

Osteoblasts and Osteocytes: Essentials and Methods

Nadja Fratzl-Zelman and Franz Varga

- 2.1 From Mesenchymal Stem Cells to Osteocytes – 18
 - 2.2 Inverse Relationship Between Osteogenic and Adipogenic Programming – 19
 - 2.3 From Osteoblasts to Osteocytes – 22
 - 2.4 The Osteocyte – 22
 - 2.5 Some Essential Facts About Osteocytes – 23
 - 2.6 Conclusion – 25
 - 2.7 Experimental Systems to Study Osteoblastic Differentiation and Behavior – 25
- References – 30

What You Will Learn in This Chapter

This chapter discusses the development of the bone-forming cells that are all descendants from the mesenchymal stem cells (MSCs). MSCs have the ability to self-renew and provide a pool for osteoprogenitors. However, MSCs can also differentiate into cells of the mesodermal cell line, which besides the bone-forming cells include chondroblasts, adipocytes, and muscle cells. Hormones, local factors, and the extracellular matrix program the MSCs into the distinct differentiation pathways. Especially, the inverse relationship between osteogenesis and adipogenesis plays a pivotal role for bone formation and maintenance of the bone. During differentiation of the osteoblastic lineage, cells pass distinct states with distinct roles in the bone-forming process, i.e., matrix synthesis and mineralization as well as regulation of bone remodeling which appears to be mainly directed by osteocytes. Moreover, osteocytes have important endocrine functions as they secrete factors into circulation that regulate other organs of the body.

In the second part of this chapter, several experimental systems to study bone cell differentiation and mineralization are presented and discussed.

2.1 From Mesenchymal Stem Cells to Osteocytes

Osteoblasts, bone lining cells, osteocytes, chondrocytes, adipocytes, myoblasts, and fibroblasts differentiate all from common precursor cells present in the non-hematopoietic compartment of the bone marrow, the *mesenchymal stem cells* (MSCs). Cells committed to skeletal cells are also referred to as adventitial reticular cells (ARCs) or CXCL12-abundant reticular (CAR) cells in the murine bone marrow [1]. MSCs reside in the trabecular space (stem cell niche) in common with hematopoietic stem cells, which are the founder cells of the hematopoietic lineage, the source for the bone removing osteoclasts. Additionally, endothelial cells and their progenitors reside in the stem cell niche as well.

Differentiation of the MSCs is regulated on the one hand by a tight interaction between the cells but also by local and hormonal factors activating or repressing gene transcription. This happens by cell surface-bound receptors, which can interact either with cell surface-bound ligands or mobile ligands. Moreover, the receptors might be located intracellularly within the cytoplasm as found with some steroid hormones like the estradiol receptor, moving into the nuclei after hormone binding or directly bound to DNA in the nucleus as the thyroid receptor. In addition, the MSCs are wrapped with extracellular matrix (ECM), which not only stabilizes the three-dimensional structure of the bone marrow but also signals to the cells. Balanced concentrations of hormonal and local factors as well as proper ECM in the trabecular space are therefore important for the development not only for the bone cells but also for hematopoiesis. It is now generally accepted that osteoblasts influence hematopoiesis and vice versa [2, 3] (■ Fig. 2.1).

Osteoblasts regulate not only differentiation and function of osteoclasts (as discussed below) but also of B-cell differentiation [4] and leukemogenesis [5].

Importantly, MSCs committed to differentiation into skeletal cells are able to differentiate into adipocytes as well. With increasing age there is a bias to adipocyte differentiation, which is manifested in accumulation of adipocytes in the bone marrow [6]. It is

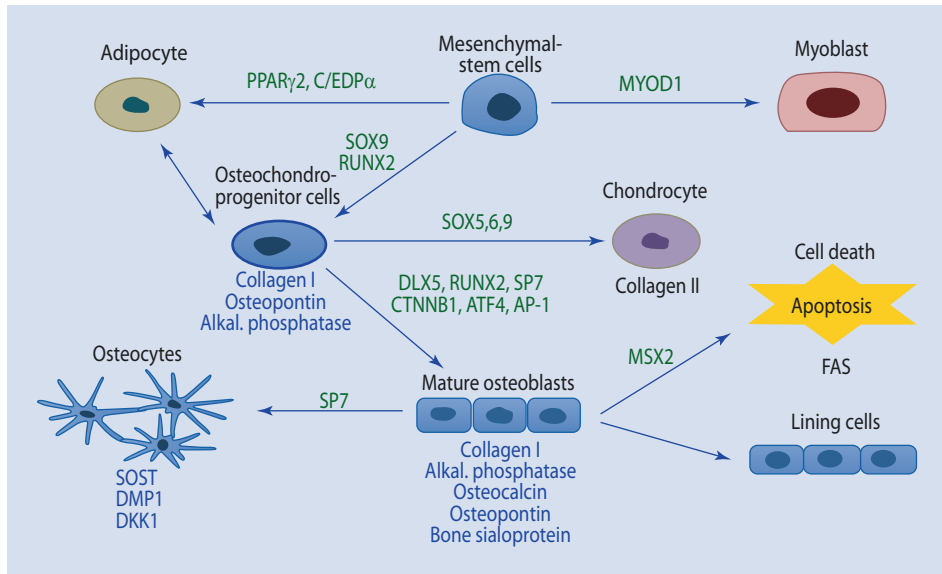


Fig. 2.1 Mesenchymal stem cells have the capacity to differentiate into several cell types (tissues). These processes are regulated by transcription factors (green). This results in expression of genes typical or specific for these tissues (blue). The surplus of mature osteoblasts, which do not become neither a lining cell nor an osteocyte, is removed by apoptosis

estimated that the bone marrow from newborn infants lacks any fat deposition, while about 70% of the adult bone marrow is occupied by fat. This process is probably irreversible and results in a reduced capacity of bone formation and hematopoiesis [1].

