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Bone cells-biomaterials interactions

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1. ABSTRACT

With the aging population, the incidence of bone defects due to fractures, tumors and infection will increase. Therefore, bone replacement will become an ever bigger and more costly problem. The current standard for bone replacement is autograft, because these transplants are osteoconductive and osteoinductive. However, harvesting an autograft requires additional surgery at the donor site that is related to high level of morbidity. In addition, the quantity of bone tissue that can be harvested is limited. These limitations have necessitated the pursuit of alternatives using biomaterials. The control of bone tissue cell adhesion to biomaterials is an important requirement for the successful incorporation of implants or the colonization of scaffolds for tissue repair. Controlling cells-biomaterials interactions appears of prime importance to influence subsequent biological processes such as cell proliferation and differentiation. Therefore, interactions of cells with biomaterials have been widely studied especially on two-dimensional systems. This review focuses on these interactions.

2. INTRODUCTION

Bone has many functions. It is the basic support system for locomotion, the site of attachment for muscles, ligaments and tendons, and it provides mechanical support for, and protection of, vital organs. The bone marrow is also a major site of hematopoiesis and an important reservoir of minerals (1). The aging of the population of the developed world over the coming years will result in a significant increase in musculoskeletal diseases (2). There are over 150 such diseases and syndromes, and they are all generally progressive and painful (3). Rheumatoid arthritis, osteoarthritis, osteoporosis, low back pain and limb trauma have the greatest social impact (3). About 5 to 10% of the 5.6 million fractures that occur each year in the USA result in slow or inefficient bone repair (4). Fractures involving large segmental bone defects (beyond a critical size) cannot self-repair spontaneously and need orthopedic surgery (5). In addition, 5 to 35% of spinal fusion operations result in nonunion (6). The World Health Organization reported recently that the direct and indirect costs (death and morbidity) of musculoskeletal conditions in the USA have more than quadrupled in just three decades to reach $215 billion in 1995 (3).

Some bone fractures and unstable bone structures are presently treated with bone grafts or bone materials (6-8). Bone substitutes should, ideally, be biocompatible, osteointegrative, osteoconductive and osteoinductive. Osteointegration implies strong interactions between the host bone tissue and the grafted materials, while osteoconduction defines the ease with which materials can be colonized by host bone cells and blood vessels. Osteoinductive materials stimulate host mesenchymal stem cells from surrounding tissues to differentiate into bone forming cells (9). There are two types of bone grafts: autografts and allografts. A graft that uses a bone biopsy taken from the patient is an autograft, while a graft using bone taken from another person is an allograft. Autografts are the gold standard for surgeons, but these grafts are associated with 8.5 to 20% of the complications encountered post surgery, including blood loss, nerve injury, infections, morbidity and chronic pain at the donor and/or acceptor site (9). Allografts are also possible causes of disease transmission and immune responses resulting in the rejection of the implanted tissue (6, 8, 10-12). However, the risk of infection with the human immunodeficiency virus from a properly screened allograft is estimated to be 1 in 1.6 million, much lower than that of blood transfusions (1 in 450 000) (9).

Researchers have tried to overcome these problems of bone grafts by finding alternative solutions using natural or synthetic biomaterials. The developments in biomaterials that have occurred since 1960 have evolved in three distinct steps (13). First generation biomaterials were developed to have the physical properties of bone tissues while minimizing immune responses during their implantation in vivo (13). Most of these biomaterials were inert, and one, titanium (Ti), is still currently used (14-16). The second generation biomaterials were resorbable or bioactive, while the third generation combines both properties by controlling cell responses at molecular level, such as biomimetic materials and nanopatterned surfaces (13, 17).

This chapter focuses on the interactions between bone cells and biomaterials, as they play a crucial role in bone repair. We will first briefly review bone histology (cells, matrix, regulating factors), and then discuss the ways in which bone cells adhere to biomaterials in two-dimensional (2D) systems. Third, we will examine the influence of biomaterial surface properties on bone cell behaviors. Finally, we will describe the way cells and biomaterials interact in three-dimensional (3D) systems.

3. BONE HISTOLOGY

Adult bones can be cortical (compact) or trabecular (cancellous) (18, 19). The main role of cortical bones is mechanical, while trabecular bones are metabolically important. Cortical bones are made up of cylindrical structures called osteons or Haversian systems. Each osteon is composed of concentric lamellae of mineralized matrix. The juxtaposition of the osteons provides the mechanical properties of the cortical bone. The Haversian canals at the center of each osteon contain capillaries, nerves and connective tissue. These canals run parallel and are separated by transverse Volkmann's canals (18, 20). Trabecular bones are found mainly in the spine, flat bones, short bones and in the extremities of long bones. Cortical bones are only 5 to 30% porous, while trabecular bones can be 30 to 90% porous (20).

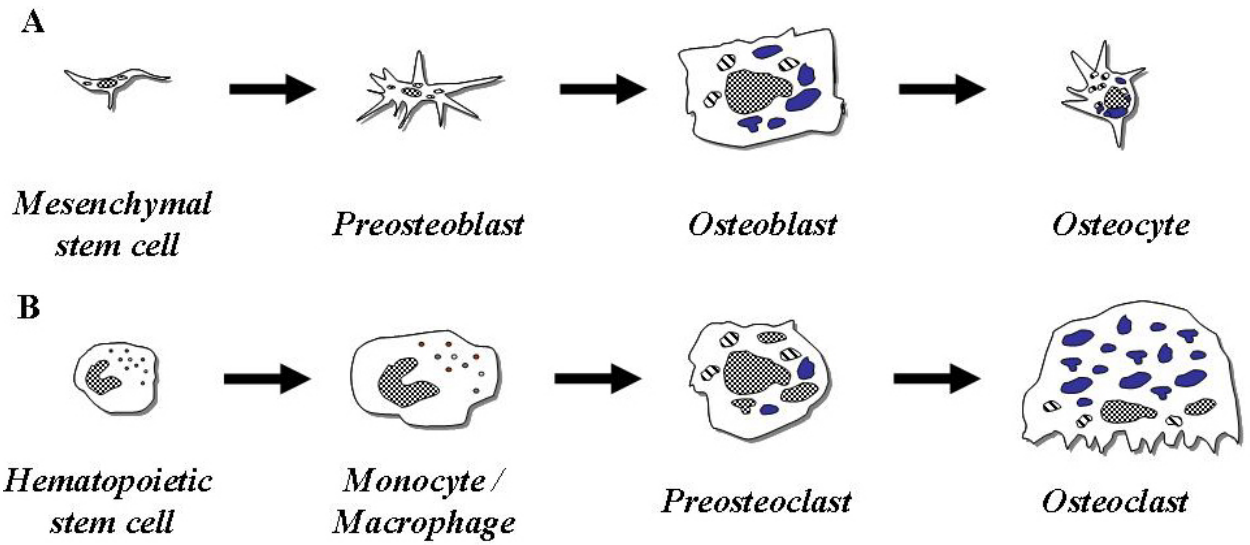

Figure 1. Osteoblast, osteocyte (A) and osteoclast (B) differentiation. Preosteoblasts start to differentiate slowly from mesenchymal stem cells in the bone marrow until they have the location and phenotype of osteoblasts. As the osteoid becomes mineralized by osteoblasts, these cells become enclosed in lacuna as osteocytes. Osteoclasts are giant multinucleate cells that differentiate from hematopoietic cells of the monocytes/macrophage lineage in the bone marrow.

3.1. Bone cells

There are three types of differentiated bone cells: osteoblasts, osteocytes and osteoclasts. Osteoblasts and osteoclasts, which have different origins and functions, are crucial for bone remodeling.

3.1.1. Osteoblasts

Preosteoblasts start to differentiate slowly from mesenchymal stem cells in the bone marrow until they have the location and phenotype of osteoblasts (Figure 1A). *In vitro*, the osteogenic differentiation from mesenchymal stem cells requires the presence of various factors including growth factors, beta-glycerophosphate, ascorbic acid and dexamethasone (21). The final differentiation into osteoblasts requires a complex cellular and molecular regulation by transcription factors such as Runx2 (also called Cbfa-1) and Osterix, a Zn finger-containing protein downstream of Runx2 (1, 22). During osteogenesis, differentiating cells contain a changing repertoir of cadherins, which are single chain integral membrane glycoproteins that mediate Ca2+-dependent cell-cell adhesions (23). Disruption of cadherins by the overexpression of dominant negative N-cadherins prevents cells differentiating into osteoblasts (24).

Active osteoblasts possess a prominent rough endoplasmic reticulum and the Golgi apparatus characteristic of protein-secreting cells. These cells are a major source of osteoid, the unmineralized organic matrix composed of collagen and various non-collagenous proteins. As the osteoid becomes mineralized by osteoblasts, these cells become enclosed in lacuna as osteocytes (25). Osteoblasts that are not enclosed in the mineralized matrix become boarding cells, which are involved in bone remodeling, or they die by apoptosis (26).

3.1.2. Osteocytes

Osteocytes make up 95% of all bone cells and they are dispersed throughout the mineralized bone matrix. The molecular cues and mechanisms underlying their entrapment within the osteoid and their differentiation from osteoblasts are still being debated (Figure 1A) [for review see (27)]. However, the entrapment of osteocytes results in profound changes in their morphology, including the reorganization of their actin cytoskeleton to give them a distinctive dendritic shape and a ~30-70% size decrease (28). Entrapment also affects their physiology by decreasing the osteoid secretion, the cellular activity and the mitochondria number (27).

Osteocytes located in lacuna are interconnected to neighboring osteocytes and cells at the bone surface through adherent junctions made of cadherins and gap junctions made of connexins (Cx) (23). Some studies have suggested that osteocytes are sensors of bone mechanical stimuli, based on the hypothesis that mechanical forces act on bone to create fluid flow through the lacunar-canicular space (cannaliculi) surrounding the osteocytes, and probably lead to deformation of the extracellular matrix (ECM) and cell membranes of the osteocytes (29). The intracellular domain of osteocyte cadherins is anchored to the actin cytoskeleton through a multiprotein complex that includes alpha- and beta-catenins (30). Disruption of cadherins mediated cell-cell adhesions causes beta-catenins to become dissociated from the cadherins tails, allowing their translocation to the cell nuclei where they modulate the Runx2 gene expression (30, 31).

Connexins are integral membrane protein with four transmembrane domains, a large intracellular loop

with intracellular C- and N-terminal ends, and two small extracellular loops. Cx43 is the major connexin in osteoblasts and osteocytes (23). Cx can assemble as either homohexameric or heterohexamiric hemichanels also called connexons (23). Cx phosphorylation at the plasma membrane changes its structure and modulates the opening and closing of connexons (32). Gap junctions are formed by joining the connexons of neighbouring cells to allow direct two-way communications via ions, small metabolites (~1 kDa) and second messengers such as Ca^{2+} and cyclic Adenosine 3'-5' Monophosphate (cAMP), which modulate bone cell functions (33, 34).

3.1.3. Osteoclasts

Osteoclasts are giant multinucleate cells that differentiate from hematopoietic cells of the monocytes/macrophage lineage in the bone marrow (Figure 1B) (35). The differentiation to osteoclasts, or osteoclastogenesis, is regulated by various factors including macrophage colony stimulating factor (cFms) and the Receptor Activator of Nuclear Factor-kappaB (NF-kappaB) Ligand (RANKL) (Figure 2). RANKL can exist as a type 2 transmembrane protein on the surface of stromal cells, osteoblasts and hypertrophic chondrocytes (1). A soluble form of it can also be secreted by osteoblasts and bone marrow stromal cells (35). RANKL binds to the osteoclast receptor RANK and activates osteoclast differentiation via the translocation of NF-kappaB into the nucleus by intermediates such as Tumor Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF6) (36). RANKL is essential not only for osteoclastogenesis, but also for the survival and activation of mature osteoclasts (35). Nevertheless, RANKL signals can be blocked by a soluble RANKL-binding decoy receptor, osteoprotegerin (OPG), produced by osteoblasts. This OPG prevents osteoclast formation (37, 38). Tartrate-Resistant Acid Phosphatase (TRAcP) is also involved in osteoclast activity and used as a marker of osteoclast phenotype. This glycoprotein which is produced by mature osteoclasts has a binuclear iron in its active site that allows hydroxyl radical formation in the presence of hydrogen peroxide. The polypeptide chain of TRAcP is also cleaved by proteases into two isoforms 5a and 5b which activate its phosphatase activity (39, 40).

Osteoclasts are responsible for bone resorption. Their cytoplasm contains a well-developed endoplasmic reticulum, a large Golgi apparatus, and many mitochondria and lysosomes. Osteoclasts are activated when they become attached to the bone matrix and form sealing zones and polarized ruffled membranes. They possess a vacuolar H^+-ATPase pump directed by a special membrane subunit delimited by a tight integrin attachment (section 4.1.) that controls the acid secretion and the mineral resorption lacuna [for review see (40)]. Osteoclasts also synthetize and secrete proteolytic enzymes such as matrix metalloproteinases (MMP) and cathepsin K, that can break down organic matrix proteins (40, 41). Degraded bone matrix products are transcytosed in vesicles to the free surface of osteoclasts and released (1).

3.2. Bone remodeling

Bone mass is regulated by bone growth and remodeling. Bone growth occurs during childhood and adolescence, while bone remodeling is a life-long process mediated by osteoclasts and osteoblasts (42). Bone remodeling is influenced by physical activity, body weight, hormones and nutritional factors including Ca^{2+} and vitamin D (1,25-dihydroxycholecalciferol) (42, 43). Bone remodeling has five distinct phases: 1) quiescence, 2) preosteoclast recruitment and osteoclast differentiation, 3) bone resorption, 4) preosteoblast recruitment and osteoblast differentiation and 5) bone formation (Figure 3) (26). At the beginning of this remodeling cycle, the quiescent bone surface is covered by inactive osteoblasts (44). The preosteoclasts then migrate to the remodeling sites where they differentiate into mature osteoclasts (26). These osteoclasts break down the bone matrix at resorption lacuna (44). The osteoclasts become detached from this lacuna once resorption is complete and die by apoptosis. Then, preosteoblasts move into the resorption lacuna, where they differentiate into mature osteoblasts. Finally, the osteoblasts synthesize a new bone matrix to fill the lacuna (26, 44).

3.3. Bone matrix

Bone has an ECM composed of organic and mineral fractions. The organic phase gives bone its flexibility, while the mineral phase provides its structure (18). The biomechanical properties of bone are a compromise between the needs for stiffness and ductility. Stiffness, the physical property of being inflexible and hard to bend, reduces strain. Ductility, the ability of bone to be deformed and absorb energy, enables it to absorb impacts and reduces the risk of fracture (18, 45).

3.3.1. Osteoid

The organic phase (22% of bone mass) is also called the osteoid or preosseous substance. It is mostly fibrillar molecules containing structural and adherent proteins plus interfibrillar molecules such as glycosaminoglycans, proteoglycans, small non-collagenous proteins and some lipids (20).

