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# VITAMIN D AND NANOSTRUCTURED SURFACES IN OSTEOBLASTIC DIFFERENTIATION OF DENTAL STEM CELLS

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# *1.Introduction*

## 1.1 Bone Tissue

Bone is a highly specialized form of connective tissue that is an internal support system in all higher vertebrates. It is a complex tissue in which the extracellular matrix is mineralized, conferring rigidity and strength to the skeleton, providing mechanical support to soft tissues, still maintaining some elasticity. In addition bone is a major source of inorganic ions maintaining blood calcium level, supports hematopoiesis and contains the brain and spinal cord.

Bone is composed of an organic matrix that is mineralized by calcium salts deposits. Type I collagen constitutes approximately 95% of the organic matrix; the remaining 5% is composed of proteoglycans and numerous noncollagenous proteins. Crystalline salts deposited in the organic matrix of bone under cellular control are primarily calcium and phosphate in the form of hydroxyapatite.

Morphologically, there are two forms of bone: cortical or compact, and cancellous or spongy. In cortical bone, packed collagen fibrils form concentric lamellae, and the fibrils in adjacent lamellae run in perpendicular planes. Cancellous bone has a loosely organized porous matrix.

Differences in the two bone types are also functional: cortical bone provides mechanical and protective functions and cancellous bone provides metabolic functions. Bone undergoes to a continuous tissue renewal, called remodelling, occurring throughout life and carried out by osteoblasts, or bone-forming cells, and osteoclast or bone-resorbing cells.

## 1.2 Bone cells

The microscopic observation of bone tissue reveals the presence of four different cell types: osteoclasts, bone resorbing cells, osteoblasts, bone forming cells, osteocytes, which are completely embedded in the bone tissue, and bone lining cells that represent a quiescent phase of osteoblasts.

Osteoblasts, osteocytes, and bone-lining cells originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursor of hematopoietic origin.

## 1.2.1 Osteoblasts and bone formation

Osteoblasts are responsible for the production of the bone matrix components and for their mineralization. Osteoblasts differentiate from their mesenchymal precursors (bone marrow stromal stem cells or connective tissue mesenchymal stem cells) during a complex process under the control of local growth factors and specific transcriptional factors that regulate the expression of certain genes and thus define the osteoblast phenotype.

RUNX2/Cbfa1 and Osterix, are required for precursor cell differentiation into the osteoblastic lineage, and the absence of either one results in a complete lack of mineralized skeleton (Komori 2005; Nakashima et al. 2002).

Active osteoblasts are present in clusters of cuboidal cells along the bone surface, usually lining a layer of organic matrix that they are producing before it is calcified (called osteoid tissue).

Osteoid tissue is a transitional tissue existing between the matrix formation and its subsequent calcification.

At the ultrastructural level, the osteoblast is characterized by a round nucleus at the base of the cell, the presence of an extremely developed rough endoplasmic reticulum, and a prominent Golgi complex reflecting the high biosynthetic and secretory activity of these cells. Cytoplasmic processes on the secreting side of the cell extend into the osteoid matrix and are in contact with the osteocyte processes in their canaliculi.

The osteoblast plasma membrane is rich in alkaline phosphatase and expresses several receptors and adhesion molecules that allow these cells to adhere to the matrix (via integrins) and to neighboring cells via junctional structures. Both gap junctions and adherens junctions are present among osteoblasts.

Osteoblasts also express receptors for several peptide and steroid hormone, as well as receptors for cytokines. They also express cytokine on their membrane, most important among these are colony stimulating factor1 (CSF-1) and RANK-L, which are involved in osteoclastogenesis.

