synthesis of bone matrix occurs. Stover et al. used the collagen1A1 promoter sequence in order to achieve osteoblastic-specific expression [88]. Expression of a marker gene regulated by the tissue specific promoter was limited to osteoblasts both in vitro in MSC cultures and in vivo in chimeric embryos. A similar approach using the osteocalcin promoter was undertaken by Abboud et al. where osteoblasts were engineered to express CSF-1 for the treatment of osteopetrosis. Maintaining expression limited for osteoblasts is important to prevent the expression in other cell types and is more required in genetic skeletal diseases or systemic metabolic diseases such as osteopetrosis and osteoporosis. For local skeletal defects, this approach is less relevant, since the required transgene expression is limited to a short period of time, and the exact type of cells that expresses it is not critical for the repair. Fine-tuning of transgene expression and temporal control on the duration of expression may be critical in the future development of gene therapy applications for orthopedic medicine. The use of tetracycline-regulated promoters to manage transgene expression was suggested [17, 40]. Using a Tet-off promoter system in the MSC line, C3H10T1/2, where tetracycline presence inhibited BMP-2 transgene expression, it was shown that engineered MSCs expressed and secreted BMP-2 only in the absence of tetracycline. The presence of tetracycline in vitro and the addition of tetracycline to the drinking water of mice transplanted with these genetically engineered MSCs completely inhibited BMP-2 expression. In vitro, BMP-2 regulation by tetracycline has resulted in the control of the engineered MSCs osteogenic differentiation. In conditions promoting BMP-2 expression, osteogenic differentiation of engineered MSCs was induced. In contrast, this was not evident when the transgene was suppressed, i.e., in the presence of tetracycline. In in vivo experiments, where engineered MSCs were transplanted both ectopically in muscle tissue and in radial nonunion bone defect, it was found that bone formation and nonunion defect regeneration were both dependent on tetracycline control. Tetracycline administrated to mice transplanted with the engineered cells completely inhibited bone formation and defect regeneration otherwise observed when tetracycline was not administered. This study demonstrated the potential of exogenously regulated promoters. Such promoters have the potential to allow the control over the duration and intensity of transgene expression and therefore to modulate in real time its biological effect. Such constructs can be used in both chronic systemic and metabolic diseases, such as osteoporosis, that may need long-term gene expression and regulation, and for local injury and temporal disease. In the latter case, regulated constructs that will not integrate permanently to the cells are preferable.

7.7 Future Prospects

As is evident from the above-reviewed studies, MSCs present a great advantage for stem cell-mediated gene therapy directed for orthopedic medicine. MSCs can be isolated from various tissues, the most common of which are bone marrow, adipose,

and muscle tissues. Although many studies on the immunoisolation based on surface markers of MSCs have been performed, the various selected MSC subtypes are indistinguishable [89]. Some evidence indicates that MSCs may even be retrieved from peripheral blood [89]. Together with molecular studies directed at finding distinct molecular markers of MSCs, future development may provide us with a reliable technique for purifying and expanding in vitro blood circulating MSCs, which are more easily retrieved.

Since most of the studies performed on nonunion defect models were highly successful, it is expected that large animal studies are to follow, possibly leading toward first clinical trials. It is expected that additional cell-meditated gene therapy studies involving other genes with osteogenic potential will be undertaken [90, 91]. As MSCs present a multi-lineage differentiation potential, further studies are expected to examine MSC advantages and relying on their ability to differentiate to various lineages for the regeneration of skeletal tissues. Investigations may evolve following other studies elucidating the signal transduction pathways of mesenchymal tissue differentiation and identifying novel genes that can trigger lineage-specific differentiation of MSCs.

The increasing understanding and recognition of the complexity of skeletal tissue formation has led to the discovery of mechanisms that support skeletal tissue development and/or regeneration. This has been exemplified by the discovery of the important role of angiogenesis in bone development and regeneration as discussed above. Moreover, this has addressed the complexity of bone regeneration and its mechanisms by expressing both BMP-4 and VEGF, and therefore achieving a synergistic effect between the two mechanisms of osteogenic differentiation and angiogenesis. Expressing several genes that are applied for different specific mechanisms in order to promote skeletal tissue development and regeneration should be in the scope of future gene therapy strategies applied for skeletal regeneration. Such complex approaches should also pave the way for the development of MSC-based therapeutic applications for systemic and metabolic bone diseases, like osteoporosis, which have several mechanisms involved in their pathophysiology.

A major hurdle to overcome in tissue engineering is an insufficient supply of oxygen to newly forming tissue. Insufficient oxygen results in cell death [92, 93] and loss or delay of cell differentiation [94], especially with regard to osteogenesis, which is dependent on vascularization and oxygen supply [95]. One option in overcoming hypoxic conditions within a tissue-engineering scaffold is to increase the level of oxygen within the scaffold by using perfluorocarbons (PFCs) such as PFTBA. PFCs have a linear relationship between oxygen partial pressure and oxygen concentration [96]. While oxygen solubility in water is only 2.2 mM, oxygen solubility in PFTBA is 35 mM, a 15- to 20-fold increase over solubility in water alone [97]. In a recent study, in which BMP2 expressing MSCs were implanted in ectopic and orthotropic sites in PFTBA supplemented fibrin gel, the positive effect of this material on bone formation was demonstrated. In the ectopic site, there were significant increases in bone formation (2.5-fold increase), cell survival, and *osteocalcin* activity in the PFTBA-supplemented groups. PFTBA supplementation significantly increased structural parameters of bone in radial bone defects and triggered a

significant 1.4-fold increase in bone volume in the spinal fusion model. Synthetic oxygen carrier supplementation of tissue-engineered implants enhanced ectopic bone formation and yielded better bone quality and volume in bone repair and spinal fusion models, probably due to increased cell survival [98].

Finally, it is expected that engineered MSCs combined with specially designed polymeric scaffolds will soon be used for skeletal tissue engineering both in vivo and ex vivo. Combining MSCs with a particular growth factor gene that directs their differentiation and that triggers the process of tissue formation is a good approach to engineering tissues. Here as well, the properties of MSCs that enable them to differentiate and express growth factors can be exploited for the purposes of tissue engineering.

To conclude, MSCs as reviewed here can have a wide range of applications for orthopedic medicine. Their differentiation ability, easy manipulation in vitro, and relatively easy accessibility from various tissues enable them to become major building blocks for the design and development of therapeutic applications to all skeletal tissues concerned in orthopedics. It is expected that the use of MSCs will expand to other tissues and will acquire an important place in regenerative medicine.