2.2 Inverse Relationship Between Osteogenic and Adipogenic Programming

After commitment the osteoblast precursor cells differentiate along the osteoblastic lineage that involves the development of *osteo-chondroprogenitor* cells representing the common precursor of osteoblast and chondrocytes. These osteo-chondroprogenitors differentiate either into *chondroblasts* or *osteoblasts*. These cells express two master transcription factors, SOX9 and Runt-related transcription factor 2 (RUNX2) that are essential for chondrogenesis and osteogenesis, respectively, and interact mutually [7–9]. RUNX2 is the master gene of bone formation (although not sufficient for osteoblast maturation) directing MSCs to the osteogenic lineage and inhibiting differentiation into the adipocyte fate. SOX9 is the transcriptional activator of chondrocyte-specific genes. In the osteoprogenitor cells, SOX9 and RUNX2 are co-expressed, whereas SOX2 represses the activity of RUNX2. In proliferating preosteoblastic cells, SOX9 is not any more expressed, and RUNX2 directs the osteogenic cells toward the mature osteoblastic phenotype promoting matrix synthesis and maturation in concert with many other factors like Osterix

and ATF4. Conversely, *Runx2*-deficient mice have a heavily disturbed endochondral bone formation, lacking functional osteoblasts and having only a cartilaginous skeleton. In addition the mice fail to form growth plate calcification since *Runx2* directs also the late stages of chondrocyte differentiation triggering chondrocyte hypertrophy. These animals are not viable and die after birth.

Osteoblasts proliferate and form the bone matrix. They start producing high levels of bone/liver/kidney alkaline phosphatase (*ALPL*). Alkaline phosphatase was described in 1923 by Robison who first suggested that the enzyme is essential for bone mineralization. This was confirmed later on by the discovery of hypophosphatasia, an inborn metabolic disorder characterized by undermineralized bone due to loss-of-function mutation(s) of the enzyme. Alkaline phosphatase is a membrane-bound glycoprotein, anchored in the outer plasma membrane of osteoblasts (and chondrocytes) capable of dephosphorylating a wide range of molecules. Deficiencies of *ALPL* lead to local accumulation of inorganic pyrophosphate (PP_i), a potent inhibitor of mineralization. Moreover, alkaline phosphatase seems necessary for the phosphorylation status of non-collagenous proteins involved also in the mineralization process (like osteopontin; see below). Nevertheless, the exact way of action of alkaline phosphatase is still not completely elucidated.

Matrix-forming osteoblasts also express osteoprotegerin (OPG), a decoy receptor for RANK-ligand that blocks osteoclast formation (for further details, see, e.g., [10]).

In addition, osteoblasts have receptors for many systemic hormones and most importantly for the traditional mineral-regulating hormones (parathyroid hormone (PTH), parathyroid hormone-like hormone, calcitonin, 1,25-dihydroxyvitamin D3, thyroid hormones, growth hormones, androgens, estrogens, etc.).

During extracellular matrix secretion, the cells synthesize collagen and their accessory proteins to form stable collagen fibrils. This includes proteins for post-translational collagen modification (procollagen-lysine, 2-oxoglutarate-5-dioxygenases, PLOD1–PLOD3) important for folding and cross-linking (lysyl oxidase, LOX). Additionally the cells produce non-collagenous proteins (NCPs) with different functions, which are integrated into the bone matrix.

Here are some important NCPs. For more details, refer, for example, to [11]:

- Growth factors like TGF β , bone morphogenetic proteins, and IGF1. These proteins are often integrated as precursors and/or associated with binding proteins and play multiple roles in cell signaling.
- Osteonectin is the most abundant NCP. It is a phosphorylated glycoprotein (35-45 kD protein) regulating collagen organization probably mediating mineral deposition.
- Proteoglycans are macromolecules containing acidic polysaccharide side chains (glycosaminoglycans) attached to a central core protein. In the bone (and skin!), the predominant proteoglycans are decorin, lumican, and biglycan belonging both to the family of secreted small leucine-rich proteoglycans (SLRPs). These proteins bind to collagen and regulate the activity of TGF- β as well as of other growth factors. Perlecan is a very large heparin sulfate proteoglycan with a core protein of over 4000 amino acids and plays an essential role for the maintenance of osteocyte functionality [12].
- Osteopontin, bone sialoprotein, dentin matrix protein 1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE) belong all to the small integrin-binding ligand N-linked glycoprotein (SILBING) superfamily. They are mostly

highly phosphorylated and play an important role in initiation and regulation of mineralization. Note that DMP1 and MEPE are important regulators of osteocyte function [13].

- Osteocalcin (or bone Gla protein) is a gamma-carboxyglutamic acid-containing 5 kD protein. Osteocalcin is exclusively expressed by mature osteoblasts, binds strongly to hydroxyapatite the mineral of the bone matrix, and is thought to have multiple functions in regulating bone turnover. More recently, it has emerged that osteocalcin is not only stored in the bone matrix but also released into the circulation, acting as a glucose metabolism regulating hormone on pancreatic B-cells to enhance insulin production and secretion and on muscle cells to increase insulin sensitivity and glucose uptake and decreasing visceral fat [14].