Osteoblasts develop in a linear sequence progressing from osteoprogenitors to preosteoblasts, osteoblasts, and osteocytes (Figure 1.1). When mesenchymal precursors are committed to the osteoblast lineage, cell growth declines and they became pre-osteoblasts showing a progressive expression of differentiation markers. Committed pre-osteoblasts are characterized by expression of alkaline phosphatase (ALP), one of the earliest markers of the osteoblast phenotype. Mature osteoblasts secrete collagen type I and specialized bone matrix proteins organized as an extracellular matrix (ECM); synthesis of the ECM contributes to cessation of cell growth leading to a matrix maturation stage when induced expression of ALP and specialized bone proteins make the ECM competent for mineral deposition. Mineralization results in upregulated expression of several non-collagenous proteins, thereby providing markers of mature osteoblast (OCN, OPN, BSP), on which calcium phosphate is deposited as hydroxyapatite crystals.

These calcium and phosphate binding proteins may function in regulating the mineral deposition, amount of the hydroxyapatite crystals, or crystal size.

Once the bone matrix synthesis has been deposited, most posteoblasts become flattened lining cells. However, a fraction of cells loose cell-cell junctions and become embedded within the matrix to become osteocytes. Remaining osteoblasts loose both adherences to cell matrix and cell-cell junction and undergo apoptosis.