Bone is mostly composed of types I and V fibril-forming collagens (46). There are 27 collagens which have at least one helical domain located in the ECM (47). This right-handed domain is made up of three left-hand alpha helixes composed of repeated (Gly-X-Y) sequences, where X and Y are frequently Pro and hydroxy-Pro residues (Figure 4) (46, 48, 49). The presence of the smallest amino acid (Gly) at every third position allows the packing of this coiled-coil structure (47). Also, the distribution of amino acids in left-hand alpha helices has a periodicity of 18 residues per turn (46, 48). Type I collagen accounts for more than 90% of bone organic mass (46) and is generally made of two alpha1(I) chains and one alpha2(I) chain (48). Electrostatic and hydrophobic interactions between collagen molecules form fibrils with diameters of 25-400 nm (46). The longitudinal distribution of polar and

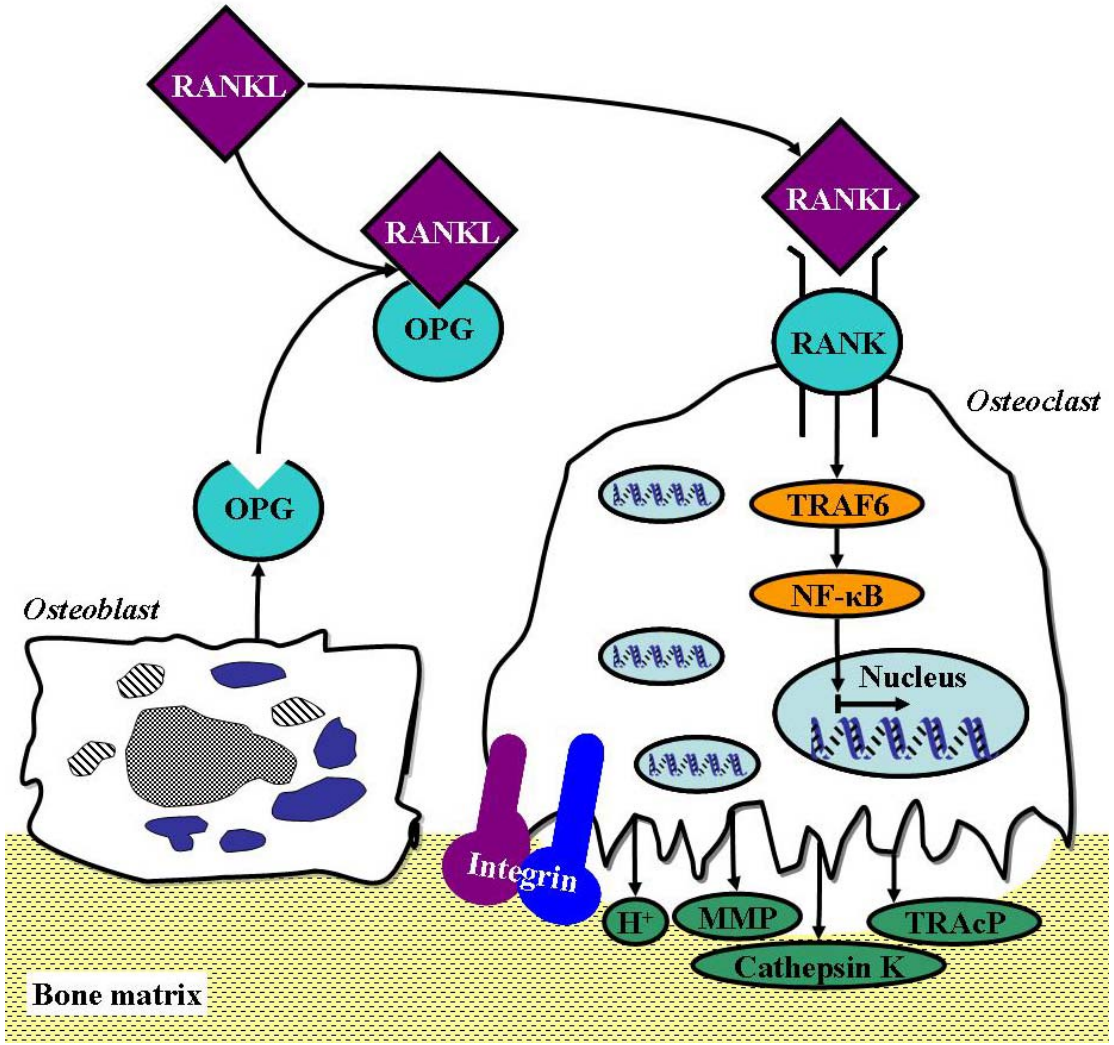

Figure 2. Osteoclast maturation is regulated by various factors, such as Receptor Activator of Nuclear Factor-kappaB (NF-kappaB) Ligand (RANKL). RANKL can exist as a soluble form and binds to the osteoclast receptor RANK. This binding activates osteoclast differentiation via the translocation of NF-kappaB into the nucleus by intermediates such as Tumor Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF6). RANKL signals can be blocked by a soluble RANKL-binding decoy receptor, osteoprotegerin (OPG), produced by osteoblasts. Vacuolar H^+-ATPase pump (H^+), matrix metalloproteinase (MMP), Cathepsin K and Tartrate-Resistant Acid Phosphatase (TRAcP) are involved in osteoclast activity. Adapted with permission from (36).

hydrophobic residues creates a characteristic banding pattern with a periodicity of 67 nm between collagen fibrils (48). Mechanical constraints determine the orientation of the collagen fibers and the subsequent bone matrix mineralization (20).

The main non-collagenous proteins in bone are osteocalcin, osteonectin and osteopontin, with osteocalcin being the most abundant (Table 1) (50). After their proliferative period, differentiating osteoblasts synthesize and secrete non-collagenous bone matrix proteins following a temporal sequence. Alkaline phosphatase is first expressed while osteocalcin and osteopontin are synthesized when the bone matrix starts to mineralize (63). Bone matrix also contains biglycan and decorin proteoglycans, which have a central protein core linked by glycosaminoglycans (60). Adhesion proteins such as fibronectin and thrombospondin are also critical for bone cell adhesion (54).

3.3.2. Mineral phase

During bone formation, each osteoblast produces 2 microns^3 ECM/day until the formation of a final layer about 10-15 microns thick (20). Mineralization begins 5-10 days after osteoid deposition (43). This process involves two steps, the nucleation of calcium phosphate crystals followed by crystal growth (48). Crystal nucleation needs saturated concentrations of Ca^{2+} and PO_4^{3-} in the interstitial fluid. This saturation is obtained through the entrapment of extracellular Ca^{2+} by osteocalcin and matrix vesicles

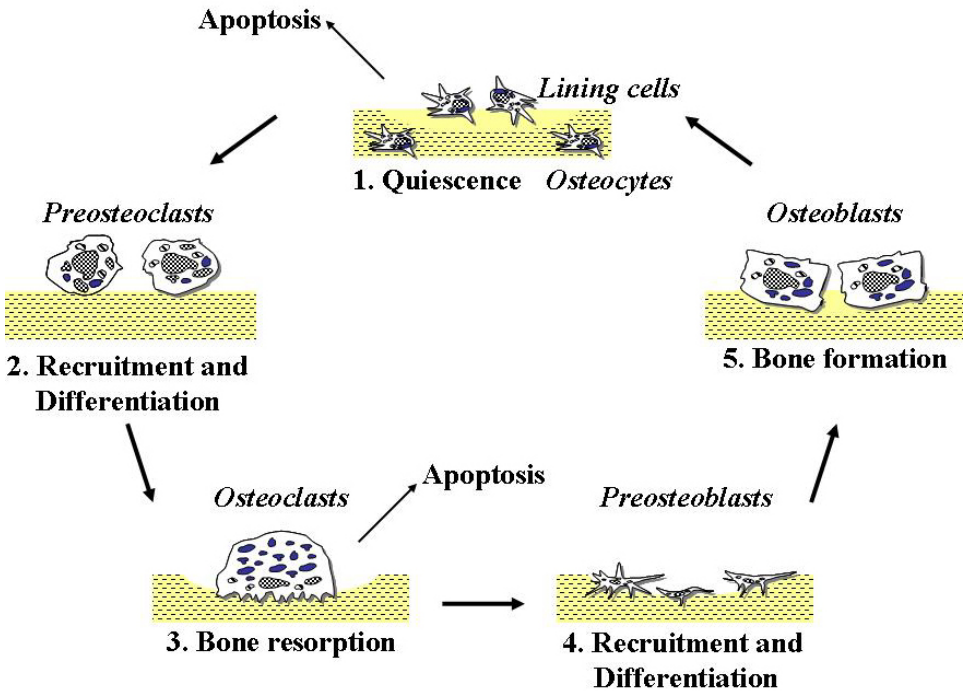

Figure 3. Bone remodeling has five distinct phases: 1) quiescence, 2) preosteoclast recruitment and osteoclast differentiation, 3) bone resorption, 4) preosteoblast recruitment and osteoblast differentiation and 5) bone formation. Adapted with permission from (26).

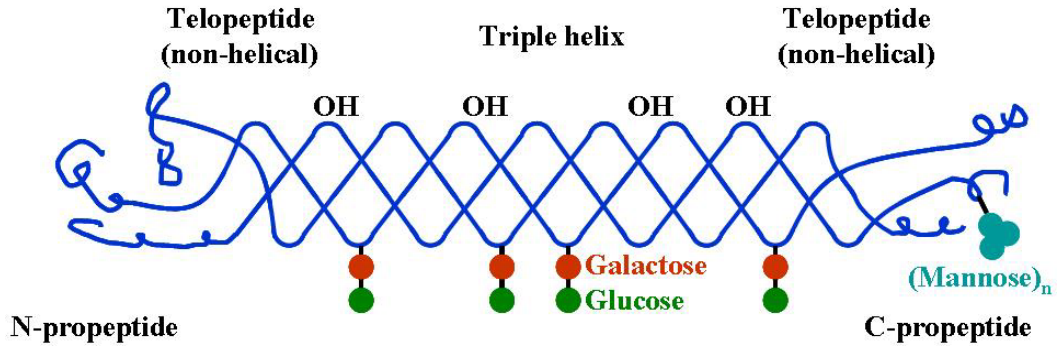

Figure 4. Structure of fibril-forming collagen. Adapted with permission from (46).

containing alkaline phosphatases, which accumulate both Ca^{2+} and PO_4^{3-} (20). While Ca^{2+} and phosphorus produce many thermodynamically unstable salts during bone mineralization, the formation of stable hydroxyapatite (HAP) crystals is favored. The 3D structure of HAP provides a large interface area (2 m^2/gram of crystal) between HAP crystals and the interstitial fluid (20). The parallel gaps between collagen fibers are filled by inorganic crystals during crystal nucleation at multiple and independent sites across a large volume of collagen fibers (48, 64). These crystals grow up to form thin plates (4 nm thick), which frequently exceed the length of these 67 nm gap zones. Crystal growth causes collagen fibrils to merge (48). The mineralization of fibril-forming collagens gives bone its biomechanical properties of load-bearing, tensile strength and torsional stiffness (Figure 5) (46).

3.4. Regulation of bone cell behavior

3.4.1. Osteoblasts

The behavior of osteoblasts is regulated by specific factors including cytokines (proteins usually released by or involved in the regulation of immune system cells), growth factors, vitamins and hormones. Cytokines such as interleukin-1 (IL-1) and TNF-alpha inhibit type I collagen production and alkaline phosphatase activity (65).

The Bone Morphogenetic Proteins (BMPs) are well known and play a crucial role in bone tissue formation. Marshall Urist observed *de novo* bone formation *in vivo* in 1965, while he was implanting demineralized bone in muscle. He discovered that BMPs gave the organic bone matrix its osteoinductive properties (66). Other growth factors, like Transforming Growth Factor beta

Table 1. Non-collagenous proteins

Proteins	Monomeric molecular weight	Effects on osteoblasts	Effects on osteoclasts	References
Specific bone proteins				
Osteocalcin	6 kDa	↓ Mineralization		50, 51
Osteonectin	35 kDa	↑ Mineralization	↑ Cell adhesion	51, 52, 53
Osteopontin	35 kDa	↑ Cell adhesion ↑ Differentiation	↑ Cell adhesion ↑ Bone resorption	51-54
Bone sialoprotein	34 kDa	↑ Cell adhesion ↑ Mineralization	↑ Cell adhesion ↑ Differentiation ↑ Bone resorption	51, 54-56
Proteoglycans				
Type I (biglycan)	42 kDa	↑ Differentiation ↑ Mineralization	↑ Differentiation due to defective osteoblasts	51, 57-60
Type II (decorin)	40 kDa	↓ Mineralization (?)		51, 60, 61
Adhesion proteins				
Fibronectin	235 – 250 kDa	↑ Cell adhesion ↑ Cell survival ↑ Differentiation ↑ Mineralization		51, 62
Thrombospondin	120 – 150 kDa	↑ Differentiation	↑ Bone resorption	51, 54

Table 2. Types I and II BMP receptors

Subfamily	BMPs	Type I receptors	Type II receptors	Signal transduction	References
BMP-2/4	BMP-2	ALK3/6	BMPRII/ActRIIA	Smad 1/5/8	
	BMP-4	ALK3/6	BMPRII/ActRIIA	Smad 1/5/8	
BMP-3	BMP-3 (osteogenin)	ALK4	ActRIIA	Smad 2/3	
BMP-7	BMP-5	ALK3/6	?	Smad1/5/8	84
	BMP-6	ALK2/3/6	BMPRII/ActRIIA	Smad 1/5/8	
	BMP-7 (OP-1)	ALK2/3/6	BMPRII/ActRIIA	Smad 1/5/8	
	BMP-8A (OP-2) BMP-8B	ALK3/6 (?)	BMPRII/ActRIIA (?)	Smad 1/5/8 (?)	85, 86
CDMP	BMP-12 (GDF-7/CDMP-3)	ALK3/6	BMPRII/ActRIIA	Smad 1/5/8	
	BMP-13 (GDF-6/CDMP-2)	ALK3/6	BMPRII/ActRIIA	Smad 1/5/8	
	BMP-14 (GDF-5/CDMP-1)	ALK3/6	BMPRII/ActRIIA	Smad 1/5/8	
Others	BMP-9 (GDF-2)	ALK1	BMPRII/ActRIIA	Smad 1/5/8	87
	BMP-10	ALK1/3/6	BMPRII/ActRIIA	Smad 1/5/8	87
	BMP-11 (GDF-11)	ALK4	ActRIIA/ActRIIB	Smad2/3	
	BMP-15 (GDF-9)	ALK5	BMPRII	Smad2/3	

ActR: Activin receptor, ALK: Activin receptor-like kinase, BMP: Bone Morphogenetic Protein, BMPRII: Bone Morphogenetic Protein type II Receptor, CDMP: Cartilage Derived Morphogenetic Protein. Adapted with permission from (83)

(TGF-beta), Insulin like Growth Factor (IGF), Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF), all regulate bone regeneration (67). Growth factors also activate the adhesion, migration, proliferation, differentiation or apoptosis of cells (68-70). Growth factors are enclosed in the bone and are released locally to regulate cell activity. For example, there is 1 – 2 µg BMPs in a kg of demineralized bone matrix (71-73). More than 20 BMPs have been identified to date (74). These molecules, which can be produced by osteoblasts, are members of the TGF-beta family. BMPs are implicated in many biological events, such as cell proliferation and differentiation, embryogenesis, angiogenesis, inflammation and tissue repair (5, 75-79). While the actions of recombinant BMPs in bone repair have been well studied, they are expensive to use because of high production costs using *E. coli* bacteria or Chinese hamster ovarian cells (67, 73).

The effects of 14 human BMPs (BMP-2 to BMP-15) have been studied *in vitro* and *in vivo*. Each BMP was produced by cells transfected with a recombinant Adenoviral vector encoding BMP (AdBMP) (80, 81). BMP-2, BMP-6 and BMP-9 increased the alkaline phosphatase activity of human pluripotent mesenchymal stem cells *in vitro* (80). These growth factors also generated the greatest alkaline phosphatase and osteocalcin responses in human C2C12 cells *in vitro*, while BMP-4 and BMP-7 were less potent. BMP-3 is an antagonist of most BMPs and inhibits this response (80, 81). The osteogenic potential of BMPs has been verified *in vivo* by injecting transformed C2C12 cells with AdBMPs into mouse quadriceps muscles. Kang *et al.* found that the most efficient and quick ossification was obtained with BMP-6 or BMP-9 followed by BMP-2 and BMP-7 (81).

Like other members of the TGF-beta family, BMPs act on cells by inducing the formation of an heterotetrameric complex composed of two type I and two type II Ser/Thr kinase receptors (Table 2) (77, 82). A total of 7 type I receptors and 5 type II receptors have been identified to date. They can bind over 30 TGF-beta family ligands (68, 69) and all have similar structures (70). They have a short extracellular domain containing a Cys residue,

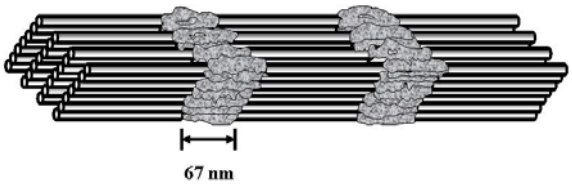

Figure 5. Mineralization of collagen fibers. The parallel gaps between these fibers are filled by inorganic crystals that nucleate at multiple sites and grow up to form thin plates, which frequently exceed the length of these 67 nm gap zones. Adapted with permission from (64).

a single transmembrane domain, an intracellular domain containing a Ser/Thr kinase and an intracellular domain containing a succession of Gly-Ser domains in type I receptors. Type I receptors bind to the central section of the BMP dimer (near alpha helixes), while type II receptors bind to the convex section of the BMP beta sheets (68). Type II receptors phosphorylate one of the Ser residue in the intracellular domain Gly-Ser of each type I receptor. BMPs interact with the complex formed by type I and type II receptors, but bind less well to type I or type II receptors alone (82). BMP-2 interacts with the type I receptors ALK-2 (ActR-I), ALK-3 (BMPR-IA) and ALK-6 (BMPR-IB), and the type II receptors BMPR-II, ActR-IIA and ActR-IIB (69, 82, 88). The amino acids in receptors BMPR-IA and BMPR-IB are 83% homologous (70).