It is important to note that metabolically active, matrix-secreting osteoblasts do not function individually but are found in clusters on the bone surface where they deposit new collagen and non-collagenous proteins within a cavity that has been previously resorbed by osteoclasts (see ► Chap. 1). Not all matrix-forming osteoblasts present on the bone surface will share the same fate:

- During matrix formation, some cells stop making matrix, are left behind the other active osteoblasts, become embedded within the (still non-mineralized!) organic matrix, and will differentiate toward the *osteocyte* phenotype.
- At the end of the matrix formation process (e.g., when the resorption cavity on the bone surface is filled), alkaline phosphatase activity declines, and some cells will become flatter and metabolically less active. These cells become *lining cells*, form tight junctions with each other, and cover the bone surface forming a natural barrier toward the bone marrow space or stem cell niche. The lining cells, although considered as postosteoblastic, are quiescent cells that retain the ability to redifferentiate into matrix-forming osteoblasts upon exposure hormones or mechanical conditions [15]. As lining cells are connected to the osteocyte via the canalicular network and gap junctions, they could also signal to osteocytes when stress and damage are sensed (see below). Other functions attributed to the lining cells are regulation of the influx and efflux of mineral ions [16] and the ability of cleaning and deposition of a thin layer of a collagenous matrix along the Howship's lacuna to enable new matrix formation [17].
- However, most of the former active osteoblasts will undergo apoptosis and express genes regulating apoptosis. Apoptosis (programmed cell death, very different from necrosis) is a regulated process to maintain bone homeostasis. It is important to realize that the balance of osteoblast proliferation, differentiation, and apoptosis determines the size of a cell population [18]. Conversely, the lifespan of the osteoblasts determines the amount of bone that is formed and can be controlled physiologically by hormones and local factors. For example, intermittent treatment with PTH prevents apoptosis of osteoblasts and osteocytes leading to an increase in bone mass. Similarly, androgens decrease the rate of apoptosis in osteoblasts and osteocytes as other bone anabolic agents like insulin-like growth factors (IGFs) do. Pharmacologic levels of glucocorticoids, however, induce apoptosis of osteoblasts and osteocytes, and this is thought to be the mechanism by which these steroids cause bone loss [19] (■ Fig. 2.2).

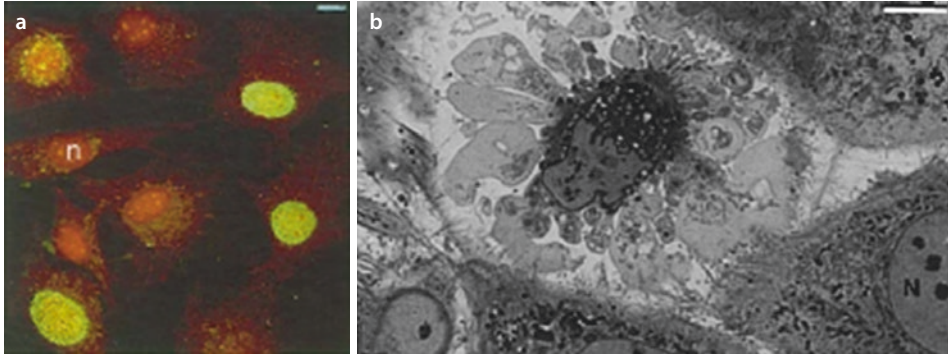


Fig 2.2 Apoptosis is an actively and genetically controlled process, which is ATP dependent. Characteristically, apoptosis leads to enzymatically controlled DNA fragmentation. **a** This fragmentation can be detected by fluorescent TUNEL assay, which uses enzymatic addition of FITC-labeled Bromodeoxyuridine (*green*) to the free 3'-hydroxyl termini of DNA fragments. The cell nuclei are stained with propidium iodide (*red*). **b** Morphologically, apoptotic cells undergo shrinkage and separation from their neighbors; plasma membrane blebblings and a characteristic form of chromatin condensation occur; there is nuclear membrane breakdown and cytolysis into condensed apoptotic bodies which are phagocytized by surrounding cells and macrophages (From Varga et al. [39], with permission from BioScientifica Ltd)

2.3 From Osteoblasts to Osteocytes

The question whether the mature osteoblast-directed mineralization *in vitro* is physiological or not might be overcome by the assumption that the mineralizing cell *in vivo* is rather the osteocyte than the osteoblast.

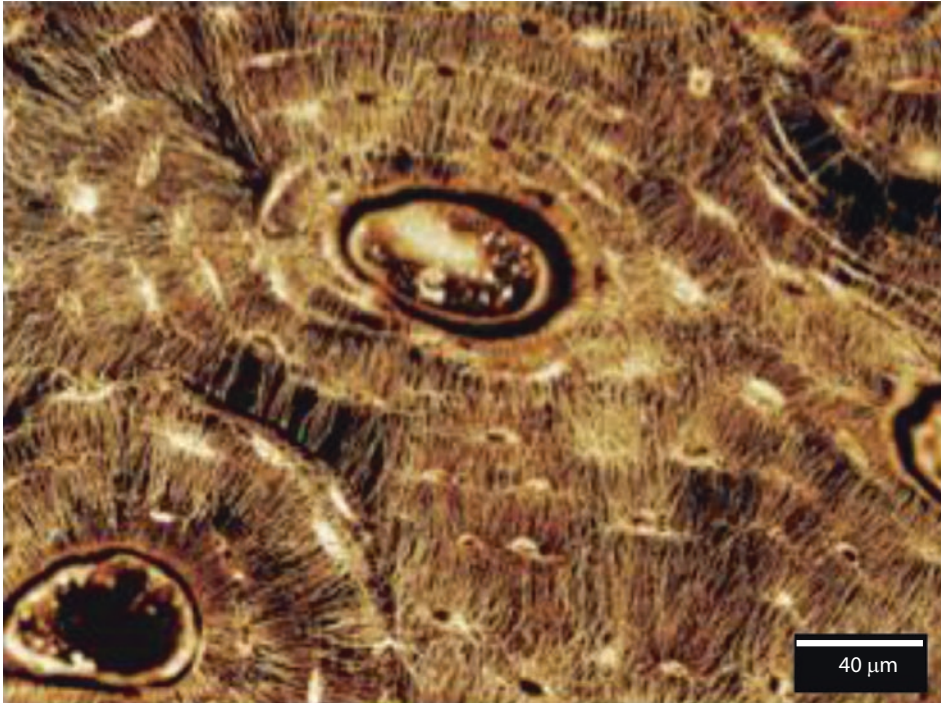
Mikuni-Takagaki et al. [20] characterized already in 1995 the different cell population in newborn rat calvaria after sequential digestion with collagenase and made the following observations:

1. The mature osteoblastic cells on the bone surface do not mineralize but rather separate themselves from the mineralization front by a 10–20 μm layer of unmineralized matrix (8–10 μm in adult remodeled bone).
2. The mineralizing cells are not (or very weakly) positive for alkaline phosphatase.
3. The initiation of mineralization coincides with the phenotypic transformation of cuboidal osteoblastic cells to stellate osteocytes (formation of dendritic processes) within the collagenous matrix, a differentiation state qualified as osteoid-osteocyte.

It has to be stated that about one decade ago, very little was known about osteocyte function. One reason is that unlike osteoblasts, the *in vitro* study of osteocytes is complicated by the fact that isolated osteocyte from bone tissue does not proliferate [20, 21]. The establishment of osteocyte-like cell lines has greatly improved the knowledge about osteocyte differentiation and function [22].

2.4 The Osteocyte

Osteocytes have been defined during decades by their morphology (cells with cytoplasmic processes) and location (cells embedded within the mineralized bone matrix). They were thought to be passive cells that become «buried alive» in the matrix formed by mature osteoblasts.



■ **Fig. 2.3** Osteocyte network in osteonal equine bone (With courtesy of the Max Planck Institute of Colloids and Interfaces, Department of Biomaterials, Golm, Germany For further details see Kerschnitzki et al. [46])

One of the first suggested functions postulated was that osteocyte senses mechanical deformation. Julius Wolff in 1867 suggested that bone adapts its external shape and internal structure in response to the mechanical forces that are required to support it. The remodeling of the bone in response to loading is achieved via mechano-transduction, a process through which forces or other mechanical signals are converted into biochemical signals (■ Fig. 2.3).

2.5 Some Essential Facts About Osteocytes

- They are differentiated cells of the osteoblastic lineage that become embedded within the mineralizing matrix.
- They share many markers with osteoblasts but do not make matrix.
- They are the most abundant (90–95%) and the longest-lived cells in the bone. Their number in the human adult skeleton is estimated to 42 billion [23].
- They are connected through dendritic processes called canaliculi (about 89 ± 25 per cell, in total human skeleton about 3.7 trillion! [23]) via gap junctions (= transmembrane channels that connect cytoplasm of two adjacent cells allowing the passage of molecules <1 kDa) [24]:
 - To each other
 - To cells on the bone surface (osteoblasts, lining cells)
 - To the bone marrow (osteoblast and osteoclast recruitment!)
 - To blood vessels (!)

- Cell body and dendritic processes form a functional network, the lacunocanalicular system, which is surrounded by an unmineralized space filled with interstitial fluid providing oxygen and nutrients to maintain cell viability in the mineralized environment.

Dendritic osteocytes convert from polygonal matrix-producing mature osteoblasts by progressing through different transitional stages and sequential expression of marker genes reflecting changes in morphology and functionality.

Osteocyte differentiation stage	Some important marker genes	Function
Young osteoid-osteocyte: Cell embedded in non-mineralized matrix, beginning to generate dendritic processes	Podoplanin (<i>PDPN</i>): transmembrane protein, the earliest known marker of osteocyte differentiation	Regulates formation of dendritic processes
	Membrane-anchored proteinase that cleaves collagen (<i>MMP14</i>)	Important for dendritic formation and morphology
Late mineralizing osteoid-osteocyte: Cell embedded within the osteoid with small, calcified spheres forming along the cell membrane toward the mineralization front	Dentin matrix protein1 (<i>DMP1</i>): secreted serin-rich acidic protein with many phosphorylation sites	Regulates osteocyte maturation, phosphate metabolism, and mineralization Inactivation mutations cause autosomal recessive hypophosphatemia and osteomalacia
	Phosphate-regulating gene with homologies to endopeptidase on the X chromosome (<i>PHEX</i>)	Regulates osteocyte maturation, phosphate homeostasis, and mineralization Inactivation mutations cause hypophosphatemic rickets (X-linked hypophosphatemia (XLH))
	Matrix extracellular phosphoglycoprotein (<i>MEPE</i>)	Regulates phosphate metabolism and mineralization. Inhibits PHEX
	Osteopontin (<i>SPP1</i>)	Negative regulator of bone mineralization
Mature osteocyte: Cell is completely embedded within the mineralized matrix Numerous dendritic processes connect the osteocytes to each other	Sclerostin, secreted factor. Highly and specifically expressed in the late osteocyte (<i>SOST</i>)	Negative regulator of bone formation through inhibition of the WNT/ β -catenin signaling pathway (regulation of transcription factors) in a negative feedback loop [25]. Treatment of mice with a sclerostin antibody leads to increased osteoblast number by converting quiescent lining cells into active osteoblasts [26] Inactivation mutations cause sclerostosis and van Buchem disease with increased bone formation