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Chapter 8
Scaffolds in Skeletal Repair

Erella Livne and Samer Srouji

The need for tissue repair is one of the major concerns of reconstructive surgery and in aging and disease. Fracture healing is regulated by osteogenic cells and growth factors. The ability to enhance healing of bone defects and fractures can contribute to prevent the complications of long-term immobilization that are especially fatal in old age. Three-dimensional scaffold provides the necessary support for cells to proliferate and maintain their differentiated function and its architecture defines the shape of the newly formed bone. At the same time the scaffold is biodegraded providing space for the newly formed tissue. Skeletal tissue such as bone is organized into three-dimensional structure (3D) in the body. The 3D scaffold can be used as a temporary device containing the osteogenic cells. This could provide the initial conditions for bone repair. Biodegradable scaffold contains committed osteogenic stem cells and growth factors which serve as a graft substitute for bone and cartilage repair. Bone marrow stem cells are selected as the osteogenic subpopulations cultured in medium supplemented with osteogenic supplements. The selected osteogenic subpopulation is identified using osteogenic markers (Alizarin red, von Kossa staining, osteocalcin, osteonectin, osteopontin immunolocalization, and mineral-hydroxyapatite (HA) deposition). Committed osteoprogenitor cells are cultured on scaffold and transplanted with growth factors in tibia segmental bone defect. The healing of the defect is examined by morphology, radiology, 3D CT, and EDS for mineral deposition. Results indicated that our 3D hydrogel scaffold supports proliferation and differentiation of bone marrow stem cells. This provides an approach for the use of bone marrow stem cells-based transplants of bone cells that will enhance bone repair, eliminate the need for additional surgical procedure, reduce unnecessary pain and complications to the patient, and shorten the hospitalization time. The biodegradable scaffold can serve as biocompatible matrix for bone marrow stem

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cells-derived osteoprogenitor cells and growth factors, and that it also provides space for bone regeneration. The biodegradable scaffold containing committed stem cells and growth factors is thus a promising surgical tool for enhancement of bone and cartilage defect reconstruction for tissue engineering in aging and disease. Fracture healing is regulated by osteogenic cells and growth factors. The ability to enhance healing of bone defects and fractures can contribute to prevent the complications of long-term immobilization that are especially fatal in old age [1]. Skeletal tissue such as bone is organized into three-dimensional (3D) structure in the body. The search for artificial bone graft can contribute to the enhancement of bone repair. Scaffolds are 3D structures used as bone graft substitute for bone repair. The (3D) structure scaffold provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the shape of the newly formed bone. At the same time the scaffold is biodegraded providing space for the newly formed tissue [2–4]. Bone is the second most frequently transplanted tissue in humans. Surgical uses of bone grafting materials include surgical intervention of osseous nonunion, restoration of the structural integrity of bone after trauma, and filling defects following bone tumor removal. Autogenous cancellous bone is considered the ideal graft material for several reasons: it is biocompatible and nonimmunogenic, it will not transmit a disease to a recipient, and it has osteogenic potential due to the presence of viable osteoprogenitor cells [5, 6]. Since cancellous bone is taken from the iliac crest, rib, fibula, or tibia, it is sometimes not available in a sufficient amount. In addition, harvesting the bone imposes potential complications of pain, blood loss, infection, and donor site instability. Another frequently used bone graft material is allogenous bone. Allograft bone is available in an unlimited supply but, unfortunately, does not have the osteogenic potential of the autogenous bone and often elicits immunological response when implanted. This leads to continued search for effective artificial substitutes for bone grafts [7, 8]. The stromal compartment bone is composed of a network of interconnected stromal cells that include mesenchymal stem cells (MSCs), distinct from the hematopoietic stem cells (HSCs). The MSCs are capable of differentiating along the osteogenic, chondrogenic, fibroblastic, and adipogenic lineages. MSCs-derived osteoprogenitor cells can be used in modern technology for tissue engineering and cell therapy. They can be used in metabolic bone diseases and orthopedic approaches when bone repair is needed. By accomplishing the task of identifying a specific osteoprogenitors, the selection of osteoprogenitor cells from the bone marrow will be available [9, 10]. The development of an osteoinductive slow-release devices containing committed bone forming cells and growth factors will minimize the need for autologous bone grafts used today for filling bone voids or gaps, and will thereby reduce the inherent risks and complications associated with the additional surgery of bone harvesting [11, 12]. Reinforcing the developed osteogenic bone graft substitute will improve the mechanical properties of the device thereby extending its potential applications to highly loaded locations in the human body. Introduction of such bone substitute into clinical practice will restore the mobility and improve the quality of life of both young and aging patients who lost bone during trauma or surgical resection [13]. Biodegradable scaffold can serve as a biocompatible matrix for bone marrow stem

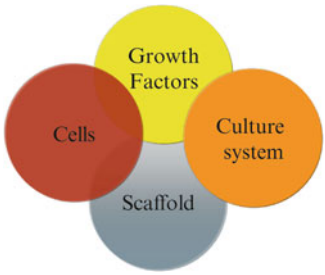

Fig. 8.1 Strategy for bone repair using cell-scaffold constructs

cells-derived osteoprogenitor cells and growth factors, and that it also provides space for bone regeneration (Fig. 8.1). The biodegradable scaffold containing committed stem cells and growth factors is thus a promising surgical tool for enhancement of bone and cartilage defect reconstruction for tissue engineering in aging and disease [14].

8.1 Bone Defect Repair and Growth Factors

The need for bone repair is one of the major concerns in bone defects, fracture healing, and reconstructive surgery [15, 16]. Bone consists of cells and extracellular matrix; the latter is comprised of 35% organic and 65% inorganic components [17]. The inorganic components are mainly calcium and phosphate as HA. The organic components of bone matrix are traditionally divided into collagen and non-collagenous proteins. Type I collagen constitutes more than 90% of the organic material in bone matrix and is the major structural protein of bone. The remaining 10%, the non-collagenous proteins, have different regulatory functions for mineralization, mediation of cell matrix-to-matrix binding, and various interactions with structural proteins such as collagen. Bone silaoprotein (BSP) is involved in calcium binding [18]. Bone growth factors consist of less than 1% of the non-collagenous proteins. The main cell types in bone include the osteoblast, osteocyte, and osteoclast. The process of bone remodeling is regulated by osteoblast–osteoclast direct signaling [19–21]. The bone growth factors exhibit their effect in local cellular environments, thereby stimulating neighboring bone cells to proliferate and increase matrix protein synthesis (paracrine effect). Likewise, the osteoblast which produces the growth factors can stimulate themselves to additional metabolic activity (autocrine effect). The total number of growth factors which are able to affect proliferation, differentiation, and secretive functions of bone-related cells is unknown [17] (Fig. 8.1). In 1965, in an experiment to recalcify cortical bone, Marshall Urist [22] made the key discovery that led to the hunt for factors responsible for bone formation. Urist's discovery led to a series of investigation to determine the putative agents in demineralized bone (DMB) that provoke autoinduction. The osteoinduction activity in bone matrix was found to be the result of non-collagenous and water