There are two pathways involved in BMP signaling (Figure 6), the well-known Smad pathway and a pathway involving TGF-beta1 Activated tyrosine Kinase 1 (TAK1) and Mitogen Activated Protein Kinase (MAPK) (89). The Smad pathway is induced by the phosphorylation by type I receptor of two Ser residues in the motif Ser-Ser-X-Ser of the C-terminating region of an intracellular protein called Receptor-regulated (or pathway-restricted) Smad (R-Smad), made up of Smad 1, Smad 5 and Smad 8 (88, 91). Two phosphorylated R-Smad molecules form a heterotrimeric complex with Smad 4, a Common-partner Smad (Co-Smad). This complex is translocated into the nucleus directly or by forming a complex with other proteins such as transcription factors (TF) in order to activate the transcription of target genes coding for early markers of the osteoblast phenotype (parathormone receptor, alkaline phosphatase, type I collagen, osteopontin, osteonectin), or late markers of the osteoblast phenotype (bone sialoprotein, osteocalcin) (20, 69, 91, 92). The Smad signal pathway is regulated by the binding of Smad 6 or Smad 7, also called Inhibitory Smad (I-Smad), to type I receptors via the intracellular domain (90). This stable binding prevents the formation of Smad complexes (91). The mechanism by which TAK1 is activated by type I receptors is still unknown. The X-linked inhibitor of apoptosis (XIAP) might mediate the signal transduction between BMP receptor and TAK1 (89).

3.4.2. Osteoclasts

Soluble cytokines, growth factors and calciotropic factors can also modulate the differentiation and functions of osteoclasts (Figure 7) (36). These regulations vary widely with ages and species (38). Several studies have shown that an essential direct factor in osteoclast differentiation is RANKL, a member of the TNF ligand superfamily of cytokines that binds to its signal transducing receptor RANK (36, 38). But this cytokine can be replaced, or its effect can be increased, by the activation of other TNF-family receptors, such as the TNF-alpha receptor. By contrast, several other cytokines including interferon gamma (IFNgamma) and IL-4 interfere with the ability of RANKL to induce osteoclast differentiation. Other interleukins such as IL-6 and IL-11 increase osteoclast generation and may replace TNF-family signals. These ILs use a co-receptor, glycoprotein 130 (GP130), to transduce their intracellular signaling, primarily through Jak/Stat pathway.

Vitamins also influence osteoclast behavior. For example, the active metabolite of vitamin D binds to a nuclear homolog of the estrogen receptor alpha, causing it to interact with the retinoid X receptor to enhance osteoclast formation *in vitro* (93). The growth factor TGF-beta can also replace RANKL in medium containing serum. It functions through its binding to the TGF-beta receptor and the subsequent activation of Smad 2 and/or Smad 3, also called Activin/TGF-beta-specific Receptor-regulated Smad (AR-Smad). These AR-Smads can interact with various downstream regulatory proteins such as the vitamin D receptor, Jun kinase and NF-kappaB (36).

Recent studies have also shown bisphosphonates to be inhibitors of the osteoclast recruitment and differentiation, since they can induce osteoclast retraction and apoptosis (94). Bisphosphonates bind strongly to mineral crystals and can inhibit ruffled border formation, thereby preventing bone resorption (94).

4. CELL ADHESIONS

Interactions between cells and biomaterials play a crucial role in tissue integrity and repair. They influence cell survival, proliferation, differentiation and migration. The integrins are the main cell receptors involve in the adhesion of cells to biomaterials (95).

4.1. Integrins

Cell surface integrins interact with biomaterials through their extracellular ligands, which can be adhesion proteins adsorbed from the serum, or ECM proteins secreted by the cells (96). These interactions can also involve adhesion peptides that have been grafted onto the surface of biomaterials (96, 97). The critical importance of ECM ligands, integrin receptors and signaling events is underscored by the sure death in early embryonic development of animal models with targeted integrin gene deletions (95). The specific types of integrins used by cells to interact with the ECM seem to be matrix specific (98). The most common binding site is the tripeptide sequence Arg-Gly-Asp (RGD). This sequence is presented in most of the bone ECM proteins, including type I collagen, osteopontin, bone sialoprotein, thrombospondin and fibronectin (95).

4.1.1. Structure

The integrins are heterodimeric alpha beta transmembrane glycoproteins. Currently, there are 18 alpha

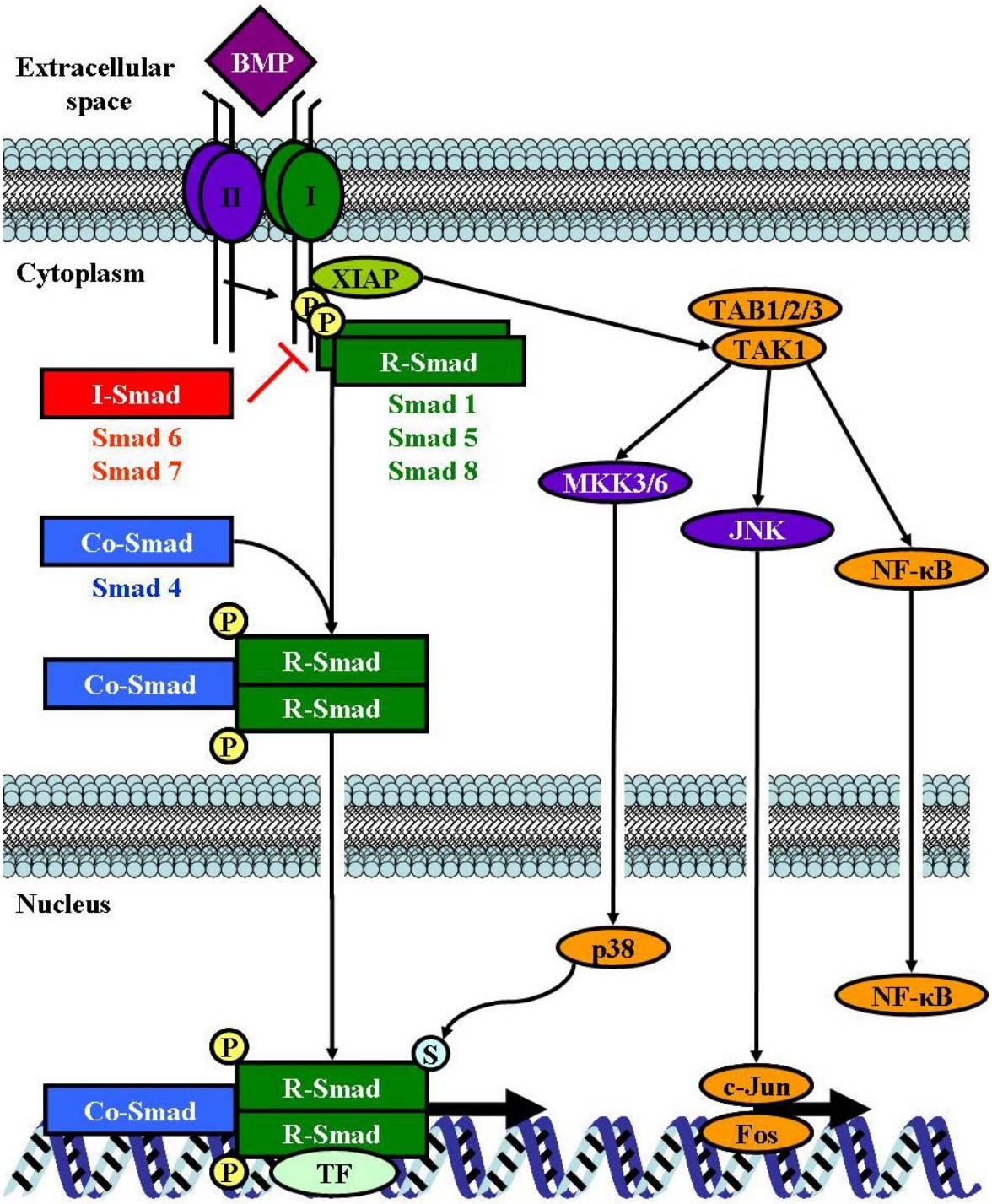

Figure 6. Bone Morphogenetic Protein (BMP) signal transduction. Smad and TGF-beta1 Activated tyrosine Kinase 1 (TAK1). The Smad pathway is induced by the phosphorylation by type I receptor of an intracellular protein called Receptor-regulated Smad (R-Smad). Two phosphorylated R-Smad molecules form a complex with a Common-partner Smad (Co-Smad). This complex is translocated into the nucleus and interacts with transcription factors (TF) to activate target genes transcription. The Smad signal pathway is regulated by Inhibitory Smad (I-Smad) that binds to type I receptors. In TAK1 pathway, the X-linked inhibitor of apoptosis (XIAP) might mediate the signal transduction and activate Mitogen Activated Protein Kinase (MAPK) pathways including MKK3/6, p38, Jun-N terminus Kinase (JNK) and Nuclear Factor-kappaB (NF-kappaB). Adapted with permission from (89, 90).

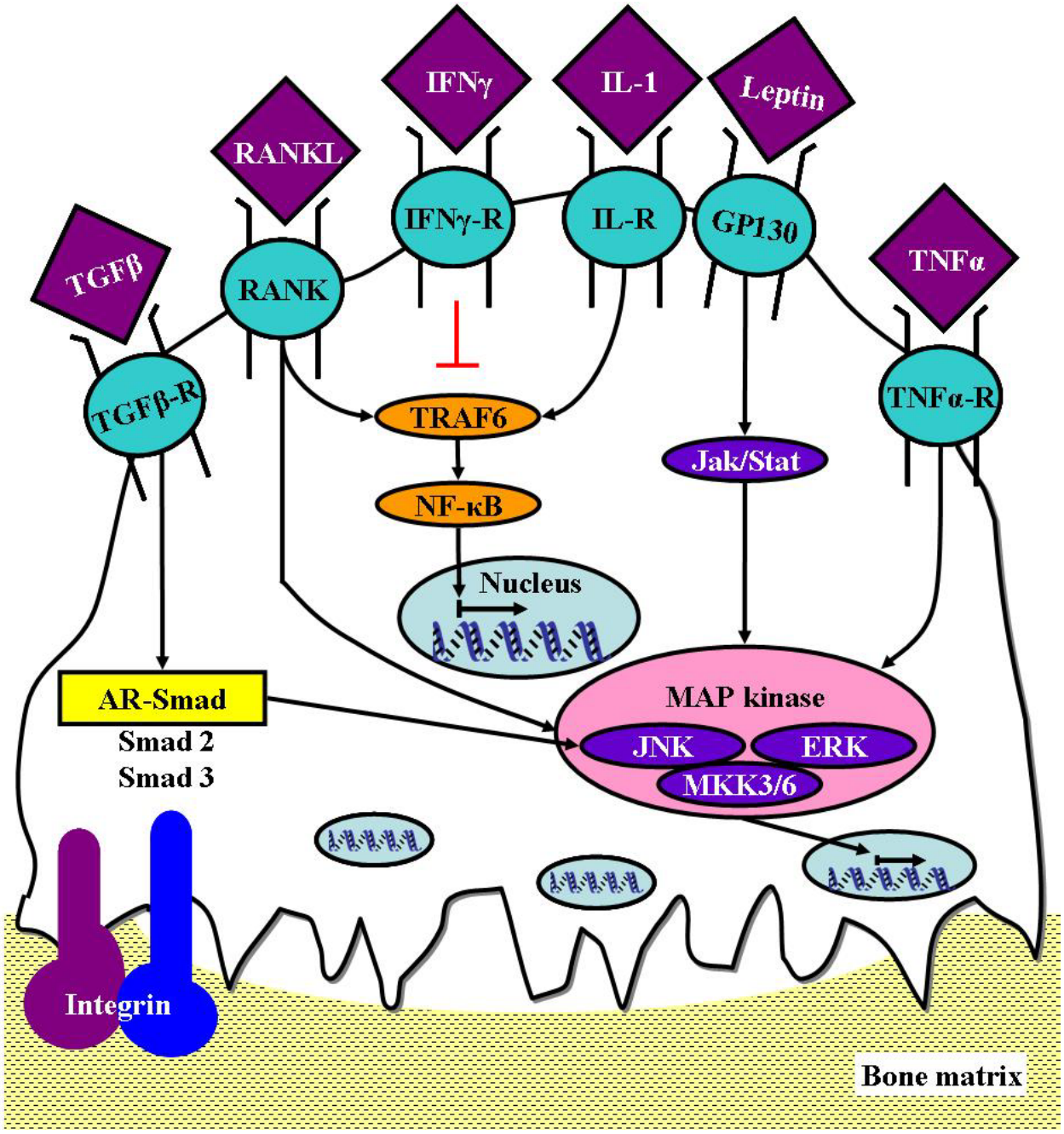

Figure 7. Osteoclast differentiation and functions are influenced by soluble cytokines and growth factors. AR-Smad: Activin/TGF-beta-specific Receptor-regulated Smad, ERK: Extracellular signal Regulated Kinase, GP: glycoprotein, IFN: interferon, IL: interleukin, JNK: Jun-N terminus Kinase, MAPK: Mitogen Activated Protein Kinase, NF-kappaB: Nuclear Factor-kappaB, RANKL: Receptor Activator of NF-kappaB Ligand, TGF: Transforming Growth Factor, TNF: Tumor Necrosis Factor, TRAF6: TNF Receptor-Associated Factor 6. Adapted with permission from (36).

and 8 beta subunits known to assemble to form 24 distinct integrins. Each of these integrins appears to have a specific nonredundant function. For example, gene knock-out experiments for each of the beta subunits produced a distinct phenotype (95). The overall shape of integrins deduced from the X-ray crystal structure of alpha v beta 3 integrin reveals a globular head connected to "rod-like legs" (Figure 8) (99, 100). Each integrin subunit also contains a flexible region called the "knee" which is involved in the active state of the integrin. The alpha and beta integrin subunits are not covalently bound together. The upper surface of the alpha integrin subunit head is formed by a 7-bladed beta propeller in close juxtaposition with a von Willebrand factor type A domain (betaA

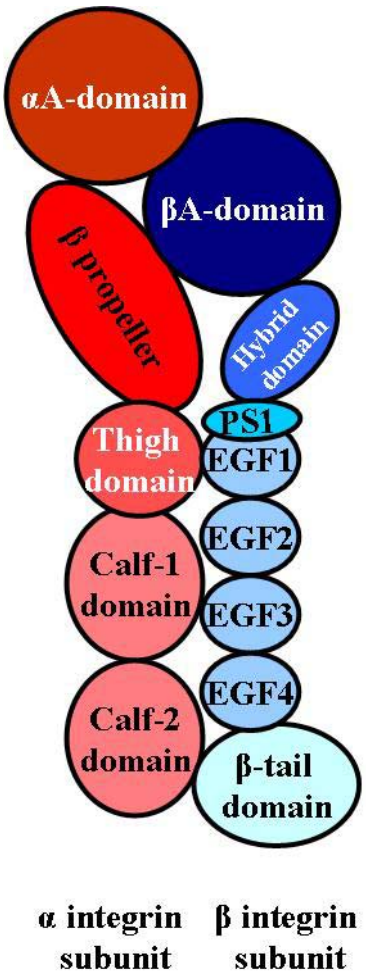

Figure 8. Schematic structure of alpha and beta integrin subunits deduced from X-ray crystallography. Adapted with permission from (99).

domain) in the beta integrin subunit (99, 100). The juxtaposition of the betaA domain, which contain a metal ion dependent adhesion site called MIDAS, and the beta propeller is the site of the ligand binding pocket. Interactions between the alpha and beta cytoplasmic tails are also critical for stabilising the association between the leg regions of these integrin subunits (101). The affinity of integrins is regulated by a mechanism that converts conformational changes from the cytoplasmic tails to head domains (101). Breaking the interactions between the alpha and beta integrin subunits, via talin binding to beta integrin subunits for example, allows repositioning of the head region to point away from the cell surface. The cytoplasmic domain of beta integrin subunits is therefore a major factor in establishing connections between the ECM and the cytoskeleton. These cytoplasmic domains contain one or two conserved Asn-Pro-X-Tyr or Asn-Pro-X-Phe motifs that interact with phosphotyrosine proteins (102, 103). The transmembrane and extracellular domains of alpha subunits are also important for the recruitment of signaling molecules such as the insulin receptor substrate (Shc) to integrins (104).

4.1.2. Integrin expression by osteoblasts and osteoclasts

Several studies have described the expression of integrins by osteoblasts and osteoclasts (105-107). Osteoblasts can bear alpha 1, alpha 2, alpha 3, alpha 4, alpha 5, alpha 6, alpha v, beta 1, beta 3 and beta 5 integrin subunits. But the synthesis of integrins depends on osteoblast differentiation (108, 109). Bennett *et al.* used immunohistochemistry to demonstrate that alpha 2, alpha 3, alpha 5, alpha v, beta 1 and beta 3 subunits are present on osteoblasts, but that alpha 2 is restricted to cells close to the bone surface and alpha v beta 3 integrins are most frequently located on osteocytes (109). The interaction of integrins with the ECM can affect signal transduction, the activation of transcription factors and the expression of specific genes in osteoblasts (105, 110).