Osteocyte differentiation stage	Some important marker genes	Function
	Fibroblast growth factor 23 (<i>FGF23</i>) Secreted factor! Most important organ is the kidney Highly expressed in <i>DMP1</i> and <i>PHEX</i> -associated hypophosphatemic rickets, chronic kidney disease, and tumor-induced osteomalacia	Reduces serum phosphate (Pi) levels by inhibiting renal phosphate reabsorption and downregulation of 1,25-dihydroxyvitamin D3 synthesis <i>The osteocyte network becomes an endocrine gland</i> [14] Inactivation mutation causes autosomal dominant hypophosphatemic rickets
	Receptor activator of nuclear factor – κ B ligand (RANKL, <i>TNFSF11</i>)	Control of osteoclastogenesis – major contribution to bone remodeling in adults [27]
	Tartrate-resistant acid phosphatase (TRAP, <i>ACP5</i>) Cathepsin K (<i>CTSK</i>)	Removal of perilacunar matrix = osteocyte osteolysis. Important in situation of high calcium demand like lactation [27]
	hypoxia upregulated 1 (ORP150, <i>HYOU1</i>)	Preserves viability of osteocytes in hypoxic environment

2.6 Conclusion

Emerging data from osteocyte function have established a new paradigm: osteocytes embedded within the mineralized bone matrix are extremely active and multifunctional cells – they control bone mineralization mainly through expression of factors that regulate phosphate homeostasis (reviewed by [22]); they secrete factors that target the kidney and muscles; they do remodel their extracellular matrix and modify their microenvironment. Osteocytes regulate bone remodeling through regulation of both osteoclasts and osteoblasts as well as their apoptosis, which is an essential tool to control skeletal damage repair [28]. Most importantly, the lacunocanalicular system appears as a highly organized network of connected osteocytes that sense mechanical strain, respond to chemical signals, and orchestrate bone homeostasis. The discovery of these multiple signaling pathways raises also the possibility to develop new therapeutic agents for skeletal diseases [29].

2.7 Experimental Systems to Study Osteoblastic Differentiation and Behavior

In their pioneering work, Friedenstein and coworkers showed for the first time that a bone marrow cell suspension contains a subset of long, plastic-adherent cells with a robust proliferative activity that will give rise to single cell-derived colonies (or colony-forming

units, CFUs) with the capacity to differentiate into the bone, chondrocytes, adipocytes, and fibroblastic cells [6, 30, 31]. Experimentally, these cells can be used to study osteoblastic differentiation and regulation. Cell behavior depends on their (micro)environment: the substrate, the degree of contact with other cells, the constitution of the medium, the oxygen tension, and more. Under optimal culture, cells proliferate and differentiate *in vitro* to form an extracellular matrix that might become later on mineralized (reviewed by, *i.e.*, [32]).

Actually, primary bone cells or cell lines from chick, rat, and mouse are widely used to study the molecular properties of the osteoblast phenotype during proliferation, differentiation, and maturation. Osteoblasts and early osteocytes can be isolated from aseptically dissected calvaria or long bones. For this purpose the bones are serially digested with collagenase. After each sequential digestion, the cell suspension is precipitated by centrifugation and, after washing the cells, suspended in culture medium and seeded into cell culture dishes; the last fraction shows the phenotype of early osteocytes.

Another experimental system is a culture of cells growing out from trabecular bone. For this purpose, small pieces of trabecular bone, *i.e.*, remnants from surgery, are placed in culture dishes containing an appropriate medium for about 3–4 weeks. Thereafter, a lawn of cells is found that can be split several times, and these cells can be frozen in liquid nitrogen for long-term storage.

Osteosarcoma cell lines with diverse differentiation stages can be used as well for studying osteoblastic cell behavior. It must be emphasized that these osteoblast-like cell lines are established from tumor and consequently the system might have some limitations. In particular, the genotype of these transformed and immortalized cell lines is often partially polyploid, and some genes are possibly overrepresented, whereas some others may be deleted or mutated. Nevertheless, these cells are very suitable for studying specific issues. The most frequently used cell lines are U-2 OS, MG-63, and SaOS-2. They differentially respond to vitamin D. While MG-63 expresses and responds to vitamin D with increase expression and secretion of osteocalcin, the marker protein of the mature osteoblast and the two other cell lines do not. Mineralization, however, was demonstrated for MG-63 and SaOS-2 [33].

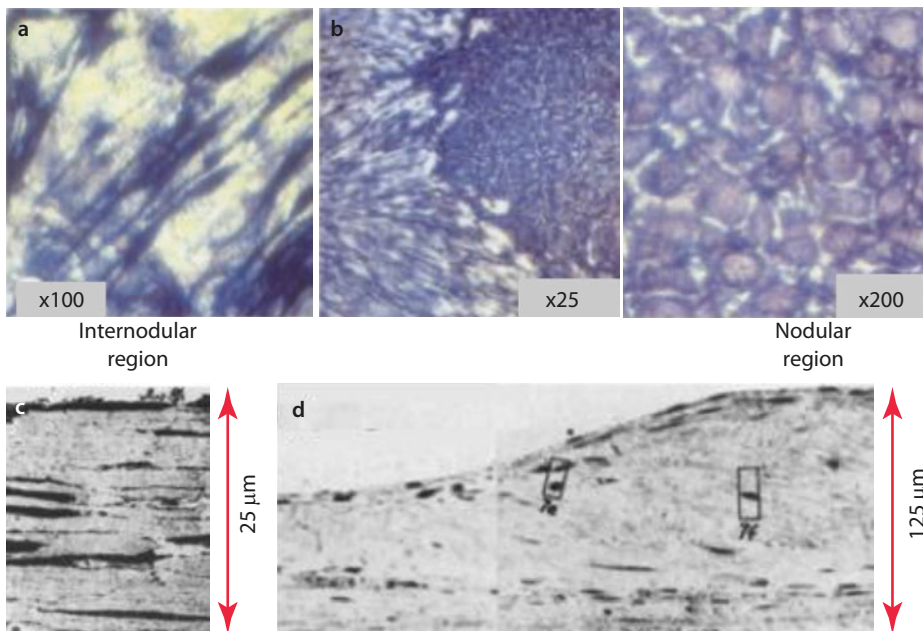
For mice, several cell lines exist showing different phenotypes and differentiation states and competence. The cell line with the curious denomination C3H10T1/C3H10T2 is an undifferentiated mesenchymal cell line with the potential to differentiate into myoblasts [34], adipocytes [35], chondrocytes, and osteoblasts [36]. This cell line is best suited to study differentiation between osteoblasts and adipocytes [35]. A more differentiated cell line on the way to osteoblasts is the stromal cell line ST2. This cell line, as suggested for stromal cells, supports the differentiation of osteoclast-like cells [37].