soluble substance coined bone morphogenetic proteins (BMPs) [23]. BMPs are members of the transforming growth factor-β (TGF-β) superfamily and regulate differentiation of various cells implicated in cartilage and bone formation during skeletal development and fracture repair [24, 25]. The role of BMPs in induction of osteoblast differentiation has been established using various preosteoblastic cell lines, such as primary cultures of calvarial osteoblasts and human and mouse bone marrow cultures. Using these models, induction of osteoblast differentiation has been described for BMP-2, -4, -5, -6, and -7 [26–29]. BMPs exert their effect by binding to a heterodimeric complex, consisting of two BMP type I receptors and two BMP type II receptors, which possesses serine/threonine kinase activity.. To date, however, it is still not clear how BMPs being potentially a big promise, failed to play a larger role in the clinical arena. Other local growth factors have proven to be important by affecting the type and the rate of fracture repair. An age-related diminished capacity of fracture repair process has been observed with the advancement of aging and an overall decrease in osteoblast function was observed with aging [30]. Nielsen et al. [31] reported on increase in bone strength of tibial fracture following local injection of TGF-β (40 ng, every second day for up to 40 days) and an ultimate load dose-dependent increase in cross-sectional area of callus and bone at the fracture line. However, in a study on distraction TGF-β had no detectable effect on bone mineral density or bone volume in the distraction gap, but increased fibrous tissue in the callus region. Another study on mid-tibial osteotomy in rabbits treated with TGF-β1 (10 ng/day for 6 weeks) resulted in increased maximal bending strength and callus formation [32]. IGF-1 is known to play a role in fracture healing; it promotes cell proliferation and matrix synthesis by chondrocytes and osteoblasts. The levels of circulating IGF-1 and bone mineral density decrease with the increase in age. Also the secretion of GH decreases with aging [33]. It has been shown that administration of IGF-1 increased bone turnover in patients with low bone mineral density. FGF-2 was also shown to stimulate callus formation which provided mechanical stability to the fracture, accelerated healing, and restored competence [34, 35]. Also increased bone mineral content and osteoblast number were observed in fracture healing in dogs treated with FGF-2 [36] and in rabbit skull defect [37] (Fig. 8.2).

Fracture healing is a process of reconstruction of the tissue. The matrix in the fracture and in the defect sites plays an effective role in the earlier restoration of the mechanical strength. A process of remodeling occurs and the molecular mediators released by the aggregating platelets and other thrombotic factors, as well as active mediators, are released by the tissue breakdown. All these structures produce many factors such as chemo-attractants, angiogenic and growth factors. The monocytes and macrophages that exist in the hematoma, or infiltrate to the infected site also produce growth factors involved in bone repair. The platelets contain several growth factors such as platelets-derived growth factor (PDGF), epidermal growth factor (EGF), TGF-β, and many others. Different blood cells (granulocytes, macrophages, and erythrocytes) migrate in waves to the fracture site [38, 39] and to supply the various factors that are in involved in bone repair process. No knowledge of the constituents of the fracture exudates is available, but it is known that the fracture

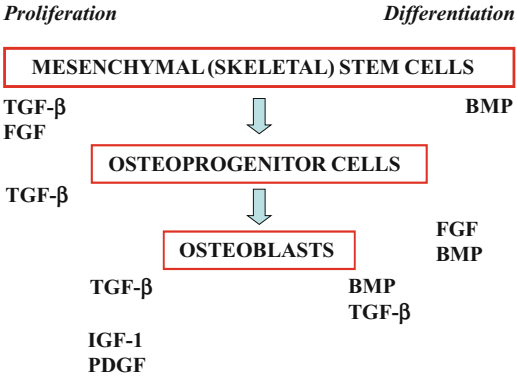

Fig. 8.2 Involvement of various growth factors in proliferation and differentiation of skeletal progenitor cells along the osteoblastic lineage

callus contains a high amount of hyaluronate in the beginning which decreases after 7–8 days. Hyaluronate promotes migration and proliferation of mesenchymal cells. It is likely that growth factors regulate both resorption and formation of bone in remodeling process. TGF-β seems to be of special importance in fracture healing and induces the typical granulation tissue [40]. In the present study, TGF-β and the combination of TGF-β + IGF-1 were shown to induce bone defect healing. It is hypothesized that growth factors and selected bone forming cells that will be incorporated in osteoinductive osteoconductive scaffold will enhance bone formation. The scaffold with appropriate biodegradability will function not only as a release matrix for the growth factors and cells, but also as a space provided for bone osseointegration affecting the firmness of the external fixation implants. The hydrogel containing growth factors and bone forming cells is thus a promising surgical tool for bone defects and for orthopedic implants osseointegration. TGF-β may stimulate bone repair by causing proliferation of osteoblasts or by stimulating mineralization as represented by expression of alkaline phosphatase [41, 42]. TGF-β influences osteoblast production of several bone proteins like osteonectin, a bone-specific 32 K protein linking mineral to collagen fibers, osteopontin, a matrix protein enhancing cell attachment, fibronectin, collagens, and proteoglycans. Two other ways in which TGF-β may enhance the formation of ECM are by stimulating the production of protease inhibitors such as plasminogen activator inhibitor (PAI), and tissue inhibitor of metalloproteases (TIMP), or by inhibiting the production of proteases such as plasminogen activator and metalloproteases. On the other hand, MMPs are needed for resorption of the initial callus and for remodeling of cancellous bone to compact bone. The process of bone resorption could serve also for the release of matrix-stored growth factors by bone resorption. Thus, osteoblasts deposit growth factors in bone and later when these growth factors are released from bone via bone resorption, the growth factors stimulate osteoblast precursors to proliferate [43, 44]. TGF-β plays also a role in the formation of new bone and bone repair by stimulation of collagen and matrix protein synthesis by bone cells and chondrocytes. It is concluded that TGF-β and TGF-β + IGF-1 were shown to induce an increase in the rate of bone defect repair process and restore the biomechanical quality of the

newly formed tissue. Finding a treatment that can induce an increase in bone mass is important to enhance osteoinduction in bone defects loss and reconstructive surgery and aging [45, 46]. Bone defect healing depends on the mechanical stability and on the actual size of an osteotomy or bone defect [47]. The relationship between biomechanical properties and bone formation during the healing of the defect revealed that after 2 weeks the biomechanical tests did not reveal yet any significant changes between the groups and the control. At the same time, the morphology did not reveal new bone formation. Moreover, the biomechanical changes of the various treatments and of the control were significantly different from the intact femur. After 4 weeks, the biomechanical properties of the bones treated with TGF-β + IGF-1 were different from the other treatments and were closer to the value of the intact femur, and at the same time morphology revealed that cancellous bone was present in the defect site [45, 48, 49]. Radiology revealed that after 2 weeks some opacity was seen in the TGF-β + IGF-1 group. It represents the very beginning of response to the growth factor, but not enough to be seen in the mechanical tests.