Osteoclasts contain alpha 2, alpha 5, alpha v, beta 1 and beta 3 integrin subunits. The alpha v beta 3 integrin is the major functional receptor on osteoclasts. It can bind to osteopontin and bone sialoprotein (106) (Table 3).

4.2. Types of cell adhesions

4.2.1. Osteoblasts

Osteoblasts can adhere to biomaterials in several ways in 2D systems *in vitro*. Integrin-mediated adhesions are molecularly heterogeneous, appearing as focal complexes, focal adhesions and fibrillar adhesions. Focal complexes are the main precursors of focal adhesions (118). They are characterized by the recruitment of vinculin and phosphoproteins and exert forces of between 1 and 3 nN/micron^2 (119). Both focal and fibrillar adhesions are involved in the processes of cell adhesion and ECM formation (120), but the morphology, size and subcellular distributions of these cell adhesions differ (118).

Focal adhesions are elongated streak-like structures (3-10 microns) that are often located at the cell periphery (121, 122). They anchor bundles of actin stress fibers (F-actin) through a plaque made up of many proteins, including alpha v beta 3 integrins and structural proteins like vinculin and talin. Signaling proteins, especially phosphotyrosine proteins such as Focal Adhesion Kinase (FAK), are directly coupled to integrin receptors clustered in focal contacts (Figure 9) (123). The clustering of integrins causes the rapid phosphorylation of the nonreceptor tyrosine kinase FAK at Tyr^{397}, as well as at two residues in the catalytic loop of the kinase domain, Tyr^{576} and Tyr^{577} (124). FAK phosphorylated on Tyr^{397} can then interact with proteins like the Src-family kinases. Such kinases phosphorylate two proteins that interact with FAK, Crk Associated Substrate (CAS) and paxillin (125). Paxillin is a 68 kDa cytoskeletal protein with no intrinsic enzymatic activity. This focal adhesion protein contains many Tyr and Ser/Thr phosphorylation sites and is believed to be a modulator of both cell adhesion and growth factor signaling pathways (126-128). Integrin-linked kinases that can interact with beta 1 integrin subunits and paxillin also have a central function in integrin signaling (129).

Fibrillar adhesions have been discovered only recently (Figure 9) (113-115). Luthen *et al.* demonstrated the presence of fibrillar adhesions in mouse MC3T3-E1

Table 3. Expression of integrin subunits in osteoblasts and osteoclasts

beta integrin subunits	alpha integrin subunits	Bone integrin ligands	Functions	References
beta 1	alpha 1	Type I collagen	↑ Osteoblast differentiation induced by BMP-2	111
	alpha 2	Type I collagen		
	alpha 3	Fibronectin, Thrombospondin		
	alpha 4	Fibronectin		
	alpha 5	Fibronectin	Fibrillar adhesion formation	112-115
	alpha 6	Laminin		
	alpha v	Fibronectin (vitronectin for cultured osteoblasts)		
beta 3	alpha v	Bone sialoprotein, fibronectin, osteopontin, thrombospondin, vitronectin	Osteoblast adhesion (*in vitro*) Focal adhesion formation in osteoblasts Major integrin in osteoclasts	38, 106, 116, 117
beta 5	alpha v	Vitronectin	Osteoblast adhesion (*in vitro*)	

BMP: Bone Morphogenetic Protein. Adapted with permission from (108)

Table 4. Principal adhesion components in 2D and 3D systems

Proteins	Functions	Focal adhesion	Fibrillar adhesion	3D-matrix adhesion	References
Receptors					
alpha 5 beta 1 integrins	Cell-fibronectin interactions	+/-	+	+	
alpha v beta 3 integrins	Cell-ECM interactions (vitronectin, fibronectin)	+	-	-	
Structural proteins					
Talin	Integrin-associated protein Integrin activation/F-actin binding Regulator of inside-out signaling Target vinculin to focal adhesion	+	+	+	135, 136
Vinculin	Integrin-associated protein Ubiquitous actin binding protein Anchoring F-actin to the cell membrane	+	-	+	137, 138
Tensin	Integrin-associated protein F-actin binding Role in signal transduction	-	+	+	
alpha-actinin	F-actin linker	+	+/-	+	
Signaling proteins					
FAK	Non receptor tyrosine kinase Role in signal transduction leading to cell cytoskeletal organization and cell spreading	+	-	+	124
Phospho-FAK Tyr^{397}	Interactions with different signaling effectors containing SH2 domains (Src family kinase) Rac and Rho regulation MAPK activation Focal adhesion strength modulation	+	-	-	139-142
Paxilin	Tyr and Ser/Thr protein Adaptor protein involved in integrin signaling	+	-	+	126, 128, 143
Cytoskeletal proteins					
F-actin	Structural component	+	+	+	

ECM: extracellular matrix, F-actin: Filamentous actin, FAK: Focal Adhesion Kinase, MAPK: Mitogen Activated Protein Kinase, SH2: Src-homology 2. Adapted with permission from (134)

preosteoblasts attached to a Ti surface (130). In comparison to focal adhesions, fibrillar adhesions are more centrally located and consist of extracellular fibronectin fibrils, alpha 5 beta 1 integrin receptors and the tensin. Tensin is a phosphotyrosine cytoplasmic protein involved in signal transduction (131). The translocation of fibrillar adhesions is highly directional, proceeding centripetally from the cell periphery towards the center and is always aligned along the long axis of the focal contacts. Multi-ligand alpha v beta 3 integrins remain within focal adhesions, while alpha 5 beta 1 integrins are translocated at 6.5 microns/h parallel to the actin microfilaments in fibrillar adhesions (112). The formation of fibrillar adhesions is closely linked to the capacity of cells to polymerize fibronectin to form ECM fibrils (112). Cell migration is also strongly linked to the assembly and disassembly of adhesions that requires a certain mobility of integrins (132, 133). Both focal and fibrillar adhesions have been described in osteoblasts on 2D surfaces. Nevertheless, Zaidel-Bar *et al.* used fibroblasts to demonstrate a considerable difference between components in 2D and 3D adhesions (Table 4) (118).

4.2.2. Osteoclasts

Unlike osteoblasts, osteoclasts adhering to a substratum, such as glass, form a unique type of matrix adhesion known as a podosome (Figure 10) (144). Podosomes are small (1 micron in diameter) and have a dense actin core surrounded by a rosette-like structure

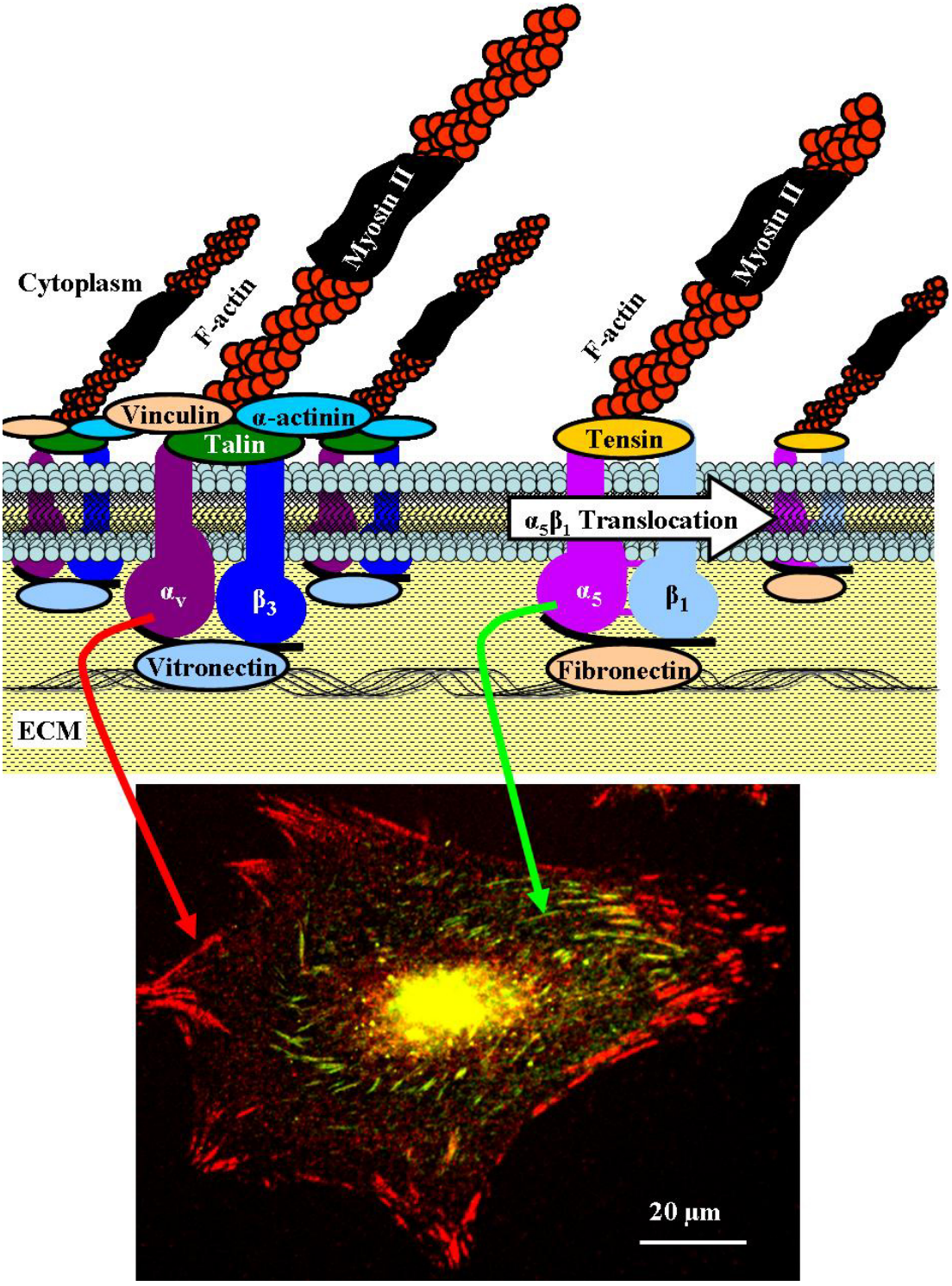

Figure 9. Organization of focal and fibrillar adhesions. Focal adhesions are elongated streak-like structures located at the cell periphery that anchor bundles of actin stress fibers (F-actin) through many proteins, including alpha v beta 3 integrins and structural proteins like alpha-actinin, vinculin and talin. Fibrillar adhesions are more centrally located and consist of extracellular fibronectin, alpha 5 beta 1 integrin receptors and tensin. The translocation of fibrillar adhesions (Fibronectin-alpha 5 beta 1-tensin complex) is highly directional, proceeding centripetally from the cell periphery towards the center. Double immunolabeling for alpha v (red) and alpha 5 (green) in cells attached to fibronectin revealed the segregation between focal and fibrillar adhesion. ECM: extracellular matrix.

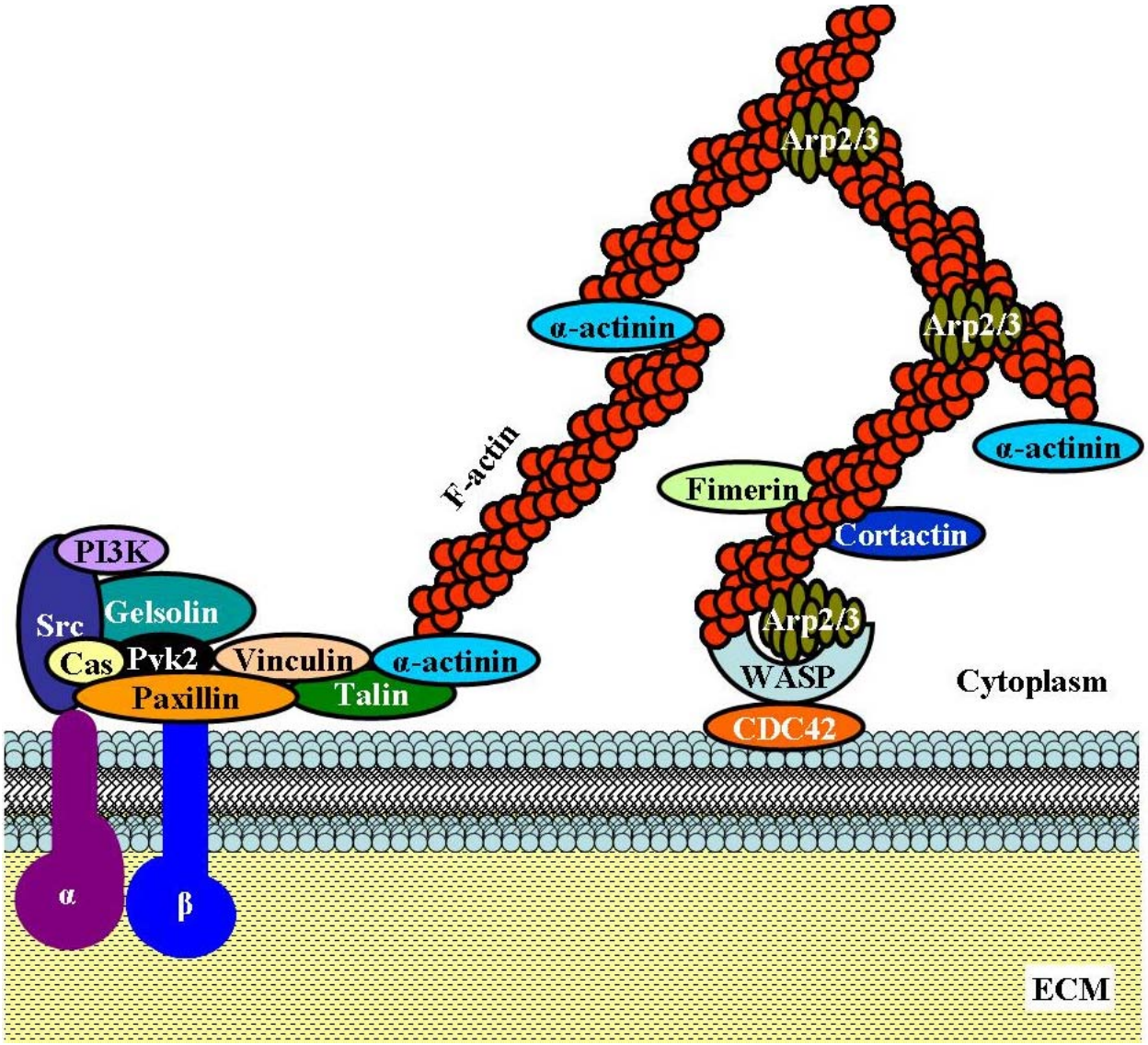

Figure 10. Osteoclast podosome architecture. Podosomes have a dense actin core surrounded by a rosette-like structure containing proteins such as alpha v beta 3 integrins, structural focal adhesion proteins (talin and vinculin), actin-associated proteins (gelsolin, alpha-actinin and actin-related protein 2/3 (Arp2/3)), tyrosine kinases (Src, Pyk2 and phosphoinositide-3 kinase (PI3K)) and RhoGTPases (Cell Division Control protein 42 (CDC42)) (38, 117). The podosome core also contains large amounts of proteins involved in actin polymerization; WASP: Wiskott-Aldrich Syndrom Protein. Adapted with permission from (145).

containing proteins such as alpha v beta 3 integrins, structural focal adhesion proteins (talin and vinculin), actin-associated proteins (gelsolin and alpha-actinin), tyrosine kinases (c-Src and Pyk2) and RhoGTPases (Cell Division Control protein 42 (CDC42) and Rho) (Table 5) (38, 117). For example, the binding of osteopontin to alpha v beta 3 integrins on osteoclasts triggers the c-Src-dependent phosphorylation of Pyk2, a member of the FAK family (117, 145). Pyk2 is a major adhesion-dependent tyrosine kinase both *in vivo* and *in vitro*. It is involved in the formation of the sealing zone and favors the recruitment of cytoskeletal proteins such as vinculin and gelsolin (146). The podosome core also contains large amounts of proteins involved in actin polymerization. Podosomes undergo a major reorganization during the maturation of osteoclasts. They become organized in ordered clusters forming belt of podosomes (147). Microtubules, made up of alpha- and beta-tubulin heterodimers in filamentous networks, also regulate the podosome organization at the end of osteoclast differentiation. Microtubules are colocalized with the upper portion of actin dots in osteoclasts on glass coverslips or calcified materials (148).