An interesting and widely used cell line is MC3T3-E1 and will be discussed here a little more in detail: the cell line was established by immortalization of newborn mouse osteoblasts and behaves similar as primary cells isolated from newborn mouse calvaria by sequential digestion with collagenase [38]. The MC3T3-E1 cells have the capacity to differentiate into mature osteoblasts as indicated by increasing expression levels of alkaline phosphatase and osteocalcin [39]. During prolonged culture time (up to 6 weeks), the cells form many cell layers with a tissue-like appearance [40, 41]. Moreover, the cells form discrete three-dimensional nodular structures consisting of collagenous matrix and covered with cuboidal alkaline phosphatase positive cells [32, 38]. It is generally assumed that the nodules become mineralized by mature osteoblasts [32].

However, MC3T3-E1 like most osteoblastic cell cultures usually do not mineralize spontaneously their extracellular matrix but generally require phosphate supplementation, to induce mineral deposition. For this purpose, 5–10 mM β -glycerophosphate (β GP) is added (generally designated as mineralizing medium) [42].

Although the process of mineralization is still controversial and yet not clarified, there are some aspects that are general consents. The mineral in the bone is hydroxyapatite, a calcium phosphate compound. The solubility product of Ca^{++} ions with phosphate ions (P_i) of the intercellular liquid favors calcium phosphate precipitation. However, mineralization is an ordered and highly regulated process where delivery of Ca^{++} and phosphate ions occurs only at the mineralization front. Therefore uncontrolled initiation of mineralization must rather be inhibited than promoted. Matrix proteins like osteopontin, SIBLING proteins, as well as enzymes regulating the local ratio between pyrophosphate (PP_i inhibits mineralization) and phosphate ions (P_i promotes mineralization) are important players that in concert modulate the physiological mineralization process [43] (■ Fig. 2.4).

Addition of β GP to MC3T3-E1 cultures provides an increase in P_i concentration, possibly by the action of enzymes synthesized by the cells, like alkaline phosphatase, promoting formation of calcium phosphate. Whether the formed mineral is hydroxyapatite or non-apatitic and ectopic (non-collagen associated) is still a matter of controversies [42] (■ Fig. 2.5).



■ **Fig. 2.4** Long-term culture for 6 weeks of MC3T3-E1 cells in the presence of ascorbate to enable collagen synthesis, which is important for osteoblastic differentiation. Alkaline phosphatase-positive cells are stained in blue. In the internodular region **a**, the predominant phenotype of the cells is a spindle-shaped fibroblastic like phenotype, while in nodular region colonies densely packed cuboidal alkaline phosphatase positive cells were observed **b**, **c** the hallmark of the mature osteoblast. Cross sections of the internodular region **c** and the nodular region **d** (right From Fratzi-Zelman et al. [41] with permission from Elsevier)

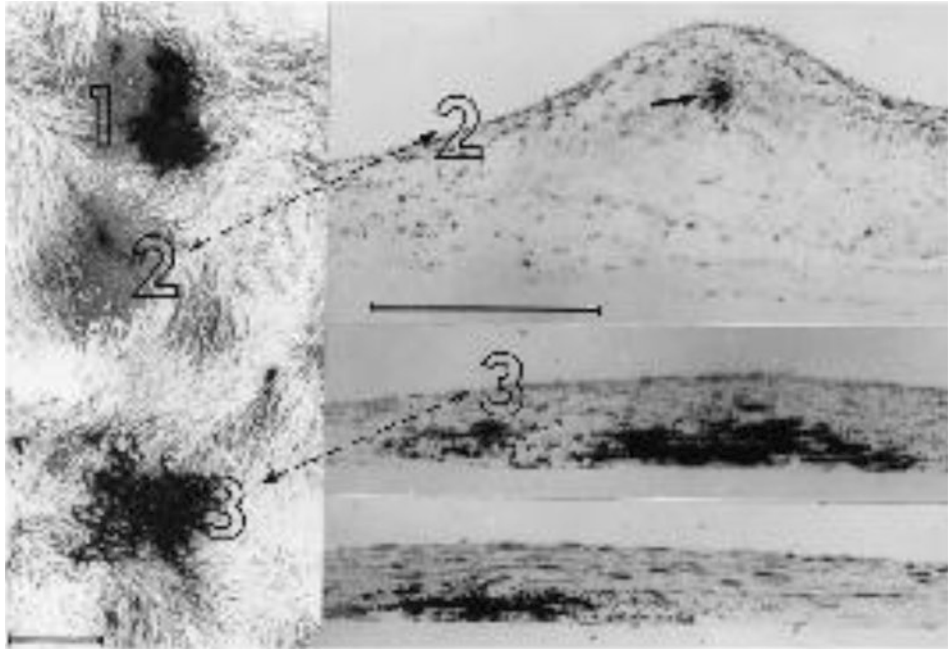
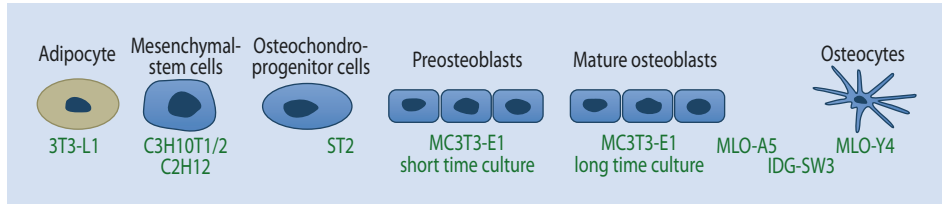