8.2 Bone Marrow Stem Cells

The human skeleton accumulates bone up to approximately age 30, after which bone is gradually lost. Bone remodeling and bone loss as a function of age are under the influence of both endogenous hormonal changes and external mechanical loads resulting from physical activity. These impart their effects through regulation of the relative activities of bone cells in particular osteoblasts and osteoclasts, which control bone deposition and resorption, respectively [50, 51]. The need for bone repair is one of the major concerns of reconstructive surgery and fractures [52, 53]. Fracture healing is regulated by osteogenic cells and systemic growth factors. To aid the healing process, it is often necessary to introduce the selective subpopulation of bone forming osteoprogenitor cells in the healing bone tissue. Bone grafts are currently being used for repair of large defects and fractures, and bone grafting should improve recovery, shorten hospitalization time, and decrease complication frequency [54, 55]. Bone marrow MSCs are multipotent cells capable of forming bone, cartilage, and other connective tissues. These cells may also provide a potential therapy for bone repair [56, 57]. It has been well documented that MSCs include actively proliferating osteoprogenitor cells [58–61]. In vitro, these cells express the osteogenic phenotype only when treated with differentiation factors such as glucocorticoids [62–64]. Maniatopoulos et al. described a culture system in which MSCs obtained from bone marrow of fetal or neonatal skeleton have the capacity to produce mineralized-like nodules in vitro when the culture medium is supplemented with dexamethasone, ascorbic acid, and β-glycerophosphate [63]. The ECM secreted by MSC in this culture system was shown to consist predominantly of type I collagen, to include osteonectin and osteocalcin, to contain bone hydroxyapatite as its mineral phase, and to express BMPs [63]. These properties are characteristic

of bone ECM produced in vivo. The differentiation of osteoblast, to bone-like tissue, has been muddled as three-step process, consisting of proliferation phase, a matrix maturation phase, and mineralization phase [65]. Adult stem cells, like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway, although this characteristic may not be as definitive as once though [14]. Adult stem cells are rare. Their primary functions are to maintain the steady-state functioning of a cell and, with limitations, to replace cells that die because of injury or disease [66]. For example, only an estimated 1 in 10,000 to 15,000 cells in the bone marrow is a HSC (blood forming) [67]. And 1 in 1:100,000 is estimated as osteoprogenitor stem cell. Furthermore, adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment [68]. Bone marrow stromal cells represent a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation [2, 69, 70]. The bone marrow appears to contain three stem cell populations—HSCs, stromal cells, and (possibly) endothelial progenitor cells. To date, it has not been possible to isolate a population of pure stromal cells from bone marrow. Panels of markers used to identify the cells include receptors for certain cytokines (interleukin-1, 3, 4, 6, and 7), receptors for proteins in the extracellular matrix (ICAM-1 and 2, VCAM-1, the α-1, 2, and 3 integrins, and the β-1, 2, 3, and 4 integrins), etc. Despite the use of these markers and another stromal cell marker called Stro-1, the origin and specific identity of stromal cells have remained elusive. Like HSCs, stromal cells arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated and identified [71].

8.3 Scaffolds and Biomaterials

There are many approaches to bone tissue engineering, but all involve one or more of the following key components: cultured stem cell, growth factors, and three-dimensional (3D) matrices. One approach involves seeding highly porous biodegradable matrices (or scaffolds), with cells and signaling molecules (e.g., protein growth factors), then culturing and implanting the scaffolds into the defect to induce and direct the growth of new bone [72, 73]. The goal for the cells is to attach to the scaffold, multiply, differentiate (i.e., transform from a nonspecific or primitive state

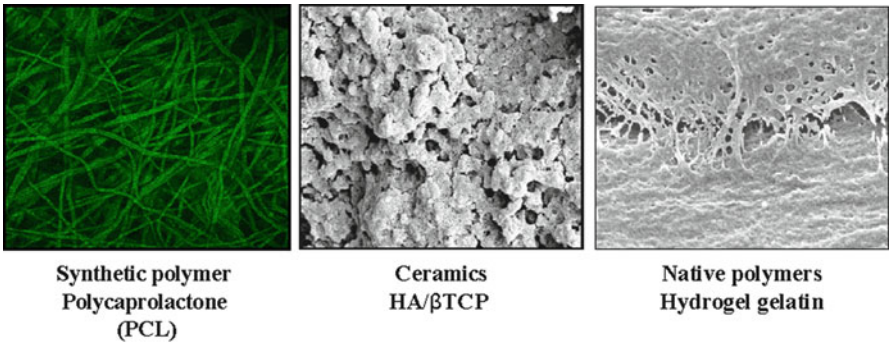

Fig. 8.3 Types of scaffolds for bone

into cells exhibiting the bone-specific functions), and organize into normal, healthy bone as the scaffold degrades. Scaffold materials for making matrices for bone tissue engineering include several classes of biomaterials: synthetic polymers, ceramics, native polymers, and composites (Fig. 8.3).

8.3.1 Synthetic Polymers

Both organic and inorganic are used in a wide variety of biomedical applications. The polymers can be biodegradable or nondegradable. Examples of biodegradable polymers include polylactic acid (PLA), polyglycolic acid (PLGA), and copolymers thereof. These polymers are broken down in the body hydrolytically to produce lactic acid and glycolic acid, respectively. Other biodegradable polymers currently being studied for tissue engineering applications include polycaprolactone (PCL), polyanhydrides, and polyphosphazenes [74–78].

8.3.2 Ceramics

These are widely used in dental applications and are being examined for bone tissue engineering applications. Two common ceramics used in dentistry and hip prostheses are alumina and HA. Alumina (Al_2O_3) has excellent corrosion resistance, good biocompatibility, high strength, and high wear resistance, and has been used for over 20 years in orthopedic surgery [79]. HA is a calcium phosphate-based ceramic and has also been used for over 20 years in medicine and dentistry [80]. HA is a major component of the inorganic compartment of bone. HA prepared commercially is biocompatible with biodegradability either absent or protracted [81, 82]. The degradation of HA can be controlled by varying the chemical structure. Tricalcium phosphate degrades much more quickly than HA [13] and has been used

for long bone defects repair in rabbits [83, 84]. Bioactive glasses have been shown to bind to soft tissue and bone. These bioactive glasses contain different ratios of Na_2O–CaO–P_2O_5–SiO_2 [85]. There are currently two commercially available glasses advertised for applications in bone sites.