In vivo, polarized osteoclasts attached to the ECM mineral phase have three distinct areas. The first, which is not in contact with the bone mineral, is the basolateral

Table 5. Principal components of osteoclast podosomes

Proteins	Functions	Localization
Receptors		
Mainly alpha v beta 3 integrins	Cell-ECM interactions	Core + ring
Structural proteins		
alpha-actinin	F-actin linker	Core + ring
Cortactin	F-actin linker Arp 2/3 complex	Core
Gelsolin	Uncapping of F-actin	Core + ring (?)
Talin	Integrin-associated proteins	Ring
Vinculin	Integrin-associated proteins	Ring
Signaling proteins		
Pyk2	Major adhesion-dependent tyrosine kinase Activator of structural protein recruitment (vinculin, gelsolin)	Ring
Paxillin	Tyr and Ser/Thr protein Adaptor proteins involved in integrin signaling	Ring
Actin regulators		
WASP	Activator of Arp 2/3 actin nucleating activity	Core
Arp2/3 complex	Actin nucleation	Core
Motor protein		
Myosin	Actin-based motor	Ring

Arp: Activator of actin-related protein, ECM: extracellular matrix, WASP: Wiskott-Aldrich Syndrom Protein. Adapted with permission from (145).

membrane. The second is the sealing zone, which is closely apposed to the bone surface. And the third is the ruffled border that faces the resorbing site (149). The sealing zone consists of a central actin belt surrounded by rings of integrins and focal adhesion structural proteins. Sealing zones (4 micron wide and 4 micron thick) and podosomes seem to have the same components. Sealing zone is made of structural units related to individual podosomes which differ mainly in actin intensity associated with the belt. Indeed, clusters podosomes seem able to condense in a sealing zone-like structure. Nevertheless, the architecture of osteoclast adhesion sites has not been elucidated at high resolution. Thus, the spatial and temporal relations between podosomes and the sealing zone structures remain controversial (147). Both the sealing zone and the pososome belt are stabilized by an increase in microtubule acetylation that is regulated by the Rho-mammalian Diaphanous-Related Formins-2/histone deacetylase-6 (Rho-mDRF2/HDAC6) pathway (150). The resorption/migration cycles of osteoclasts are associated with repeated 3D depolarization/polarization cycles of the sealing zones (144).

4.3. Signal transduction for osteoblasts and osteoclasts

Many inactive integrins can be present on the surface of cells. In this state, they do not bind to ligands and do not transduce signals (Figure 11 A, B and C) (95, 151). The affinity of an integrin and its avidity are two distinct features of integrin activation. The affinity regulates the initial interaction of an integrin with its ligand, while its avidity is due to the clustering of these receptors. The cytoplasmic domain of the beta subunit can interact with cytoplasmic proteins to stabilize the active state of integrins bound to ECM proteins. Integrins have no enzymatic activity and signals are transduced by the clustering of the receptors and the subsequent recruitment of intracellular signaling proteins such as FAK or Pyk2 (126, 152). In addition, homo-oligomerization of the alpha and beta transmembrane domains can occur to stabilize the clustering of the receptors.

The integrins are therefore important for the regulation of other pathways, in addition to their architectural function. They transmit mechanical stresses across the plasma membrane, so cytosolic signal transduction is initiated when they bind to extracellular ligands (outside-in signaling), but their binding affinity is also regulated intracellularly (inside-out signaling) (133). Two major pathways, the Extracellular signal Regulated Kinase (ERK) from the MAPK pathway and the Rho family GTPases, are also involved in these mechanisms (153). The ERK MAPKs are the most extensively studied subfamily of MAPKs. The MAPK family can be divided into three groups depending on the motif in their activation loops. The ERK/MAPKs have a Thr-Glu-Tyr motif, the p38 proteins have a Thr-Ala-Tyr motif and the Jun-N terminus Kinases (JNK) have a Thr-Pro-Tyr motif [for review see (154)].

4.3.1. Mechanical stress

Two mechanisms could be involved in the transduction of mechanical stress through integrins. The force applied to integrins might result in more integrin cluster formation and cytoskeletal rearrangement following protein recruitment. Tension may also alter some components of focal adhesions and modulate the activities of intracellular enzymes (119). For example, steady fluid flow increases the synthesis of the beta 1 integrin subunits by human osteoblasts (155) and activates alpha v beta 3 integrin (156). Nevertheless, the exact molecular pathway activated by shear stress and mediated by integrins in bone cells is not fully understood. FAK is believed to be a key factor in the integration of mechanical signals (119). Cyclic strain can induce the phosphorylation of the Tyr in FAK and paxilin; this is mediated by stretch-induced c-Src (157-159). In addition, FAK must be phosphorylated for stretch-induced activation of ERK and p38 (160). MAPK pathway is believed to be an important transducer of oscillatory stress in bone cells (119, 161). Fisher *et al.* found that blocking alpha v beta 3 integrin with antibodies reduced the activation of ERK, JNK and NF-kappaB by shear stress

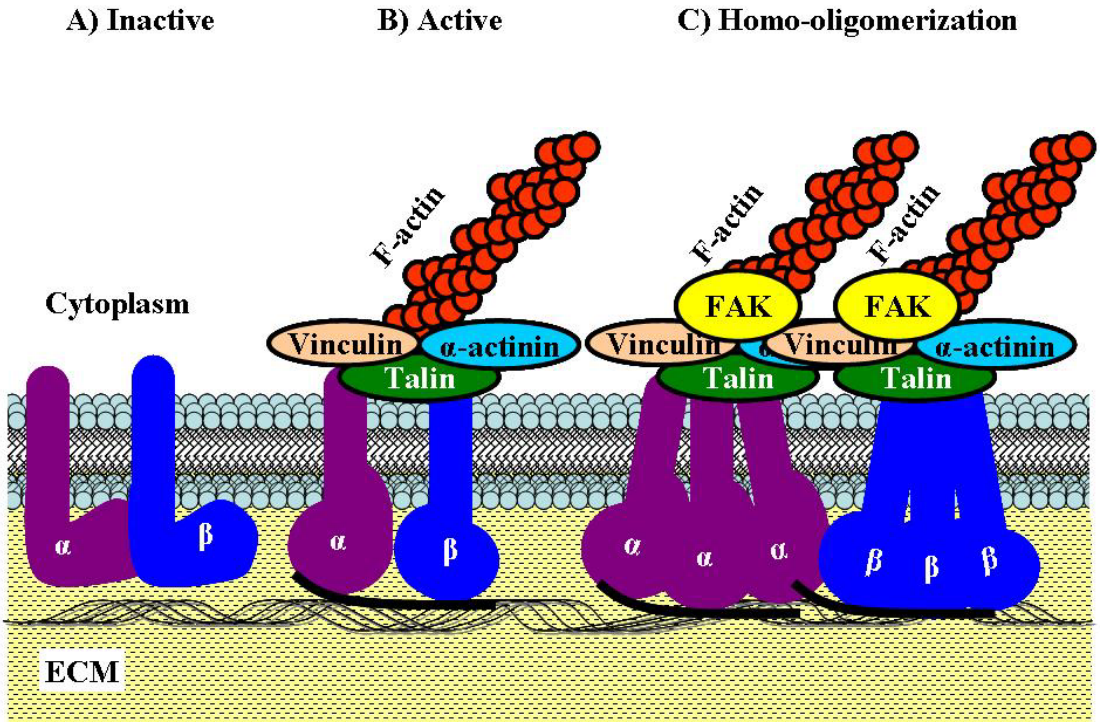

Figure 11. Schematic view of the integrin states. inactive (A), active (B) and homo-oligomerization (C). In inactive state, integrins do not bind to ligands and do not transduce signals (A). The affinity of an integrin and its avidity are two distinct states of integrin activation. The affinity regulates the initial interaction of an integrin with its ligand (B), while its avidity is due to the clustering of these receptors. In addition, homo-oligomerization of the alpha and beta transmembrane domains can occur to stabilize the clustering of the receptors (C). ECM: extracellular matrix, FAK: Focal Adhesion Kinase, F-actin: Filamentous actin. Adapted with permission from (151).

(162). The involvement of tyrosine phosphatase is also highlighted by the fact that the reinforcement of adhesion in response to physical force applied to beads coated with fibronectin is prevented by tyrosine phosphatase inhibitors (163).

4.3.2. ERK MAPK

The binding of integrins to the ECM enhances the activation and autophosphorylation of the receptor tyrosine kinase as well as the efficiency of the intracellular cascade comprising Raf-1, MEK and ERK. The ERK/MAPK cascade begins with the activation and autophosphorylation of receptor tyrosine kinase. The phosphoryl binding sites are then recognized by Src-homology 2 (SH2)-domain adaptor proteins like Growth factor receptor-bound protein 2 (Grb-2) and Shc. Grb2 can recruit son of sevenless (SOS), an exchange factor for Ras GTPase. The activation of Ras leads to the recruitment of Raf-1, the first kinase that activates MEK1 and MEK2, and hence the phosphorylation of ERK1 (p44) and ERK2 (p42). Activated ERK is translocated to the nucleus, where it can phosphorylate its various targets, including nuclear receptors and cofactors leading to gene transcription (154).

Integrin binding is necessary for the translocation of activated ERK to the nucleus. The ERK1/2 in bone stromal and osteoblast-like cells must be mechanically activated for some responses to strain (164). Takeuchi *et al.* have demonstrated that the binding of integrins to the ECM triggers the activation of MAPK, which is necessary for the differentiation of MC3T3-E1 preosteoblasts (165). But the involvement of the MAPK pathway in differentiation remains controversial, since inhibiting this pathway can also favor osteoblast differentiation (166).

4.3.3. Rho GTPases family

Stimulation of integrins by inside-out signaling involves the separation of the alpha and beta cytoplasmic tails that leads to activation of the ligand binding site in their head (95). Members of the Rho family of GTPases like Rho, Rac and CDC42 are involved in inside-out signaling in both osteoblasts and osteoclasts. A total of 22 Rho GTPases are presently known to be encoded by the human genome (167) and 8 of them (Rac1, Rac2, RhoA, RhoB, RhoG, TC10, TCL and CDC42) can be activated by receptor tyrosine kinases (168). Rho GTPases are targeted to membrane compartments by lipid modification like prenylation and they are active when they have a bound Guanosine Triphosphate (GTP). Their activation is regulated by Guanine nucleotide Exchange Factors (GEF), which promote the exchange of Guanosine Diphosphate (GDP) for GTP. In contrast, GTPase activating proteins trigger GTP hydrolysis (167).

Rho GTPases can activate two molecules, Wiskott-Aldrich Syndrom Proteins (WASP)/Verprolin-homologous proteins (WAVE) and Diaphanous-Related Formins 1-3 (DRF1-3) that are involved in actin polymerization. The WASP/WAVE proteins directly stimulate actin polymerization via an Actin-related protein 2/3 (Arp2/3) complex, while DRF1-3 activates the nucleation and extension of unbranched actin filaments, so avoiding the termination of actin polymerization by capping proteins (167). Rho GTPases can also inactivate the actin depolymerization factor, cofilin, by phosphorylation through the Rho-associated kinase (ROCK) and LIM kinase activation (167, 169, 170). Rho family GTPases like CDC 42 and Rho are also crucial for osteoclast activity (171). Rho regulates the activation of both 4-phosphate 5-kinase and phosphatidylinositide 3-kinase (PI3K), which control the concentrations of phosphatidylinositol 4,5 bisphosphate and phosphatidylinositol 3,4,5 trisphosphate. These second messengers regulate the actin cytoskeleton via their binding to gelsolin, alpha-actinin or vinculin (172). Rho can also increase the number of podosomes in mouse osteoclasts by modulating the motility of alpha v beta 3 integrin, thereby regulating the osteoclast actin ring (173). However, inhibition of Rho does not prevent podosome formation (150).

4.4. Influence of ECM-integrin interactions on cell behavior

Several studies indicate that the binding of integrins in osteoblasts or osteoclasts to the ECM is important during the development of bone or the turnover of mature bone (165, 174).

4.4.1. Cell differentiation

There must be interactions between the ECM and integrins for osteoblast differentiation (105, 110). Studies in which integrins were blocked with antibodies have shown that alpha 2 beta 1 integrin signaling is required for the induction of alkaline phosphatase in MC3T3-E1 preosteoblasts (165). Disrupting the binding of alpha 2 beta 1 integrin to collagen also prevents the expression of osteoblast-specific genes, such as osteocalcin, since alpha 2 beta 1 integrin activates Runx2 (175). Furthermore, Damsky *et al.* have shown that blocking interactions between fetal rat calvaria osteoblasts and fibronectin prevents the formation of mineralized nodules *in vitro* and delays the expression of bone-specific genes like the gene encoding osteocalcin (176).

Integrins are essential for the breakdown of bone by osteoclasts. Osteoclasts that lack alpha v beta 3 integrins are dysfunctional. The alpha v beta 3 integrins possess two locations during bone resorption: activated integrins are mainly located in the ruffled border, whereas those with a basal conformation are responsible for the adhesive properties of the sealing zone (174). Other studies have shown that treating osteoclasts with echistatin, a desintegrin, or the GRGDS peptide, which binds to alpha v beta 3 integrins, prevents osteoclast adhesion and causes attached osteoclasts to retract (177).

4.4.2. Crosstalk between the integrin and growth factor pathways

Integrins can regulate cell differentiation triggered by growth factors like those of the TGF-beta superfamily. Many osteoblast responses *in vitro* to soluble growth factors depend on the adhesion of the cells to substrata via integrins. The alpha 2 beta 1 integrin-mediated interaction with type I collagen is necessary in early osteoblast differentiation induced by BMP-2 (111). Integrins containing the alpha v subunit also seem to be essential for BMP-2 activity (178). Antibodies that specifically block alpha v beta 3 and alpha v beta 5 integrins are able to prevent the stimulation of alkaline phosphatase activity by BMP-2 in cultures of human osteoblasts (178). A recent study has shown that some growth factor receptors may directly form a complex with integrins (179). Similarly, cFms is located in the actin ring together with alpha v beta 3 integrin in osteoclasts. This association is the result of signaling pathways being activated following the binding of ligand to cFms (179).

5. INFLUENCE OF BIOMATERIAL PROPERTIES ON CELL BEHAVIOR IN 2D SYSTEMS

Bone ECM proteins are not simply structural proteins, they also mediate the physiological state of cells by modulating their immediate environment. Stimulation of the attachment, proliferation and differentiation of the bone cells depends in part on the surface properties, chemical composition, electrostatic charge, geometrical configuration, texture and roughness, of the biomaterials (180). Much of the current research on cells-biomaterials interactions has been done on osteoblasts, and little information is available on the responses of osteoclasts (181).

5.1. Bone substitutes

The biomaterials used in bone repair can be made of inorganic materials, natural polymers, synthetic polymers, or even composites (Table 6) (4, 9, 182). But not all bone biomaterials currently in use are osteoinductive (9). In addition, the major problem with bone substitutes is their mechanical properties (elastic modulus, fatigue, permeability) compared to bone (cortical bone elastic modulus is ~15 000 MPa and that of trabecular bone is ~500 MPa) (Table 7) (211).