Fig. 2.5 Six-week-old cultures of MC3T3-E1 treated with BGP for the last 2 weeks. The culture was stained with the azo-dye to localize ALPL-positive cells (*gray*) and with von-Kossa staining for simultaneous visualization of mineral deposition (*black*). 1 Nodule with high mineral content. 2 Nodule with low mineral content. 3 Internodular region with mineral deposition. Left overview, scale bar = 1 mm right: cross section, scale bar = 0.2 mm (From Fratzl-Zelman et al. [42] with permission from Elsevier)

Osteocytic cells are highly differentiated cells embedded within the bone matrix and therefore more difficult to isolate and to maintain in culture. In principle, they can be isolated from aseptically dissected long bones of 3- or 4-day-old mice as well. Therefore, bones are serially digested with collagenase, and the isolated cells are recovered by centrifugation and seeded on dishes (see above); the last fraction shows the phenotype of early osteocytes. In fact these osteocytes were found to adhere rapidly to glass or plastic substrates, forming numerous cytoplasmic processes and making contact with other osteocytes [20, 21]. However, these postmitotic cells become rapidly overgrown by the mitotic fibroblast-like cells that were co-extracted from the bone, making further functional analyses very difficult. But there are now recent reports on successful isolation and culture of human osteocytes [44, 45].

Studying osteocytes in culture became much easier with the establishment of osteocyte-like cell lines. Actually, three characteristic osteocyte-like cell lines derived from mouse long bones are available: the post-osteoblast, preosteocyte-like cells MLO-A5 that spontaneously mineralize in culture, the MLO-Y4 cells that mimic a rather mature osteocyte phenotype, and more recently the IDG-SW3 cell line that expresses characteristic markers from late osteoblast to mature osteocyte phenotype *in vitro*. The study of these cell lines provided within the last few years a wealth of data showing that osteocytes are



■ **Fig. 2.6** Nearly every phenotype of the osteoblastic differentiation cascade can be approached by an established cell line

extremely active cells with a complex developmental biology and multiple functions in bone metabolism [22].

Taken together, the accurate knowledge about osteoblast phenotype and differentiation shows that the choice of an appropriate cell line is crucial for a correct experimental setup (■ Fig. 2.6).

Take-Home Message

- Osteoblasts derive from mesenchymal stem cells of the bone marrow, differentiate in a highly regulated fashion, and secrete an extracellular matrix consisting mainly of type I collagen and a small amount of non-collagenous proteins. Active, matrix-secreting osteoblasts undergo one of the three fates: the great majority dies by apoptosis, which is an important regulatory process to maintain bone homeostasis, and some remain quiescent on bone surfaces as flat lining cells, while about 5–20% differentiate into osteocytes.
- Osteocytes are the most abundant cells of the bone tissue. As they become embedded within the bone matrix, they undergo a characteristic transformation from a cuboidal to a stellate cell through the formation of multiple dendritic processes called canaliculi and start to mineralize the surrounding matrix. Canaliculi and osteocytic cell body (also called osteocyte lacunae) form a highly functional network, which allows communication among osteocytes, with osteoblasts and lining cells.
- Osteocytes secrete factors that target also osteoclasts and hormones that affect other organs by endocrine mechanisms. The many functions of osteocytes include mechanosensing, regulation of phosphate metabolism, bone formation, and bone resorption.
- Cell cultures of osteoblasts and osteocytes allow to determine biological functions; however, one has to be aware that all *in vitro* models have strengths and limitations.

Acknowledgments The authors are very grateful to Univ. Prof. Dr. Klaus Klaushofer, Director of the Ludwig Boltzmann Institute of Osteology, for the continuous support and many discussions about bone biology and its relevance for the clinic.

The work was supported by the AUVA (Research funds of the Austrian workers compensation board) and by the WGKK (Viennese sickness insurance funds).