8.3.3 Native Polymers

These are extracellular matrix proteins commonly exploited as bone graft materials. Collagens, which comprise a majority of proteins in connective tissue such as skin, bone, cartilage, and tendons, are popular candidates for such circumstances, and various collagen-based products are currently under development [86–88]. The organic phase of bone is principally type I collagen. When bone is demineralized with hydrochloric acid, the method used by most commercial venders, the bone derivative is largely type I collagen and a minimal percent mixture of ECM components, cell debris, and soluble signaling molecules that are resistant to acidic demineralization [89–91]. The format for the DBM can be either a range of particulate matter, blocks, or strips.

8.3.4 Composites

The composites of ceramics and polymers are also widely studied. Composites can result in substitutes with properties between each of the respective materials [92]. For example, bovine collagen has been manufactured with HA. Collagraft is HA and bovine type I dermal collagen (95%) and type III collagen (5%). Collagraft is used for orthopedic, non-load bearing sites [93]. Bone repair is thought to be one of the first major applications of tissue engineering. At present, efforts are being made to encourage the growth of new bone, using novel matrices, growth factors, gene therapy, and stem cells [8]. Today bone grafts from elsewhere in the body to repair major damage from accidents or disease are being used, but the quality and quantity of bone is not sufficient and repair is not always achieved. Molecular scaffolds made of collagen and HA are used for small divots but are not useful for larger defects [94]. Biocompatible polymers containing growth factors were also studied, gene-cell therapy are being tested; however, cells carrying therapeutic genes are short lived [24, 94].

8.4 Bioreactors

Static cultures do not mimic the dynamics of the in vivo environment found in bone, namely the mechanical stimulation caused by hydrostatic pressure and shear stress. These factors do not affect the behavior of osteocyte at several levels [95].

Furthermore, it has been also demonstrated that mechanical stress could also upregulate Cbaf-1/runx2 expression [96].

The cultivation of cell monolayer in culture dishes to multiply the initial cell number has various disadvantages. The supply of oxygen becomes critical when the diffusion distance comes wider than 100–200 μm, and the diffusion can be improved by stirring the culture medium. The design and development of bioreactors are for sure solutions to overcome the above-referred problems [97]. Various types of bioreactors have been tested for their utility in bone tissue engineering. Two systems have been preferentially used, spinner flasks and rotating wall vessel reactor. The spinner flasks provide better migration of cells and supply of nutrients [5]. The approach of cell cultures scaffold in bioreactor will provide the optimal conditions for 3D structure scaffold/cell as bone.

8.5 Scaffold and Growth Factors for Segmental Bone Repair

Bone regeneration induced by TGF-β and IGF-1-containing hydrogel scaffold was investigated using a rat tibia defect model. An external fixation device was used before induction of the bone defect, thus enabling a controlled segmental bone defect to be created in the already fixed tibia. Soft X-rays of the defects in TGF-β-treated animals, taken after 2 weeks from start of treatment, revealed some radiopacity, indicative of newly formed mineralized bone. It has been demonstrated previously that TGF-β is ionically complexed with the hydrogel scaffold and was released from it [11, 87]. A similar effect was reported for bFGF released from hydrogel [98]. Other studies have shown that growth factors released from scaffolds induced similar responses, but only after 8 weeks [76]. Enhanced healing of bone defects is a challenge to surgery and requires a combination of the osteoinductive effect of growth factors and the conductivity of scaffolds [99]. Enhanced bone formation and bone healing could lead to improved results in surgical procedures [100, 101]. In our hydrogel system, TGF-β and IGF-1 were released from hydrogel as a result of hydrogel biodegradation. When hydrogel degrades too quickly, it neither retains its growth factors allowing in growth of soft tissue in the defect, nor does it induces bone regeneration, while hydrogel that degrades too slowly could impede the formation of new bone [88].

8.6 Scaffold Biodegradation

It has been reported that metalloproteinases (MMPs) are present in bone tissue [102]. It is possible that proteases such as MMPs capable of degrading the hydrogel scaffold are involved in biodegradation of the hydrogel and bone remodeling [103]. TGF-β has been reported to enhance fixation and ingrowth of ceramic [20] and HA [21] coated implants. However, further research is needed to establish the optimal conditions for bone defect healing in long bones.

It is concluded that scaffold containing growth factors with appropriate biodegradability could function not only as a release matrix for growth factors but also as a site for bone osseointegration that affects the firmness of the external fixation implants. Therefore, scaffold containing growth factors appears to be a promising surgical tool for the treatment for orthopedic bone repair.

8.7 Bone Tissue Engineering

Bone repair is a process of reconstruction of the bone tissue in the area of injury. This process is mediated by bone forming osteoprogenitor cells, growth factors, and three-dimensional cell matrixes at the site of injury [104, 105]. The decrease in skeletal bone formation and rate of fracture repair observed with aging in bone defects and in osteoporosis has been suggested to be due to a decrease in the growth factors and reduced numbers of the osteogenic progenitors [50]. The need for bone repair is one of the major concerns in bone defects, fracture healing, and reconstructive surgery. The ability of selected bone forming cells or TGF-β1 and IGF-1 incorporated into gelatin hydrogel to induce bone regeneration were evaluated in a previous study [46]. The use of PLA–PLGA copolymer-gelatin sponge containing rhBMP-2 induced effective bone regeneration in a rat mandible defect model [78]. TGF-β and IGF-1 incorporated into hydrogel scaffold were released from the scaffold as a result of biodegradation. When the scaffold degrades too quickly, it does not retain its growth factors, thus allowing ingrowths of soft tissue at the defect site, and does not induce bone regeneration. Scaffold that degrades too slowly could impede the formation of new bone [98]. The scaffold has to be degraded in vivo allowing the slow release of its incorporated growth factors. It could thus serve as a slow-release device. At present, efforts are being made to encourage the growth of new bone using novel matrices, growth factors, and stem cells [8]. Growth factors are important mediators of bone regeneration, but in vivo growth factors are short lived. In order to increase the availability of growth factors at the site of bone healing, the use of growth factors together with scaffolds has been introduced. Various carriers such as guanidine-extracted DBM matrix, polymeric or ceramic implants, bone grafts, or human recombinant osteogenic protein-1 containing growth factors were tested and shown to result in induced bone repair in various systems [7, 106–108]. IGF-1 incorporated into type I collagen gel enhanced nasal defects healing, and TGF-β incorporated into acid gelatin hydrogel enhanced healing of rabbit skull defects [109–111] as well as in others [112–114]. In order to further increase the osteogenic potential of scaffold-based implants, a cell therapy approach is used to incorporate osteoprogenitor cell derived from bone marrow stem cells (MSCs) in the scaffold to enhance bone repair. Cell-scaffold constructs are used for testing the functionality of vivo bone repair by selected osteogenic subpopulation of bone marrow stem cells (MSCs). The results are validated using specific osteogenic markers [115, 116]. Culture of sufficient numbers of such osteogenic cells and growth factors could conceivably be used with scaffold for bone