5.1.1. Inorganic materials

Osteoconductive inorganic materials include HAP ($Ca_{10}(PO_4)_6(OH)_2$), tricalcium phosphate ($Ca_3(PO_4)_2$, TCP), porous coralline, calcium phosphate cements, octocalcium phosphate, apatite-wollastonite and bioactive glass (215). Theses ceramic materials can be produced by synthesis or by conversion from natural bone structures such as coralline (216). They can be abrasive and therefore it is important to avoid using them in uncontained defects adjacent to articular surfaces (217). HAP has a stoechiometry similar to that of the mineral phase. The Ca/P ratio is about 1.66 in the organic bone matrix (20), while it is about 1.67 for HAP and 1.5 for TCP (218). Calcium phosphate ceramics are the most commonly used in clinical applications because of their biocompatibility,

Table 6. Characteristics of biomaterials

Materials	Advantages	Disadvantages	Types	References
Inorganic Materials	Biocompatible Osteoconduction Osteointegration Similar to bone Resorbable or non-resorbable Affinity with BMPs	Osteoinduction Brittle Difficult to mold in 3D Exothermic	HAP	183, 184
			TCP	185
			Porous coralline	185
			Calcium phosphate cement	186, 187
			Octocalcium phosphate	188
			Apatite-wollastonite	189
			Bioactive glass	2
			Ti	14-16
Natural polymers	Biocompatible Osteoconduction Osteointegration Affinity for growth factors	Osteoinduction Pathogen agents transmission Difficult sterilization	Hyaluronic acid	187
			Alginate	190
			Collagen	7, 191
			Starch	192
			Chitosan	193
Synthetic polymers	Osteoconduction Osteointegration Reproductible manufacture Readily tailored controlled release properties Easy sterilization	Breakdown products Cell recognition Osteoinduction Possibility of protein denaturation by solvents or crosslinker	Oligo(PEG fumarate)	194
			Poloxamer	195
			Poly(alpha-hydroxy acids)	196
			PLA	193, 197
			PGA	198
			Poly(ortho ester)	199
			Polyanhydride	200
			Polyphosphazene	201
			Polyphosphonate	202
Composite materials	Use a variety of materials	Complex manufacturing process	Collagen – Bioactive glass	203, 204
			Collagen – HAP – Alginate	205
			Starch – Bioactive glass	206
			PLA – Chitosan	193
			PLA – PEG – HAP	207
			PLA – PEG – *p* dioxanone	8, 208
			PLGA	209
			PLGA – Bioactive glass	11
			PLGA – PEG	210

HAP: hydroxyapatite, PEG: poly(ethylene glycol), PGA: poly(glycolide), PLA: poly(lactide), PLGA: poly(DL-lactide-*co*-glycolid), TCP: tricalcium phosphate. Adapted with permission from (4, 9)

Table 7. Bone tissue and biomaterial mechanical properties

Materials	Compressive strength (MPa)	Tensile strength (MPa)	Elastic modulus (MPa)	Fracture toughness (MPa $m^{1/2}$)	References
Human cortical bone	130 – 180	50 – 151	12 000 – 18 000	6 – 8	211
Human trabecular bone	4 – 12		100 – 500		211
Human collagen	0.2 – 0.5		44 – 96		212
Bioactive glass (45S5 Bioglass)	~ 500	42	35 000	0.5 – 1	
HAP	> 400	~ 40	~ 100 000	~ 1	
Porous HAP (82 – 86%)	0.2 – 0.4		0.8 – 1.4		213
Ti			110 000		214
Co-Cr alloy			230 000		214

HAP: hydroxyapatite. Adapted with permission from (182)

osteoconductive and bioactive properties. Furthermore, they allow direct chemical binding to bone when implanted (219). However, HAP is resorbed very slowly, while TCP is broken down 10 to 25 times faster (181).

Bioactive glasses, especially 45S5, are important calcium phosphate ceramic composites. 45S5 have been approved by the USA *Food and Drug Administration* and their composition (in weight %) is 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5 (2). The presence of CaO and P_2O_5 in these glasses explains their bioactivity, defined as the ability of a material to develop a surface layer containing hydroxycarbonate apatite, similar to the bone mineral phase (2, 220). Their ionic products and surface modifications favor the proliferation and differentiation of osteoblasts, while they also stimulate the production of the principal phenotypic markers (220-226). However, 45S5 have no effect on osteoclast formation or bone resorption (227).

Because of its resistance to deformation, non-degradable Ti is also currently used in bone repair and hip prosthesis (214). For example, bone marrow cells attached to Ti fiber meshes can differentiate into osteoblasts *in vitro* (228). Nevertheless, some experiments *in vivo* have highlighted the importance to improve the osteogenic properties of Ti fiber meshes using protein or HAP coatings (14-16; 229).

5.1.2. Natural and synthetic polymers

Natural polymers are formed of polysaccharides (hyaluronic acid, alginate, starch, chitosan) or proteins (collagen, fibrin) (7, 191, 193). These polymers have osteoconductive properties, but their mechanical weakness

and their possible rapid breakdown under biological conditions limit their clinical applications.

Synthetic polymers include poly(ethylene glycol) (PEG), poloxamer, poly(alpha-hydroxy acids), poly(ortho ester), polyanhydride, polyphosphazene and polyphosphonate. For example, poly(alpha-hydroxy acids) regroup poly(glycolide) (PGA), poly(lactide) (PLA) and their copolymers of poly(DL-lactide-*co*-glycolide) (PLGA) (4, 196). These polymers have been widely used to construct porous scaffolds because of their great versatility and capacity for resorption (196). The cell behavior generated by biodegradable polymers is also affected by their chemical composition, molecular weight and crystallinity (230). Polymer degradation can lead to the release of products such as catalysts, additives, byproducts and residual monomers that can trigger an inflammatory response and affect the adhesion, survival and proliferation of cells. However, the rate at which these polymers break down can be modulated by varying the polymer ratio of PLA or PGA for example (4). Despite poor mechanical properties of PLGA polymers, recent studies have shown that such scaffolds can be produced with oriented microarchitectural features designed with initial mechanical properties comparable to those of trabecular bone (231, 232).

5.1.3. Composite materials

Composite biomaterials that combine synthetic polymers with inorganic ceramics are particularly interesting for preparing engineered scaffolds. They have adequate biological and mechanical properties. These composites can also mimic the properties and morphology of both cortical and trabecular bone (8, 205, 206, 208).

Growth factors may be added to bone substitutes to modulate cell activity and favor bone formation. Most clinical trials used growth factors of the BMPs family, especially BMP-2 (79, 80). Since growth factors have short biological half-lives *in vivo*, it is therefore important to protect these proteins from degradation using delivery systems. In addition, such systems can release low concentration of active growth factors, preventing toxic and cancerigenic effects of supraphysiological doses (67). Gene and cell therapies also allow growth factors production and release (4, 6, 8, 67, 233, 234). Many parameters influence the release of growth factors from a delivery system, such as the surface chemistry and charge of the scaffold, its geometry, volume, porosity, hydrophobicity, cristallinity, the rate at which it is degraded and the ease of manufacturing (233, 235, 236). The release of growth factors may be controlled by diffusion, external stimulation, enzymatic/chemical reaction or a combination of these (67, 233).

5.2. Surface properties

The events that lead to the integration of biomaterials into bone take place primarily at the bone-biomaterial interface. These events require the initial adhesion of cells and then their spread over the surface through the ECM proteins (230). The adhesion of cells to a biomaterial and their subsequent behaviors depend on surface properties of the biomaterial, such as topography, wettability, charge, chemistry and surface energy. These all influence the conformation, orientation and quantities of adhesion proteins like vitronectin or fibronectin that are adsorbed and thus mediate the interactions between cells and the implanted material (237, 238). Clearly, biomaterials influence the attachment, adhesion and spreading of cells, but the exact surface characteristics necessary for optimal interactions with bone cells remain to be elucidated.

5.2.1. Topography

Electron beam lithography, colloidal particle adsorption, microcontact printing, new polymer preparations (blends and the synthesis of di and triblock *co*-polymers) and self-assembled monolayers are all used to study the interactions between cells and biomaterials at the micro and nanometer levels (239). These techniques can be used to control the topographic properties of biomaterials, such as micrometer or nanometer ridges, grooves and distributed features (pits, islands, holes) (239, 240).

Nevertheless, the impact of micrometer-sized rough surfaces (arithmetic mean roughness (Ra) > 1 micron) on bone cell attachment remains controversial. Some studies have found that rough surfaces increase cell attachment (241, 242), while others have found no effect (243). However, empirical observations of medical implants have shown better bone-implant interactions when implants with a high surface roughness are used (244). Nanoscale surface topographies can also be important for cell adhesion and spreading. Lim *et al.* used thin PLA-polystyrene films with pits 14 to 45 nm deep and found that shallower nanopits caused human fetal osteoblasts to spread significantly more and attach much better than did a flat PLA surface (245).

The surface topography of materials can also influence protein adsorption (238) and the integrins on the cell surface, so regulating the formation of both focal and fibrillar adhesions (130, 246). For example, the specific alpha v integrin subunits involved in the formation of focal adhesions and the phosphorylation of signaling proteins (FAK) are significantly increased in human fetal osteoblasts adhering to 14 and 29 nm deep pits compared to cells on 45 nm pits and flat PLA (245). Primary human osteoblasts growing on rough Ti-Al-V alloys have no alpha 3 integrin subunits, while cells growing on rough Co-Cr-Mo alloys have no beta 3 integrin subunits (107). Diener *et al.* examined the number, size and dynamic behaviors of focal adhesions in MG-63 osteoblastic cells cultured on Ti surfaces with different roughnesses (246). They found that material surface influenced the mobility of the focal adhesions and thus impaired the ability of the cells to dynamically organize and remodel the ECM. Luthen *et al.* have also demonstrated that the alpha 5 and beta 1 integrin subunits do not form fibrillar adhesions in cells grown on a Ti surface blasted with corundum particles. These fibrillar adhesions are crucial for fibronectin fibrillogenesis (130).

The surface topography can also regulate cell proliferation, differentiation and activity (247). These cell behaviors can be enhanced by the surface microstructure (248, 249). Thus the expression of various osteoblast genes

is affected by the Ti surface. The expression of about 10% of the genes is modified over three-fold when they are on a sand-blasted Ti surface (Ra = 4 microns) compared to cells on a smooth surface (Ra = 0.6 micron) (243).

5.2.2. Chemistry and wettability

Recent studies have used *in vitro* self-assembling monolayers with a terminal PEG, OH, COOH, NH_2 and CH_3 functions to evaluate the effect of surface chemistry and wettability on protein adsorption and cell behavior (237, 250-253). These substrata have been used to tailor material surfaces to control the molecular composition and the resulting properties of the surfaces (254, 255). PEG and OH groups give wettable surfaces, COOH and NH_2 functions give moderately wettable surfaces while CH_3 groups produce hydrophobic surfaces (250). Protein adsorption is a complex, dynamic process involving hydrophobic interactions, electrostatic forces, hydrogen bonding and van der Waals forces (256). The adsorption of fibronectin onto self-assembling monolayers was determined by radiolabeling; the relative adsorption varied with the surface function as follows OH < COOH < NH_2 < CH_3, which is well correlated with the increasing surface hydrophobicity (257). Fibronectin is composed of two 230-250 kDa monomers linked by disulfide bridges. Each monomer contains three types of charged globular modules, called type I, type II, and type III domains. Because of these charged domains, more flexible and extended conformations have been found on hydrophilic surfaces, while stronger binding and more rigid conformations are produced on hydrophobic substrata (258-260). Consequently, the secondary structure of fibronectin undergoes greater denaturation on hydrophobic surfaces than on hydrophilic ones (261).

The adhesion and function of osteoblasts are strongly influenced by the terminal chemistry of alkyl thiol self-assembling monolayers (262). The surface chemistry modulates the adhesion strength of MC3T3-E1 preosteoblasts and the subsequent matrix mineralization (252, 254, 263). Thus, self-assembling monolayers bearing OH or NH_2 terminating groups favor mineralization more than do monolayers bearing COOH and CH_3 groups (252, 263). The high mineralization by cells on both OH and NH_2 surfaces is correlated with enhanced alpha 5 beta 1 integrin binding and FAK activation (252).

5.2.3. Charge

The surface wettability of biomaterials is only a partial indication of adsorbed protein activity since this parameter cannot discriminate among neutral, positive or negative hydrophilic surfaces (254). Manipulations of the surface charge can also regulate protein adsorption and therefore cell attachment (108, 263). Fibronectin has an isoelectrical point of 5.5 and an overall negative charge under physiological conditions, and this influences its adsorption to charged surfaces (264). Hence the amounts of fibronectin adsorbed onto NH_2 and COOH self-assembling monolayers are similar at low coating concentrations while more fibronectin can be adsorbed onto the positively charged NH_2 surface before it is saturated than onto the negative charged COOH surface (263).

Rat calvarial osteoblasts cultured on positively and negatively charged polymers have completely different morphologies. Patterned surface chemistry was used to show that bone calvarial cells spread only on positively charged areas in the first 30 min, and only extend to negatively charged regions after 2 days (265). The same was found using positive charged hydrogels. Hydrogels made of 2-hydroxyethylmethacrylate and PEG-dimethacrylate are normally neutral and relatively inert to cell attachment. But their copolymerization with 2-methacryloxyethyltrimethyl ammonium chloride form positively charged hydrogels and copolymerization with sodium 2-sulfoethylmethacrylate gives negatively charged hydrogels (266). Osteoblasts attached and spread more on positively charged hydrogels than on neutral or negatively charged ones (266).

5.3. Biomimetic materials

An important improvement in bone tissue engineering is to enhance ability of biomaterials to interact with cells, mimicking the role of the proteins of the ECM. Proteins like fibronectin, osteopontin, vitronectin, laminin and collagen are all used to produce functional biomaterials (110, 267, 268). However, these proteins are extracted and purified from non-human species, which enhances the risk of undesirable immune responses and infections. Current biomimetic strategies focus on the use of short bioadhesive oligopeptides on non-fouling surfaces (96).

5.3.1. Homogeneous peptide-modified surfaces

Small peptides have recently been successfully covalently immobilized on the surface of biomaterials (269, 270). A variety of materials, such as cellulose (271), alginate gel (272), PEG (273) and calcium phosphate ceramics (274), have been used. The most often used sequence (RGD) for making biomaterials functional increases the attachment, growth and differentiation of osteogenic precursor cells (275, 276). Other peptides such as fragment of BMP (277) or the heparin binding domain of the bone sialoprotein (278) have been also covalently immobilized on surfaces. But little is known about the signal transduction induced by these functionalized biomaterials, especially the crosstalk between integrins, growth factors and hormones pathways (279) (Table 8).

The covalent immobilization of peptides on polymer surfaces requires functional groups such as OH, COOH or NH_2 [for review see (97)]. Many polymers without such functional groups can be attached using an additional technique like blending, co-polymerization or chemical/physical treatment. Most RGD peptides are linked to polymers via a stable covalent amide bond between an activated surface COOH groups, through carbodiimide chemistry, and the N terminus of the peptide. The major challenge with such a linkage is to protect before grafting the other functional groups of the peptide, especially COOH groups at the RGD C-terminus and Asp side chain, and NH_2 group of the Arg side chain (97). Several techniques can be used to characterize peptide grafting, depending on the surface properties of the material (287). Ligand density is measured using low throughput methods that require specialized equipment or radioactivity. Current

Table 8. Peptide-grafted materials

Materials	Peptides	Cell types	Cellular responses	References
Self-assembling monolayers				
3-aminopropyl triethoxysilane	RGDS versus RGES (negative control)	MC3T3-E1 preosteoblasts	↑ Adhesion ↑ Osteocalcin ↑ Osteopontin ↑ Bone sialoprotein	280
N-((3-trimethoxy-silyl)-propyl) diethylene triamine	RGDS versus RDGS (negative control)	Osteoblasts Fibroblasts	↑ Osteoblast attachment ↑ Fibroblast attachment	278
	KRSR versus KSSR (negative control)	Osteoblasts Fibroblasts	↑ Osteoblast attachment	
Quartz	Combination of Ac-CGGFHRRIKA-NH_2 Ac-CGGNGEPRGDTYRAY-NH_2	Human osteoblasts	↑ Attachment ↑ Spreading ↑ Mineralization	281, 282
Hydrogels				
PEG	RGD-PHSRN	Neonatal rat calvarial osteoblasts	↑ Proliferation ↑ Differentiation	283
Oligo(PEG fumarate)	DVDVPDGRGDSLAYG and GRGDS	Osteoblasts Fibroblasts	↑ Only fibroblast proliferation with GRGDS	284
Poly(*N*-isopropyl-acrylamide-*co*-acrylic acid)	Combination of Ac-CGGFHRRIKA-NH_2 Ac-CGGNGEPRGDTYRAY-NH_2	Rat calvarial osteoblasts	↑ Attachment ↑ Spreading ↑ Proliferation	285
Alloys				
Ti-6Al-4V	Linear RGD and cyclo-DfKRG (cyclo-(Asp-DPhe-Lys (mercaptopropionyl)-Arg-Gly)	Human bone marrow stromal cells	↑ Adhesion at short time	286
Ceramics				
Inorganic particles	GTPGPQGIAGQRGVV (type I collagen)	Human osteoblasts	↑ BMP mRNA ↑ Alkaline phosphatase mRNA	274

BMP: Bone Morphogenetic Protein, PEG: poly(ethylene glycol)

techniques include ellipsometry, X-ray photoelectron spectroscopy, Fourier transform infrared spectroscopy, surface plasmon resonance, radiolabeling and fluorescence-based methods (97, 287). The microdistribution is detected using atomic force microscopy or 2D time-of-flight secondary ion mass spectrometry, but this remains challenging (97).