References

1. Bianco P. Bone and the hematopoietic niche: a tale of two stem cells. *Blood*. 2011;117(20):5281–8.
2. Scadden DT. Nice neighborhood: emerging concepts of the stem cell niche. *Cell*. 2014;157(1):41–50.
3. Bethel M, Chitteti BR, Srouf EF, Kacena MA. The changing balance between osteoblastogenesis and adipogenesis in aging and its impact on hematopoiesis. *Curr Osteoporos Rep*. 2013;11(2):99–106.
4. Wu JY, Purton LE, Rodda SJ, et al. Osteoblastic regulation of B lymphopoiesis is mediated by Gs[alpha]-dependent signaling pathways. *Proc Natl Acad Sci U S A*. 2008;105(44):16976–81.
5. Kode A, Manavalan JS, Mosialou I, et al. Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature*. 2014;506(7487):240–4.
6. Chen Q, Shou P, Zheng C, et al. Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death Differ*. 2016;23(7):1128–39.
7. Karsenty G. Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet*. 2008;9:183–96.
8. Akiyama H, Kim JE, Nakashima K, et al. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci U S A*. 2005;102(41):14665–70.
9. Zhou G, Zheng Q, Engin F, et al. Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc Natl Acad Sci U S A*. 2006;103(50):19004–9.
10. Khosla S. Minireview: the OPG/RANKL/RANK system. *Endocrinology*. 2001;142(12):5050–5.
11. Sroga GE, Vashishth D. Effects of bone matrix proteins on fracture and fragility in osteoporosis. *Curr Osteoporos Rep*. 2012;10(2):141–50.
12. Thompson WR, Modla S, Grindel BJ, et al. Perlecan/Hspg2 deficiency alters the pericellular space of the lacunocanalicular system surrounding osteocytic processes in cortical bone. *J Bone Miner Res*. 2011;26(3):618–29.
13. Staines KA, MacRae VE, Farquharson C. The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling. *J Endocrinol*. 2012;214(3):241–55.
14. Fukumoto S, Martin TJ. Bone as an endocrine organ. *Trends Endocrinol Metab*. 2009;20(5):230–6.
15. Kim SW, Pajevic PD, Selig M, et al. Intermittent parathyroid hormone administration converts quiescent lining cells to active osteoblasts. *J Bone Miner Res*. 2012;27(10):2075–84.
16. Neve A, Corrado A, Cantatore FP. Osteoblast physiology in normal and pathological conditions. *Cell Tissue Res*. 2011;343(2):289–302.
17. Everts V, Delaisse JM, Korper W, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J Bone Miner Res*. 2002;17(1):77–90.
18. Varga F, Luegmayr E, Fratzl-Zelman N, et al. Tri-iodothyronine inhibits multilayer formation of the osteoblastic cell line, MC3T3-E1, by promoting apoptosis. *J Endocrinol*. 1999;160(1):57–65.
19. Bodine PV. Wnt signaling control of bone cell apoptosis. *Cell Res*. 2008;18(2):248–53.
20. Mikuni-Takagaki Y, Kakai Y, Satoyoshi M, et al. Matrix mineralization and the differentiation of osteocyte-like cells in culture. *J Bone Miner Res*. 1995;10(2):231–42.
21. van der Plas A, Nijweide PJ. Isolation and purification of osteocytes. *J Bone Miner Res*. 1992;7(4):389–96.
22. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell ... and more. *Endocr Rev*. 2013;34(5):658–90.
23. Buenzli PR, Sims NA. Quantifying the osteocyte network in the human skeleton. *Bone*. 2015;75:144–50.
24. Batra N, Kar R, Jiang JX. Gap junctions and hemichannels in signal transmission, function and development of bone. *Biochim Biophys Acta*. 2012;1818(8):1909–18.
25. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med*. 2013;19(2):179–92.
26. Kim SW, Lu Y, Williams EA, et al. Sclerostin antibody administration converts bone lining cells into active osteoblasts. *J Bone Miner Res*. 2016;32(5):892–901.
27. Xiong J, O'Brien CA. Osteocyte RANKL: new insights into the control of bone remodeling. *J Bone Miner Res*. 2012;27(3):499–505.
28. Jilka RL, Noble B, Weinstein RS. Osteocyte apoptosis. *Bone*. 2013;54(2):264–71.
29. Plotkin LI, Bellido T. Osteocytic signalling pathways as therapeutic targets for bone fragility. *Nat Rev Endocrinol*. 2016;12(10):593–605.
30. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp*. 1988;136:42–60.
31. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell*. 2008;2(4):313–9.
32. Aubin JE. Regulation of osteoblast formation and function. *Rev Endocr Metab Disord*. 2001;2(1):81–94.

33. Fedde KN. Human osteosarcoma cells spontaneously release matrix-vesicle-like structures with the capacity to mineralize. *Bone Miner.* 1992;17(2):145–51.
34. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 1987;51(6):987–1000.
35. Ahrens M, Ankenbauer T, Schroder D, Hollnagel A, Mayer H, Gross G. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell Biol.* 1993;12(10):871–80.
36. Wang EA, Israel DI, Kelly S, Luxenberg DP. Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors.* 1993;9(1):57–71.
37. Udagawa N, Takahashi N, Akatsu T, et al. The bone marrow-derived stromal cell lines MC3T3-G2/PA6 and ST2 support osteoclast-like cell differentiation in cocultures with mouse spleen cells. *Endocrinology.* 1989;125(4):1805–13.
38. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J Cell Biol.* 1983;96(1):191–8.
39. Varga F, Rumpler M, Luegmayer E, Fratzl-Zelman N, Glantschnig H, Klaushofer K. Triiodothyronine, a regulator of osteoblastic differentiation: depression of histone H4, attenuation of c-fos/c-jun, and induction of osteocalcin expression. *Calcif Tissue Int.* 1997;61(5):404–11.
40. Luegmayer E, Varga F, Frank T, Roschger P, Klaushofer K. Effects of triiodothyronine on morphology, growth behavior, and the actin cytoskeleton in mouse osteoblastic cells (MC3T3-E1). *Bone.* 1996;18(6):591–9.
41. Fratzl-Zelman N, Horandner H, Luegmayer E, et al. Effects of triiodothyronine on the morphology of cells and matrix, the localization of alkaline phosphatase, and the frequency of apoptosis in long-term cultures of MC3T3-E1 cells. *Bone.* 1997;20(3):225–36.
42. Fratzl-Zelman N, Fratzl P, Horandner H, et al. Matrix mineralization in MC3T3-E1 cell cultures initiated by beta-glycerophosphate pulse. *Bone.* 1998;23(6):511–20.
43. Zhou X, Cui Y, Han J. Phosphate/pyrophosphate and MV-related proteins in mineralisation: discoveries from mouse models. *Int J Biol Sci.* 2012;8(6):778–90.
44. Prideaux M, Schutz C, Wijenayaka AR, et al. Isolation of osteocytes from human trabecular bone. *Bone.* 2016;88:64–72.
45. Shah KM, Stern MM, Stern AR, Pathak JL, Bravenboer N, Bakker AD. Osteocyte isolation and culture methods. *Bonekey Rep.* 2016;5:838.
46. Kerschnitzki M, et al. The organization of the osteocyte network mirrors the extracellular matrix orientation in bone. *J Struct Biol.* 2011;173:303–11.