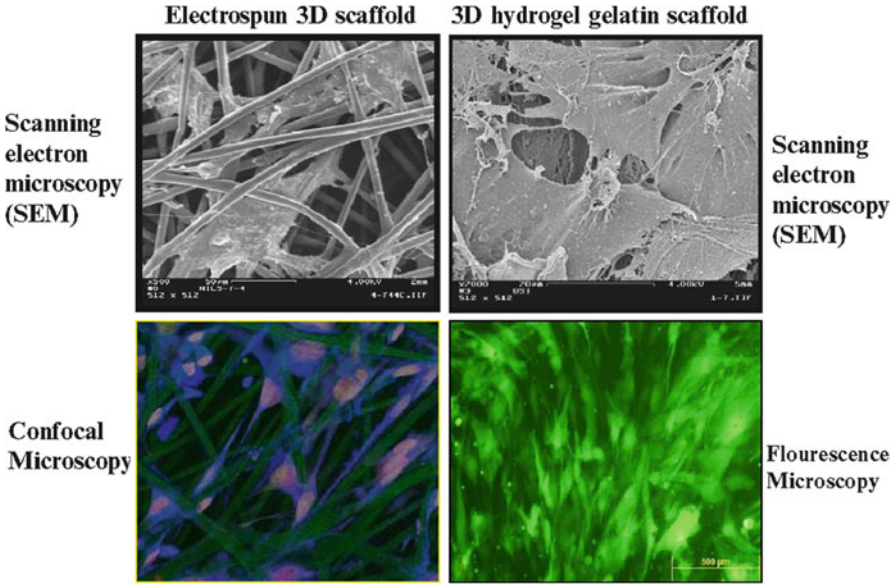

Fig. 8.4 Cell-scaffold constructs for bone repair

tissue engineering to repair bone in aging and in bone transplantation (Fig. 8.4). The methods used for in vitro selection of the osteogenic subpopulation from MSC cultures and the methods used to incorporate them in scaffold are crucial for the successful transplants for the future use in tissue engineering bone repair [5, 57, 90, 117–119]. The scaffold should be biocompatible for selected osteogenic cells and to provide support for proliferation and differentiation demonstrated by osteogenic markers. This approach can contribute to future development of an in vitro designed implant for in vivo bone repair. Scaffold should be biocompatible, osteoconductive, biodegradable, and osteinductive but not immunoreactive. Three-dimensional scaffolds should provide the necessary support for cells to proliferate and maintain their capacity to differentiate. The transition from the 2D culture system to the 3D scaffold provides a system that imitates the natural 3D structure of the body tissues and specifically the structure of bone. Three-dimensional scaffolds containing bone marrow-derived osteoprogenitors can be used within transplants in order to enhance bone repair. The complex construct is intended to mimic the native in vivo microenvironment, and this necessitates construction of bioactive scaffolds which are also capable of supporting vascularization as well as cell proliferation and osteogenic differentiation. Preclinical animal tests are a crucial step before conduction of the actual clinical trials. The preclinical tests are aimed for validation of the functionality of the transplanted cells, for safety tests, and for assessment of nonimmune reactivity of either the cells or the scaffold in the designed transplanted cell-scaffold constructs.

8.8 In Vivo Preclinical Tests

Animal testing constitutes a step midway between in vitro studies and human clinical applications. In vivo preclinical testing is crucial for the proof-of-functionality of the designed cell-scaffold constructs. Small animals used in preclinical studies are mice, rats, and rabbits. Mice are the most common animal models for molecular testing because their genome has been completely sequenced, and moreover immune-deficient mice strains (Nude, SCID beige) are used for testing human cells transplants in preclinical testing [4]. There are a number of different types of bone diseases and injuries. Known bone conditions include bone defects either cranial defect or long bone segmental defect as well as osteopenia (osteoporosis), osteonecrosis, bone fractures, and osteotomies (Fig. 8.5).

The most common tests are aimed for the repair of large voids, osteotomies, and critical size defects (CSDs) of bone. CSD was first described by Schmitz and Hollinger [52] as: "the smallest intraosseous wound that does not heal by bone formation during the lifetime of the animal," and later as: "defect which has less than 10% of bony regeneration occurring within the lifetime of the animal" [120]. The biofunctionality tests do not need to be evaluated in a complex biological and biomechanical environment reproducing clinical-like situations. Simple tests such as animal implantation in ectopic/heterotopic (subcutaneous, intramuscular), orthotopic (calvaria) sites allow in vivo evaluation of biocompatibility, osseointegration, osteoconductive, and osteogenic potential. On the contrary, preclinical evaluations rely on animal models simulating the clinical situation in which the bone replacement material will be used. The available models include long bone defect models, the radial, ulnar, femoral, and tibial bone defects, and post-transplants evaluations radiology, histology, μCT, and imunohistochemistry are performed for assessment of the results (Fig. 8.6)