The RGD sequence can bind to several integrins, including alpha v beta 3 (282), alpha v beta 1 and alpha v beta 5 (288). But other integrins, such as alpha 5 beta 1, require specific domains in addition to the RGD sequence for effective integrin-ligand interactions. The alpha 5 beta 1 integrins bind to the RGD motif on the 10^{th} type III repeat of fibronectin in the presence of the Pro-His-Ser-Arg-Asp (PHSRN) synergy domain on the type III repeat (289). The synergistic RGD-PHSRN sequence produces better osteoblast cell adhesion and spreading than do PEG gels bearing RGD alone (283). The RGD sequence is used in either its linear or cyclic form (290, 291), but the linear peptides are more easily inactivated by enzymatic cleavage than are cyclic peptides (97). Cyclic peptides also support integrin specificity and cell adhesion better than linear ones (288). The attachment of cells to RGD peptides depends on the peptide density. A high surface density of RGD leads to the formation of focal adhesions and cell spreading (97). A RGD peptide density of 1 fmol/cm^2 enables fibroblasts to spread, while a density of 10 fmol/cm^2 support the formation of focal adhesions and actin stress fibers (292). The peptide density can also regulate cell differentiation. For example, density of the peptide CGGNGEPRGDTYRAY must be above a threshold of 0.62 pmol/cm^2 to ensure matrix mineralization by osteoblasts (293).

The sequence Asp-Gly-Glu-Ala (DGEA) extracted from collagen is specifically recognized by alpha 2 beta 1 integrin (294). It modulates the Ca^{2+} signaling pathway in human osteoblasts and fibroblasts (295, 296). DGEA blocks interactions between alpha 2 integrin subunit and type I collagen and doing so decreases rat osteoblast function (297). However, poly(acrylamide-*co*-ethylene glycol/acrylic acid) polymers modified by attaching CGGDGEAG did not support the short term osteoblast adhesion as well as long term attachment or proliferation (281). Other peptides derived from type I collagen such as GTPGPQGIAGQRGVV (298) and GGYGGGPC$(GPP)_5$GFOGER$(GPP)_5$GPC (299) have also been used. The peptide containing GFOGER sequence is recognized by alpha 2 beta 1 integrin and promotes human fibrosarcoma cell adhesion and spreading as well as primary rat bone marrow stromal cell differentiation (299, 300).

Both the bone sialoprotein Phe-His-Arg-Arg-Ile-Lys-Ala (FHRRIKA) sequence and the fibronectin Pro-Arg-Arg-Ala-Arg-Val (PRRARV) peptide increase osteoblast and macrophage attachment (285, 301, 302). Various linear and cyclic peptides extracted from the primary heparin-binding site of Human Vitronectin Precursor (HVP), including (339-364)HVP, (339-351)HVP, (351-364)HVP, (351-359)HVP and cyclic(351-359)HVP, also favor osteoblast adhesion, but are less effective than RGD (303). Interactions between cell-membrane heparin sulfate proteoglycans and the heparin binding sites on ECM proteins are more specific to osteoblasts (304, 305). Therefore, new strategies have been developed to create small peptides involved in these interactions (304). For example, Lys-Arg-Ser-Arg (KRSR),

a heparan-sulfate binding peptide, is recognized by osteoblasts, while fibroblasts do not interact with it (278, 305, 306).

5.3.2. Mixed peptide surfaces

The effect of some peptide combinations depends on the cell types involved. For example, a combination of RGD and FHRRIKA favors the adhesion of rat preosteoblasts (282), while decreasing the adhesion of mesenchymal stem cells (307). By contrast, RGD and KRSR do not act in synergy on the attachment of mesenchymal stem cells or human osteoblasts (305, 307).

A major challenge in biomimetic material development remains the control of the density, spatial distribution and stability over time of the grafted molecules (282). Another concern is the sterilization of the manufactured product. Traditional materials used as bone substitutes are heat-sterilized, which eliminates pathogenic organisms by destroying their enzymes. The alternatives to heat treatment include glow discharge plasma, ethylene oxide treatment and gamma irradiation ($> 3\times10^{19}$ Hz) (308). But these are difficult to use with biomimetic materials. For example, gamma irradiation can cause oxidation or the accumulation of free radicals. Ultraviolet radiation is also used, but its possible effects on peptides are still under investigation (308).

6. INFLUENCE OF BIOMATERIAL PROPERTIES ON CELLS IN 3D SYSTEMS

One major concern about the cells-biomaterials interactions in 2D systems is that they do not reflect the behavior of cells *in vivo* (309, 310). 2D and 3D systems differ mainly in the type of cell adhesion (98) and cell polarization (311, 312). The formation and study of bone tissue in 3D polymer scaffolds have received considerable attention in recent years (313, 314). One major drawback of these scaffolds is the limited transport of nutrients, O_2 and the waste removal. Consequently, cells colonize only the scaffold surface, since they become necrotic in deeper sites (315). Nevertheless, some strategies such as fluid flow can be developed *in vitro* to increase the scaffold colonization by cells and the bone matrix formation (316). The ideal 3D bone graft scaffold is therefore one that has a high surface/volume ratio to enable cell attachment and the exchange of nutrients not simply by diffusion (317, 318). This scaffold should also act as a framework on which cells can proliferate and differentiate (317-319). Lastly, the scaffold should be degraded at a rate similar to that of new tissue formation (320).

6.1. Mechanotransduction

Mechanical stress modulates the architecture of bone remodeling. The bone is an optimized structure with a high strength and minimal weight (321). External mechanical stimuli are believed to cause transient waves and shear forces that travel through the cannaliculi of bone. Some authors also suggest that oscillating interstitial fluid flows over charged bone crystals generate electric field (133). The first source of this fluid shear force is pressure differentials in the circulatory system, while the second is environmental factors (e.g. physical exercise). In their microenvironment, cells react to mechanical stimuli, including shear stress, pressure, strain in ECM and electric field, modulating their shape and behavior (322).

6.1.1. Effects of the 3D systems on integrins

Integrins are the proteins most obviously implicated in the transduction of mechanical stress, since they anchor the cell cytoskeleton to the ECM (119). Other mechanical sensors may also be involved, such as membrane channels (connexins in osteocytes and osteoblasts), and cytoskeleton and membrane structures (membrane calveolin) [for review see (133)].

Most studies on the behavior of integrins in 3D systems have used fibroblasts (112, 323, 324) and endothelial cells (325). There are several differences between the interactions of integrins with biomaterials in 2D and 3D systems. One of the major differences is the introduction of a new type of cell adhesion, 3D matrix adhesion (see Table 4 in section 4.2) (98). For example, paxillin expression is decreased in 3D cultures of human mesenchymal stem cells with a concomitant change in its localization (326). Another striking difference between 2D and 3D concerns the assembly of focal adhesions. The focal adhesions normally involve plaque proteins such as alpha v beta 3 integrins, vinculin, paxillin, and FAK, whereas fibrillar adhesions are composed mainly of alpha 5 beta 1 integrins and tensin (113). However, the alpha 5 integrin subunits and paxillin are colocalized within a fibronectin 3D matrix (323). Furthermore, integrins must be activated *in vitro* for the assembly of fibronectin fibrils in 2D, while this activation is not required in 3D (327). The binding of integrins to collagen is also different in 2D and 3D systems. In fibroblasts, the predominant integrin involved in 2D systems is alpha 2 beta 1, while alpha 5 beta 1 is the main receptor in 3D systems (323). By contrast, human mesenchymal stem cells increased expression of alpha 2 beta 1 integrins in 3D poly(ethylene terephthalate) scaffold compared to 2D, contributing to the highly organized ECM collagen fibrils. In addition, these cells expressed alpha v beta 3 only in 2D (326).

6.1.2. Effects of shear stress and cyclic strain on cell behavior

Shear stress has a variety of effects on bone cells, influencing their adhesion, proliferation, differentiation and apoptosis. In bone, cells are typically exposed to shear stress of 0.8 to 3 Pa (133). The production of prostaglandin E_2 (PGE2), cAMP, and Inositol 1,4,5-Triphosphate (IP3) levels are increased in cells under shear stress (328). Expression of cyclooxygenase-2 (COX-2) and subsequent PGE2 production are linked to mechanically induced bone formation (329). Shear stress also increases nitric oxide (NO) production by various cell types. NO, as well as cAMP and IP3, may be involved in communication between bone cells and inhibit the action of osteoclasts during bone remodeling. Thus, shear stress can also be an important factor in the interactions of bone cells (328, 330).

Table 9. Effects of shear stress and cyclic strain on bone cells

Cells types	Physical stimuli	Effects	References
Mesenchymal stem cells			
Rat bone marrow stromal cells	Fluid shear 0.01 – 0.03 Pa for 16 days	No effect on proliferation ↑ Mineralization	331
Preosteoblasts			
MC3T3-E1	Fluid shear 1.2 Pa for 1 h	↑ COX-2 ↑ Focal adhesions containing beta 1 integrin subunit	332
	Fluid shear 0.5 to 2 Pa for 1 – 3 h	↓ Cx43 ↓ Cx45	333
	Fluid shear ≤ 1 Pa for 0 – 9 h	↑ COX-2	334, 335
	Oscillatory fluid flow 1.1 Pa (0.5 Hz) for 24 h	↑ Osteopontin ↑ COX-2	336
	Oscillatory fluid flow 0.4 Pa (3 Hz) or 0.6 Pa (5 Hz) for 0.16 h	↑ NO production ↑ PGE2	337
Osteoblasts			
Mouse	Fluid shear 1 Pa for 1 h	↑ RANKL ↑ cAMP	338
Rat	Fluid shear 0.1 Pa for 4-16 days	No effect on proliferation ↑ Mineralization ↑ Differentiation	339
	Fluid shear 0.6 Pa for 0-12 h	↑ NO production	340
Human	Fluid shear 2 Pa for 0.5 h	↑ Proliferation ↑ Differentiation ↑ ERK phosphorylation ↑ beta 1 integrin subunit mRNA	155
	Fluid shear (0.6 Pa) and oscillatory fluid flow (0.3 Pa (5 Hz)) for 1 h	No effect on proliferation ↑ NO production ↑ PGE2	341
	Oscillatory fluid flow ~0.7 Pa (5 Hz) for 0.08 – 24 h	↑ NO production	330
Osteocytes			
Mouse MLO-Y4	Fluid shear 0.5 to 2 Pa for 1 – 3 h	↓ Cx43 ↓ Cx45	333
	Fluid shear 1.6 Pa for 0.5 – 2 h	↑ PGE2 ↑ Cx43	29
	Fluid shear (1.6 Pa) and oscillatory fluid flow (0.08 Pa (5 Hz)) for 24 h	↑ Cx43	342
	Oscillatory fluid flow 0.4 Pa (3 Hz) or 0.6 Pa (5 Hz) for 0.16 h	↑ NO production ↑ PGE2	337
	Oscillatory fluid flow 1.1 Pa (0.5 Hz) for 24 h	↓ Osteopontin ↑ COX-2	336
Chicken	Fluid shear 1.6 Pa for 0.5 – 2 h	↑ PGE2	29
	Fluid shear (0.5 Pa) and oscillatory fluid flow (0.02 Pa (5 Hz)) for 0.08 – 1 h	↑ NO production ↑ PGE2	343
Preosteoclasts			
Rat bone marrow-derived preosteoclast-like cells	Fluid shear 1.6 Pa for 6 h	↑ NO production ↑ PGE2	344
Osteoclasts			
Rat	Fluid shear 0.9 – 2.63 Pa for 0-2h	↓ Carbonic anhydrase II mRNA	345
Mouse osteoclasts generated from ST-2 and RAW 264.7	Fluid shear (0.8 – 3 Pa) and oscillatory fluid flow (0.1 Pa 1 Hz)) for 0 - 72h	↓ RANKL/OPG mRNA	346

cAMP: cyclic Adenosine 3'-5' Monophosphate, COX-2: cyclooxygenase-2, Cx: Connexin, ERK: Extracellular signal Regulated Kinase, NO: nitric oxide, OPG: osteoprotegerin, PGE2: prostaglandin E_2, RANKL: Receptor Activator of NF-kappaB Ligand

Shear stress modulates osteoblast proliferation (Table 9) (329, 347, 348), while cyclic strain inhibits the proliferation of human mesenchymal stem cells, activating ERK1/2 and p38, but not JNK (349). Pulsatile flow (gradient in fluid shear) can also activate ERK1/2 in rat and human osteoblasts and increase their proliferation (347, 155). The effects of shear stress on bone cell apoptosis have not been fully reviewed. However, shear stress inhibits the apoptosis induced by TNF-alpha in MC3T3-E1 preosteoblasts and rat primary osteoblasts (350), but not in chicken osteoblasts (351). Shear stress also inhibits the apoptosis of chicken osteocytes induced by serum or TNF-alpha (351, 352). Lastly, shear stress causes apoptosis of mature mouse osteoclasts (353).

The differentiation of bone cells is also regulated by shear stress. Cyclic strain activates ERK1/2 and p38 in human mesenchymal stem cells. Activated ERK1/2 stimulates matrix mineralization, while activated p38 has the opposite effect. The experimental inhibition of p38 in these cells results in more differentiation in osteoblasts (349). Fluid flow also increases matrix mineralization, alkaline phosphatase activity and osteopontin secretion by primary rat bone marrow stem cells into a Ti-fiber scaffold more than does static conditions (316). Similary, human osteoblasts subjected to shear stress increase ECM formation and alkaline phosphatase activity (354).

Table 10. 3D scaffold fabrication

Techniques	Pore size (microns)	Materials	Fiber diameter (microns)	Porosity (%)	References
Porous scaffolds					
Solvent casting (salt leaching)	45 – 800	PLA, PGA, PLGA, TCP, hyaluronic acid		57 – 98	
Emulsion freeze-drying	11 – 500	Collagen, silk fibroin		62 – 99	
Gas-forming	70 – 500	Silk fibroin, PLGA, poly(propylene fumarate)		51 – 93	317
Rapid prototyping	300 – 900	Poly(propylene fumarate)		0 – 60	359
Nanofiber porous scaffolds					
Thermally induced phase separation	10 – 100	PLA	0.05 – 0.5	80 – 99	360, 361
Electrospinning		Collagen, chitosan, PLGA chitin, gelatin, silk protein, elastin-mimetic peptide, fibrinogen, casein, DNA	0.003 – 5		362-366
Self-assembling peptides	0.005 – 0.2	Synthetic designed peptides	0.007 – 0.01		367-369

PGA: poly(glycolide), PLA: poly(lactide), PLGA: poly(DL-lactide-*co*-glycolid), TCP: tricalcium phosphate. Adapted with permission from (313)

Shear stress also influences osteoclast behavior. Osteoclastogenesis is reduced by shear stress through a decrease in RANKL and increased OPG synthesis in an *in vitro* mouse model (346). Shear stress also decreases the transcription of carbonic anhydrase II in rat osteoclasts (345). This enzyme is needed for inorganic matrix resorption (355).

6.2. Scaffolds

6.2.1. Fabrication processes

Various techniques can be used to create micro- and nano-porous scaffolds, including solvent casting (particulate leaching), emulsion freeze-drying, gas forming, rapid prototyping, thermally induced phase separation, electrospinning and self-assembling peptides (356). However, particulate leaching, solution casting and melt molding result in random architectures with uncontrolled internal structure. Heterogeneities in 3D scaffolds produce inadequate nutrients and O_2 distribution and waste removal. Ultimately, it results in poor tissue growth, with most growth at the periphery of the scaffold (348, 357). New techniques such as rapid prototyping (also known as fused deposition modeling) using Computer-Aided Design (CAD) have now overcome these problems, allowing control of the internal architecture (pore size, pore distribution and porosity) of the scaffolds (358) (Table 10).

Solvent casting-particulate leaching is a simple technique using water, soluble particles (e.g. salt crystal or porogen) and a polymer solution. The mixture is cast in a mold and the water and particles are removed from the polymer scaffold by evaporation, lyophilisation and leaching (370). The particle/polymer ratio influences pore size and porosity, while particle shape controls the pore architecture (371). This process has several inconveniences, including poor pore interconnection and difficult particle removal from complex 3D scaffolds (361, 372).

In emulsion freeze-drying, a polymer is dissolved in organic solvent and an emulsion is made with water. Cooling this emulsion sequesters the liquid phase to form the polymeric structure. The liquid phase is then removed from the scaffold by freeze-drying. One drawback of this technique is the absence of pore interconnectivity (361, 373).

In gas-forming technique, solid polymer disks are exposed to high gas pressure (normally CO_2), which saturates the polymer with the gas. The rapid release of gas makes the polymer thermodynamically unstable, so that gas bubbles can become nucleated and grow in the polymer. This technique produces limited pore interconnectivity (10-30%) and non-porous surfaces (313, 317, 374).

Rapid prototyping relies on the use of a 3D printer (similar to an ink jet printer) to create a specific scaffold architecture by depositing successive layers of polymer. This technique, developed at the Massachusetts Institute of Technology (375), uses CAD softwares to control parameters such as the speed, flow rate and spatial deposition of the polymeric material. Manjubala *et al.* have developed a 10 mm high cylinder of composite chitosan-HAP with 50% porosity and 500 micron pores (358), but the scaffolds create by rapid prototyping currently have limited resolution and poor mechanical properties (376).