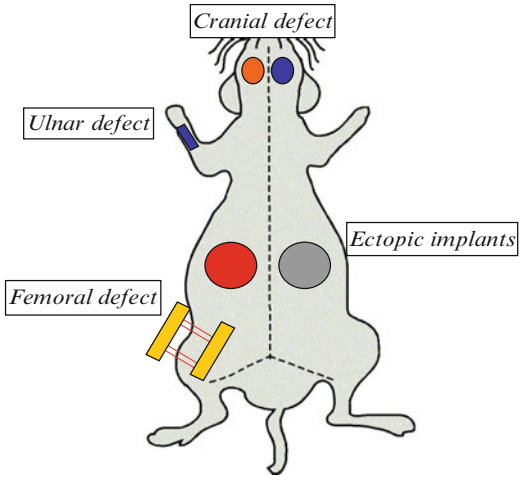

Fig. 8.5 Animal models for bone repair

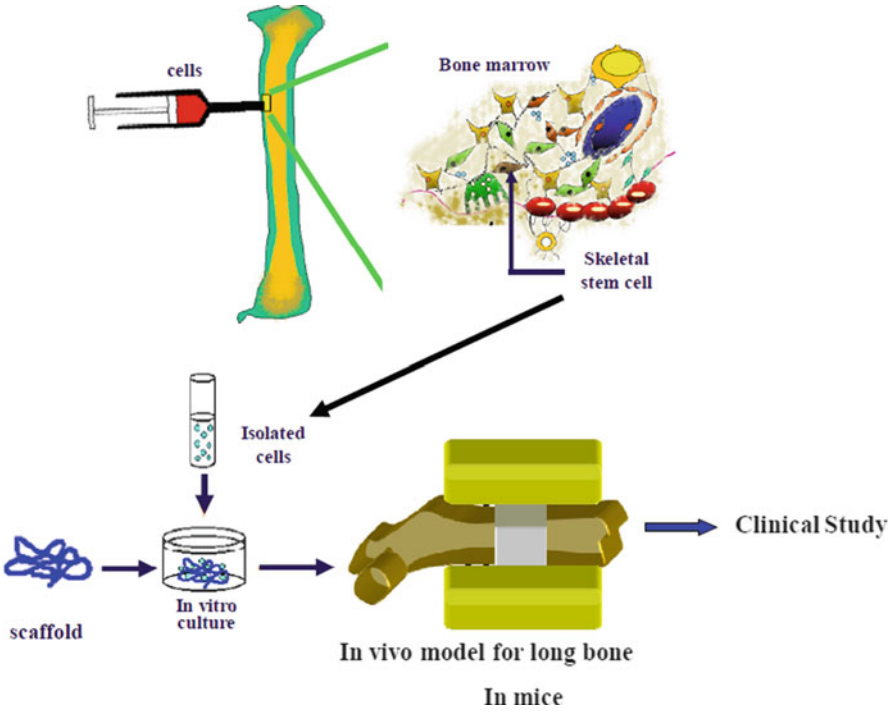

Fig. 8.6 Experimental model

The approach for the use of MSCs-based implants of bone cells to enhance bone repair will eliminate the need for additional surgical procedure, reduce unnecessary pain and complications to the patient, and shorten hospitalization time. The stromal compartment of the cavities of bone is composed of a net-like structure of interconnected MSCs. Stromal cells are closely associated with bone cortex, bone trabecules, and the hematopoietic cells. The MSCs-derived osteoprogenitor cells can be used in modern technology for tissue engineering and cell therapy. They can be used in metabolic bone diseases and orthopedic approaches, when bone repair is needed. By accomplishing the task of identifying of specific osteoprogenitors, the selection of osteoprogenitor cells from the bone marrow will be available [9, 121] (Fig. 8.3). The hypothesis is that osteoprogenitor cells derived from MSCs implanted in a biological scaffold can enhance the repair of bone defects, and will accelerate fracture healing specially in aging and bone disease. These cells are under the effects of growth factors and the 3D supporting microenvironment of the bone marrow. It has been reported that supplementation of bone marrow stromal cells cultures with FGF-2 resulted in prolonged lifespan of bone marrow stromal cells to more than 70 doublings and maintained their differentiation potential accompanied by an increase of their telomerase size [122]. MSCs from adult bone marrow are multipotent cells which can differentiate into fibroblastic, osteogenic, myogenic, adipogenic, and reticular cells. These cells may also provide a potential therapy for bone repair.

These selected cells were shown to produce a bone-specific matrix that was positive for osteocalcin. The matrix synthesized by the selected osteoprogenitors also stained positively with Alizarin Red S and von Kossa indicating both the synthesis of bone primary osteoid matrix and the subsequent mineralization of the matrix. Osteoprogenitors derived from MSCs were shown to express specific bone markers at various stages of the culture. Osteogenic differentiation stages could be divided into three periods that coincide with days in culture. The development of an osteoconductive slow-release device containing committed bone forming bone cells and growth factors will minimize the need for autologous bone grafts used today for filling bone voids or gaps and will thereby reduce the inherent risks and complications associated with the additional surgery of bone harvesting. Reinforcing the developed osteogenic bone graft substitute will improve the mechanical properties of the device, thereby extending its potential applications to highly loaded locations in the human body. Introduction of such bone substitute into clinical practice will restore the mobility and improve the quality of life of both young and aging patients who lost bone during trauma or surgical resection. One of the technical problems in conducting in vivo experiments on rat is the difficulty to aspirate a bone marrow without scarifying the animal, and for that reason preliminary in vivo results were not introduced. To solve this obstacle, larger animal such as rabbit was used. In rabbits it was possible to aspirate bone marrow and to culture it on the hydrogel, our primarily results on mandible defect revealed bone regeneration in critical size defect. It is hypothesized that growth factors and selected bone forming cells that will be incorporated in osteoinductive osteoconductive scaffolds will enhance bone formation. The hydrogel with appropriate biodegradability will function not only as a release matrix for the growth factors and supporting MSCs, but also as a space provided for bone osseointegration affecting the firmness of the fixation implants. The hydrogel containing growth factors and bone forming cells is thus a promising surgical tool for bone defects and for orthopedic and maxillofacial surgery.

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Chapter 9
Bone Reaction to Implants

David Kohavi

9.1 Introduction

Endosseous insertion of an artificial orthopedic or dental material induces an extensive tissue reaction at the implant–bone interface. Formation of a bone–implant attachment has been regularly reported. Bone repair in these instances is portrayed in several patterns. Healing depends on systemic and local conditions, inter alia, bone status, surgical technique, implant surface, biomechanical properties, and forces used. Osseointegration is defined as a direct structural bonding between bone tissue and implant surface. Clinically, such implant attachment produces a firm, asymptomatic fixation maintained in bone under functional loading. In other instances, healing is completed by fibro-integration, namely, implants are surrounded by fibrous connective tissue, showing an evident clinical mobility when loaded [1–6]. In osseointegration, light microscopy (LM) reveals insignificant amounts of fibrous tissue at the bone–implant interface; all in all, bone formation is characterized by attachment to the largest part of implant surface. Utilization of titanium alloy (Ti) implants revealed an optimal capability for osseointegration. Consequently, Ti is considered material of choice in orthopedic and oral implants. Additionally, this has been supported by biomechanical studies that showed insignificant mobility of Ti implants [7–10]. Implant stability is affected by biomechanical properties of the adjacent bone. Cortical bone allows a more stable mechanical anchorage of the implant than trabecular bone [8, 11]. Structural and mechanical changes due to impaired bone may well be responsible for reduced stability of implants [12]. Different surgical techniques have shown a significant effect on implant fixation in trabecular bone [13]. A close contact between implant and bone