Thermally induced phase separation involves dissolving the polymer at high temperature and separating the liquid-liquid or liquid-solid phases by lowering the solvent temperature. The remaining solvent is removed by sublimation to give the porous polymeric scaffold. Pore morphology is controlled by the nature of the polymer and solvent, their concentrations and the phase separation temperature (317). This technique creates scaffolds with good mechanical properties (360).

The inexpensive electrospinning process creates polymer fibers or non-woven fibrous meshes (362, 365, 366). The polymer solution is first held at a capillary tip by surface tension. A high voltage (10-20 kV) is applied to cause charge repulsion within the polymer solution to overcome the surface tension. The resulting stretched polymer is driven toward a rotating collector, but the solvent of the electrospun polymer evaporates before it

reaches the collector, creating a pure long thin polymer fiber (several km) (377). The concentration, viscosity and flow rate of the polymer solution and the collector voltage, distance, composition and geometry all change the resulting fiber morphology. Various natural polymer solutions can be used, but finding the right solvent remains a great challenge (365).

Recent developments in nanofiber scaffolds include the use of self-assembling peptides. Those synthetic peptides undergo spontaneous assembly into nanofibrous scaffold with pore sizes of 5-200 nm and fiber diameters of 7-10 nm (367-369). Self-assembling peptide scaffolds have many advantages. The design and modification of peptides (changing single amino acids) is inexpensive and rapid. They provide a better defined composition than do alternatives like Matrigel. In addition, pure peptides with known motifs can be used to study controlled gene expression and signaling. Furthermore, there is no need for chemical cross-linkers (378). However, although self-assembling peptides can create much thinner fibers than other scaffold-building methods, designing the peptides make it a more complicated option overall. Nanofiber scaffolds can be made with the self-assembling peptides RADA16 (Ac-RADARADARADARADA-x-$CONH_2$), where different sequences can be inserted at the x position. Interestingly, Horii *et al.* have created RADA16 nanofiber scaffolds bearing the osteogenic growth peptide sequence, osteopontin cell adhesion domain or a peptide containing two RGD motifs. These three scaffolds produced greater MC3T3-E1 preosteoblasts proliferation and osteogenic differentiation than did a RADA16 scaffold without inserted peptides (379).

6.2.2. Architecture

The ingrowth of bone tissue and mineralized ECM properties in 3D scaffold depend on the pore size, porosity and pore interconnection density and size (380). The 3D architecture of the scaffold influences its functions and the success of its implantation *in vivo*. Pore size influences cell motility and invasion of the scaffold. Also, the pore size modulates fluid shear stress exerted on a cell. The highest fluid shear stress occurs at the center of each pore in a scaffold, while the lowest stress is at the pore surface (348). Most cells cannot migrate through pores smaller than 5-10 microns (315, 381). However, human osteoblasts (20-30 microns) can pass through pores bigger than 2 microns (319). Cells can also migrate inside a mesh of nanofibers with limited pore diameter (< 1 micron), possibly by pushing against the fibers during their migration, although the exact mechanism is still not clear (270, 381).

Specific pore sizes are optimal for some tissues and applications (382). Since the diameter of the osteon pore in human bone is a few hundreds microns (383), the optimal bone ingrowth *in vivo* requires a pore size of 100-400 microns. For example, human osteosarcoma cells (MG-63) proliferate more on polycaprolactone scaffold with 380-405 micron pores, while a scaffold with 290-310 micron pores is optimal for new bone formation (382). Larger pores (> 1 000 microns) favor the formation of fibrous tissue [for review see (214)]. However, the optimal pore size for bone repair is still under investigation. Some studies have reported good bone ingrowth with pore sizes as small as 50 microns (384, 385), while others have found pore sizes of less than 200 microns to impair bone ingrowth *in vivo* (386). Nevertheless, the use of pore size in the range of 200 microns seems recommended, since cells will not grow deeper without an external supply of nutrients and O_2 (387-390).

Currently, porosity of 60% for nonbiodegradable scaffold is the minimal requirement to allow bone formation. Lower porosity impairs diffusion of nutrients, O_2 and waste products. Scaffolds having higher porosity can be prepared, but it alters their mechanical properties (380). Pore interconnection is also necessary for vascularization and cell migration (358). Recent studies show that minimal interconnection pore size is about 50 microns to allow osteoid tissue ingrowth (391). In addition, high interconnectivity between pores favors a uniform cell distribution as well as high nutrients diffusion out from the scaffold (317).

So, the ideal scaffold should have pores of about 200 microns to support cell adhesion and proliferation and the deposition of mineral matrix (317, 392). A high porosity (> 90%) and good pore interconnection facilitate the mass transport of nutrients (393, 394) and support cell proliferation deep within the scaffold through vascularization (395). However, the mechanical properties are reduced in highly porous scaffold, even in metal scaffolds (214).

6.2.3. Nanostructure

The normal 3D environment of the cell is formed by a nanoscale network of ECM (396). Current methods of scaffold fabrication such as electrospinning (365), thermally induced phase separation and self-assembling peptides can create matrices of nanosized fibers (363). Nanofibrous PLA scaffolds increase protein adsorption, especially that of serum fibronectin and vitronectin, and increase the adhesion of preosteoblastic MC3T3-E1 preosteoblasts about 1.7 fold. These cells produce more alpha v beta 3 and alpha 2 beta 1 integrins and have greater FAK phosphorylation after 24h (397).

The nanoscale environment can also influence cell spreading, survival, proliferation and differentiation. Osteoblasts on carbon nanofibers with decreasing diameters (200 down to 60 nm) adhere and proliferate better, and have more alkaline phosphatase activity and ECM secretion (398).

6.3. Vascularization

Nutrients and O_2 can only diffuse 150 microns from the nearest capillary in tissues (399) while they are transported through the scaffold in static cultures by passive molecular diffusion alone, over distances of 100 to 1 000 microns (400, 401). Vascularization could facilitate cell survival, activity and tissue growth within the scaffold. The importance of vascularization in bone tissue is underlined by the fact that suppressing vascularization *in*

vivo results in thicker growth plates and impaired trabecular bone formation (402). However, there are still unanswered questions regarding the rules controlling angiogenesis (403). Scaffold vascularization may be improved *in vitro* by changing culture conditions (cyclic strain, growth factors), tissue pre-vascularization (co-culture, microvessel implantation) and scaffold modifications.

6.3.1. Cell culture conditions

Endothelial cells are influenced by cyclic strain and the deformation of blood vessels under physiological conditions (404). These cells can also spontaneously form capillary-like structure in gels *in vitro* (405-407). Strain can increase the thickness of these capillary-like structures (404). Applied cyclic strain to endothelial cell cultures in scaffolds enhances their proliferation (404, 408) while inhibiting their apoptosis (409). Cell culture conditions can also be changed to increase vascularization by adding direct angiogenic factors such as VEGF and FGF or indirect factors such as PDGF and TGF-beta to the medium. VEGF is a cytokine that increases the endothelial cell proliferation, migration and can inhibit their apoptosis (410, 411). FGF and PDGF also promote endothelial cell proliferation and migration (412, 413). In contrast, low concentrations (0.1 – 1 ng/mL) of TGF-beta stimulate endothelial proliferation *in vitro,* while higher concentrations (5 – 10 ng/mL) inhibit cell growth (414).

6.3.2. Vascularization prior to implantation

The co-culture of endothelial and bone cells *in vitro* could be an interesting avenue. Co-culture of 2% Human Umbilical Vein Endothelial Cells (HUVEC) and human mesenchymal stem cells forms a 3D prevascular network (415). But such networks are not formed when higher concentrations of endothelial cells (50% HUVEC) are grown with human osteoblasts (416).

Implanting fragments of vessels isolated from patients or other sources could be a way to vascularize a scaffold *in vitro*. It has been demonstrated that isolated fragments of intact microvessels retain some angiogenic potential *in vitro,* even in the absence of blood flow. Fragments of both rat and human microvessels can form new blood vessels within 4-5 days in collagen gels (403, 417). Those new blood vessels respond to VEGF (418). Rat microvessels in type I collagen gel implanted in mice interconnect with the host mouse vascular system in only 3 days. And these vessels mature to form a vascular bed containing identifiable arteries, arteriols, capillaries, venules and veins after 28 days (403). However, this method of vascularization requires the recruitment of host endothelial cells and is therefore time-consuming (415). Time is an important factor in *de novo* vascularization *in vivo* since cell survival and invasion within the scaffold is limited without vascularization.

Increasing the scaffold pore size favors the rate of neovascularization. The vascularization of a scaffold with 120-200 micron diameter pores grew at 1 mm/week when implanted in rats (419), while a scaffold with 500 micron diameter pores became vascularized at the rate of 5 mm in 5 days (420).

6.4. Bioreactors

The density of the cells within a scaffold may be increased using bioreactors. The bioreactors provide the basic needs of cells growing in a 3D scaffold (reproducible physiological and biomechanical parameters) by overcoming the limitation of nutrient and O_2 diffusion (421). Several types of bioreactors can be used with a scaffold: spinner flasks, rotary vessels and perfusion systems (387). 2D or 3D perfusion bioreactors produce homogenous cell populations throughout the scaffold. But the scaffold size is limited by the effect of fluid shear stress, which damages or detaches cells from the scaffold. The scaffold can be modified to counteract those effects by increasing the scaffold pore size, but this reduces the effective cell culture area and scaffold mechanical strength (315). Cells can also be cultivated in rotating wall vessel bioreactors (RWV) developed by National Aeronautics and Space Administration (422). These stimulate microgravity and the construct is kept in constant movement without making contact with the bioreactor walls (314). This lack of contact increases fluid flow through the scaffold and reduces the stagnant boundary layer surrounding it (315). Unfortunately, scaffold size is limited in RWV. Another concern is the microgravity applied to the scaffold. MC3T3-E1 preosteoblasts cells cultured for 24 hours in an RWV have a decreased expression of osteogenic markers (Runx2, osteocalcin and alkaline phosphatase transcripts), suggesting that cell differentiation is inhibited (423). In contrast, Rucci *et al.* used rat osteoblast-like cells and found that microgravity increased these markers above their concentrations in comparison to conventional culture (424). Thus, the use of bioreactors to improve homogeneous cell populations in a scaffold is often limited by the scaffold's architecture and size. There is presently no single ideal type of bioreactor for bone tissue.

7. CONCLUSION

Bone remodeling is a complex dynamic process that gives bone tissue its biomechanical and biological characteristics. Osteoblasts and osteoclasts play a key role in this life-long process. Substitutes used to repair bone defects must have biomechanical properties similar to those of human cortical bone (compressive strength 130-180 MPa, elastic modulus 12-18 GPa) or trabecular bone (compressive strength 4-12 MPa, elastic modulus 100-500 MPa). Also, they must be biocompatible, osteoconductive, osteoinductive and osteointegrative. Bone substitutes may be inorganic materials, natural or synthetic polymers, or composites. Inorganic materials are osteintegrative and have good mechanical properties, but they are brittle and resorbed only slowly. Natural polymers have good biocompatibily, but limited mechanical properties. Synthetic polymers can have mechanical properties similar to those of trabecular bone, but their biodegradation byproducts can have undesirable biological effects. Only composite materials with growth factors (such as BMPs) currently produce bone substitutes that have both appropriate bone osteoinductive and biomechanical properties. The osteoconductive and osteointegrative properties of these composite materials can also be increased by using biomimetic strategies, such as grafting

specific adhesion peptides (RGD) derived from bone ECM proteins onto the biomaterial surfaces.

8. PERSPECTIVES

Development of bone substitutes similar to autografts (the actual gold standard) requires a better understanding of bone cell responses induced by biomaterials. The interactions between cells and biomaterials mediated by ECM protein-integrin bindings are extensively studied because these contacts transmit mechanical stimuli which program subsequent cell behavior, such as proliferation and differentiation. Unfortunately, most studies on bone cells in 2D systems use osteoblasts, although both osteoblasts and osteoclasts are critical for bone repair or remodeling. Osteoblasts attached to bone substitutes present focal complex, focal and fibrillar adhesions, while osteoclasts only develop podosomes or sealing zone. Biomaterial surface properties (topography, functional groups, wettability, charge) modulate the ECM protein adsorption regulating the formation of focal and fibrillar adhesions. These cell-surface interactions thus influence the signal transduction, especially specific kinase phosphorylation.

Current studies demonstrated different cell behavior between 2D and 3D systems. Therefore, cells-bone substitute interactions must be studied since cell adhesion within 3D are still far from completely understood. Another important challenge in the development of bone substitutes is to enable cells to colonize the entire scaffold, survive and function there. Since transport of nutrients, O_2 and waste is limited to 0.1–1 mm by passive diffusion, careful scaffold design and fabrication requires the control of architectural features (pore size and nanostructure) that modulate bone cell adhesion, proliferation and differentiation. Furthermore, scaffold vascularization improvement and the use of new bioreactors will also promote the efficiency of bone substitutes.

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Abbreviations: 2D: two-dimensional, 3D: three-dimensional, ActR: Activin receptor, ALK: Activin receptor-Like Kinase, Arp2/3: Actin-related protein 2/3, AdBMP: Adenoviral vector encoding BMP, AR-Smad: Activin/TGF-beta-specific Receptor-regulated Smad, BMP: Bone Morphogenetic Protein, BMPRII: Bone Morphogenetic Protein type II Receptor, CAD: Computer-Aided Design, cAMP: cyclic Adenosine 3'-5' Monophosphate, CAS: Crk Associated Substrate, CDC42: Cell Division Control protein 42, CDMP: Cartilage Derived Morphogenetic Protein, cFms: macrophage colony stimulating factor receptor, Co-Smad: Common-partner Smad, COX-2: cyclooxygenase-2, Cx: Connexin, DGEA: Asp-Gly-Glu-Ala, DRF: Diaphanous-Related Formin, ECM: extracellular matrix, ERK: Extracellular signal Regulated Kinase, F-actin: Filamentous actin, FAK: Focal Adhesion Kinase, FGF: Fibroblast Growth Factor, FHRRIKA: Phe-His-Arg-Arg-Ile-Lys-Ala, GDP: Guanosine Diphosphate, GEF: Guanine nucleotide Exchange Factor, GP: glycoprotein, Grb-2: Growth factor receptor-bound protein 2, GTP: Guanosine Triphosphate, HAP: hydroxyapatite, HVP: Human Vitronectin Precursor, HUVEC: Human Umbilical Vein Endothelial Cell, IGF: Insulin like Growth Factor, I-Smad: Inhibitory Smad, IFN: interferon, IL: interleukin, IP3: Inositol 1,4,5-Triphosphate, JNK: Jun-N terminus Kinase, KRSR: Lys-Arg-Ser-Arg, MAPK: Mitogen Activated Protein Kinase, mDRF: mammalian Diaphanous-Related Formin, MMP: matrix metalloproteinase, NF-kappaB: Nuclear Factor-kappaB, NO: nitric oxide, OPG: osteoprotegerin, PDGF: Platelet-Derived Growth Factor, PEG: poly(ethylene glycol), PGA: poly(glycolide), PGE2: prostaglandin E_2, PI3K: phosphoinositide-3 kinase, PHSRN: Pro-His-Ser-Arg-Asp, PLA: poly(lactide), PLGA: poly(DL-lactide-*co*-glycolid), PRRARV: Pro-Arg-Arg-Ala-Arg-Val, R-Smad: Receptor-regulated (or pathway-restricted) Smad, Ra: arithmetic mean roughness, RANKL: Receptor Activator of NF-kappaB Ligand, RGD: Arg-Gly-Asp, ROCK: Rho-associated kinase, RWV: Rotating Wall Vessel bioreactor, SH2: Src-homology 2, Shc: insulin receptor substrate, SOS: son of sevenless, TAK1: TGF-beta1 Activated tyrosine Kinase 1, TCP: tricalcium phosphate, Ti: titanium, TNF: Tumor Necrosis Factor, TRAcP: Tartrate-Resistant Acid Phosphatase, TRAF6: TNF Receptor-Associated Factor 6, TGF-beta: Transforming Growth Factor beta, VEGF: Vascular Endothelial Growth Factor, WASP: Wiskott-Aldrich Syndrom Protein, WAVE: WASP/Verprolin-homologous protein, XIAP: X-linked inhibitor of apoptosis

Key Words: BMP, Bone substitute, Extracellular matrix, Integrin, Osteoblast, Osteoclast, Osteoinduction, Osteoconduction, Peptide, Signal transduction

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