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does not necessarily serve to enhance osteogenesis. On the other hand, wide space of more than 500 μm is predictive of delay and reduction in the quality or quantity of the newly formed bone [14–20]. Excessive load on the implant may enhance fibrous membrane formation and displacement at the bone–implant interface preventing osseointegration [21–26]. The principal mechanisms essential for osseointegration could be compared to those occurring in fracture repair and involve a cascade of various cellular and extracellular events [22]. The insertion of an implant is in effect an excision-injury within bone tissue, often accompanied by an increase in temperature [23]. Surgical procedure is followed by blood clotting and inflammatory reaction at implant surface. Cellular infiltrate consists of polymorphonuclear granulocytes, monocytes, macrophages, osteoclasts, and osteoblasts that migrate into the tissue adjacent to the implant. The implant surface adsorbs blood-derived proteins [24–26]. Exposure of Ti implants to plasma is followed by an immediate coating of its surface by a thin proteinaceous film. Albumin, fibronectin, fibrinogen, and IgG are the main constituents [27–30]. The extent of plasma protein adsorption to the surface is an essential indicator for implant biocompatibility [28]. Cellular attachment to biomaterial surface ensues following the interaction of adsorbed soluble proteins to cell-surface-integrins. The protein type and amount may affect cellular proliferation, differentiation, and migration [29]. Increased levels of plasma fibronectin (pFN), a high molecular weight extracellular matrix glycoprotein, are evident in early phases of cell growth and attachment [30, 31]. In osteoblast regulation, pFN activates signaling pathways of gene expression, cell-cycle progression, matrix mineralization, and apoptosis [32, 33]. Plasma protein adhesion to implants and the interaction with blood cells are required for osteoconduction [34]. Biomaterial surfaces coated by pFN showed an enhanced focal adhesion of osteoclasts, essential to improve cell spreading and cytoskeleton organization as compared to non-coated surfaces [35–39]. Serum albumin, constituting circa 60% of human plasma protein, serves as a carrier for molecules of low water solubility, including various hormones and ionic calcium. Albumin-bound lipids regulate cytoplasm calcium levels and stimulate osteoblast proliferation [40]. Protein adsorption is dependent on implant surface chemistry, structure, and morphology [41–43]. It has been shown that cell attachment and proliferation are surface roughness sensitive. Ergo, surface modifications of titanium are of main interest in the study of osteoconductivity of implants [44]. Protein adsorption appears to be roughness-dependent and human serum albumin is preferentially adsorbed on the smooth Ti surfaces, while fibronectin and total protein manifest increased binding to rough Ti surfaces. On surfaces with rough micro-topographies, osteoblasts were shown to secrete factors that enhance their differentiation and decrease osteoclasts formation and activation [45]. Nano-texturing of Ti surfaces offers an improved cell attachment, influencing cell density and morphology and regulating early expression of bone proteins [46, 47]. Confocal microscope studies revealed a significantly higher amount of albumin on the acid-etched and blasted surfaces as compared to machined and acid-etched surfaces [35]. The early cellular response to Ti implants involves deposition of non-collagenous layer on the implant surface by osteogenic cells. This is similar to the observation of bone cement lines and lamina limitans [33, 48–50].

Morphological studies reported heterogeneity in implant–bone interface. However, the early non-fibrillar, calcified layer presented a high similarity in all implants despite the different type of surfaces. Migration of mesenchymal stem cells (MSCs) is followed by colonization of the implant surface. MSCs differentiate into osteoblasts that secrete a 0.5-μm-thick fibrous collagen layer. Ionic calcium and phosphorus regulate cell adhesion and mineralization, resulting with new bone formation on the implant surface [51–54]. The interaction of red blood cells, fibrin, and platelets with the implant surface may modulate migration and differentiation [55]. Osteoblast and osteoclast activity is observed at the implant surface from day 1 of insertion [56–61]. Newly formed bone at the Interface of the surgically created implant cavity shows high similarity to the one observed in bone wound healing. The mineralized matrix is subject to resorption by osteoclasts. "Cement lines" implicated in cellular attachment, 0.2–5-μm wide, are composed sulfated polysaccharide complexes and osteopontin demarcate the transition between bone resorption formation [33, 57–61]. Cement lines at bone implant interface bear resemblance to analogous ones detected in bone remodeling [33]. On the first week of implantation, osteoblasts deposit collagen matrix directly on cement line formed on implant surface [33, 49, 61–65]. The early deposition of new calcified matrix on the implant surface is followed by formation of woven spongiosa and mature lamellar trabecular bone [23, 24]. Bone marrow provides mononuclear precursors of osteoclasts for trabecular remodeling [65]. Woven bone formation occurs on the implant surface and establishes initial continuity, albeit poor mechanical competence [66]. Remodeling of primary young bone allows its replacement by highly mineralized lamellar and trabecular bone with improved biomechanical properties. Mature bone has been found around different types of Ti implants 3 months after implantation [66, 67]. Two orientations of bone formation were observed at the implant interface, toward the implant surface and starting at the implant surface [65]. Vascular disruption and osteoclast activity are implicated in implant separation. [67, 68]. In osseointegration of metallic implants, bone healing did not start on the implant surface, and bone was shown to develop toward the implant [51, 69–73]. Changes at the titanium–bone interface have been detected with LM, transmission electron microscopy (TEM), and micro-computed tomography (μCT). These showed that Ti implants induced an immediate cellular alignment on bone and implant surfaces. On day 14, new bone was found in direct contact with the implant. TEM images showed flat osteoblasts with rich rough endoplasmic reticulum along the surfaces of the implant and the preexisting bone secreting collagen and beginning of calcification. μCT images on day 13 after implant insertion showed new bone formation. Surface roughness of titanium implants affects various determinants of osseointegration such as protein adsorption, osteoblast attachment and subsequent proliferation and differentiation, extracellular matrix production, alkaline phosphatase activity, peri-implant bone formation, and primary implant stability. Roughness may also influence two local factors, TGFb1 and PGE2, acting as autocrine regulators on the osteoblasts and modulating the activity of osteoclasts [74–76]. It has been suggested that the roughness-dependent regulation of osteoblast proliferation, differentiation, and production of local factors is mediated by integrin receptors that regulate

phosphokinase C (PKC) and A (PKA) through phospholipase C (PLC) and A2 (PLA2) pathways [77]. Generally, at the micrometer grade level of evaluation, moderately rough surfaces favor peri-implant bone growth better than smoother or rougher surfaces [78–81].

Coating of titanium implant surface with hydroxyapatite (HA) or other calcium phosphate compounds may accelerate peri-implant osteogenesis and provide a mechanical barrier to metal ion release or titanium particles detachment [82–86]. Glass-ceramic coating was shown to increase implant bonding, and it was suggested that newly formed collagen tends to attach to the chemically active surface of these biomimetic materials [87]. Bone formation increases at beta-tricalcium phosphate (TCP) cylinders when compared to HA ceramics with the same pore size. Among different pore sizes, a pore size above 80 μm has been shown to improve bone attachment in both HA and TCP materials. HA-coated implants showed earlier bone formation when compared to titanium surfaces as tested by removal torque tests and histomorphometric analyze [88–90]. In addition rough surfaces increase interface contact area contributing to implant primary stability [91–93].

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