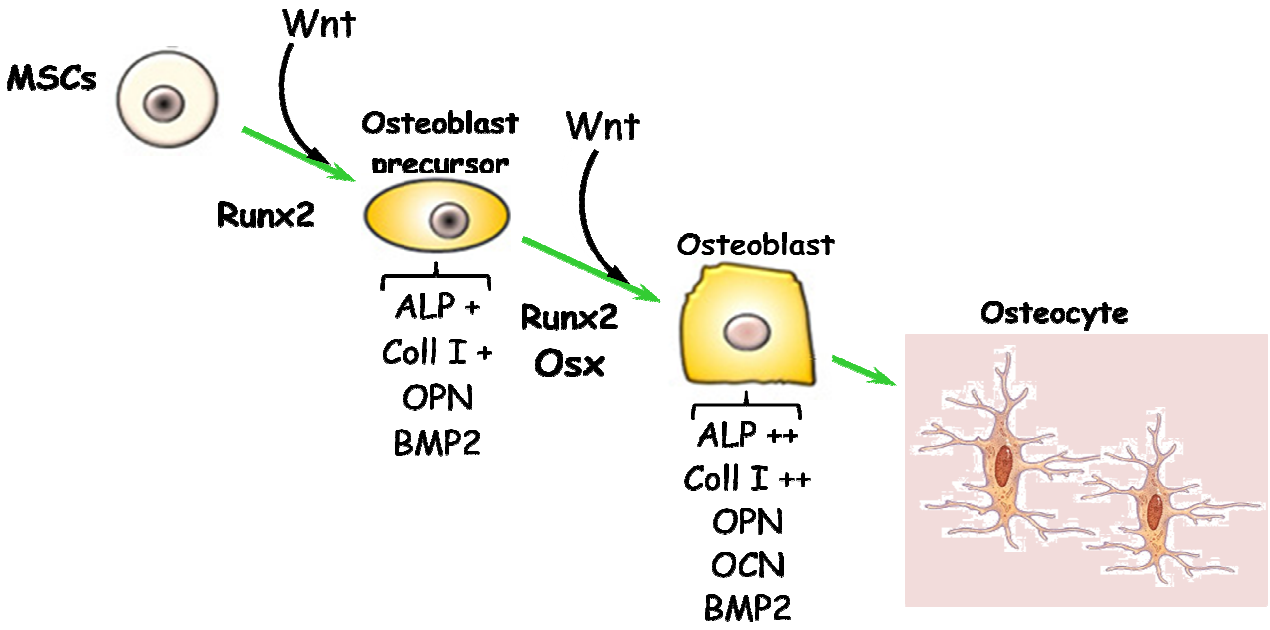


**Figure 1.1. Osteoblastogenesis.** Osteoblastogenesis from MSCs is a multistep differentiation process regulated by various signals, such as that of Wnt proteins, which activate a series of transcription factors in the various phases of osteoblastogenesis. The first transcription factor that must be activated to have the commitment of MSCs in pre-osteoblast is RUNX2. Committed cells, then, begin to express certain enzymes such as ALP, they begin to produce extracellular matrix bone proteins such as Coll I, OPN, and autocrine molecules such as BMP-2, subsequently there is the activation of another transcription factor, Osx, which cooperates with RUNX2 promoting the transition to mature osteoblast that continues to produce these proteins and begins to synthesize osteocalcin, which is necessary for the deposition of the inorganic matrix.
Mature osteoblasts begin to lay at first the organic component of the matrix and then the inorganic part synthesizing crystals of calcium phosphate similar to hydroxyapatite, osteoblasts then remain imprisoned in the matrix produced by themselves becoming osteocytes.

### 1.2.2 Two ways to make bone

Osteoblasts participate in two distinct mechanisms of bone formation: intramembranous and endhocondral ossification, both starting from mesenchymal condensation (reviewed by (Karsenty and Wagner 2002; Yang and Karsenty 2002; Zelzer and Olsen 2003)).The former, occurring mostly in the head region and clavicle, involves the condensation of mesenchymal cells followed by their differentiation into osteoblasts and secretion of osteoid to form bone spicules; osteoblasts fill in the space between spicules with the continued secretion of osteoid.

In most parts of the body the ossification is endochondral: condensed mesenchymal cells shortly differentiate along the chondrocyte pathway and form the cartilage template. Sox 9 is required for

chondrocyte cell fate determination, inactivation of Sox 9 abolishes cartilage formation and endochondral ossification (Akiyama et al. 2002).

Condrocytes undergo a further differentiation step, become hypertrophic and die, while mesenchymal cells differentiate in osteoblasts and mature in the periphery of the cartilage (pericondrium) to form bone collars. Mesenchymal cells located on periosteal surfaces and within bone marrow stroma provide a source of osteoblasts which act in concert with osteoclast to model bone during growth and maintaining bone architecture during adulthood.

## 1.2.3 Osteocyte

The osteocyte is a terminally differentiated osteoblast within the bone matrix and is thought to be responsible for its maintenance (Buck et al., 1996). Osteocytes were originally bone-forming cells, which became trapped in the bone matrix that they produced and later became calcified.

Each osteocyte occupies a space, or lacunae, within the matrix and extend filopodial process through canaliculi in the matrix to contact processes of adjacent cells and cells on the bone surface, where they engage in direct contact and communication by means of gap junctions. A young osteocyte has most of the ultrastructure of the osteoblast from which derives, an older osteocyte is located deeper within the calcified bone, shows a decreased bone volume and glycogen accumulation in the cytoplasm. These cells have been shown to synthesize new bone matrix at the surface of the osteocytic lacunae, which can subsequently calcify. These cells may also play a role as mechanosensors and in the local activation of bone turnover.

## 1.2.4 Osteoclast

Osteoclasts are the principal, if not exclusive, bone-resorbing cells and their activity has a profound impact on skeletal health. The osteoclast is a large, multinucleated cell, member of monocyte/macrophage family, which is characterized by its polar organization (induced by contact

with bone) and expression of specific proteins such as Cathepsin K. The most notable feature of the polarized osteoclast is its ruffled membrane, which represents the resorptive organelle of the cell. Formation of the osteoclast ruffled membrane is dependent on contact with bone and is only apparent when the cell degrades the skeletal matrix (Blair et al. 1989). Bone degradation is initiated by formation of an actin rich sealing zone that isolate the resorptive microenvironment from the extracellular space. Cathepsin K residing in vescicles is secreted in an exocytic process into the resorptive space and degrades the organic matrix of bone.

### 1.2.5 Osteoblast differentiation

The progression of osteoblast maturation requires a sequential activation and suppression of genes encoding the phenotypic and functional proteins of the osteoblast population.

Signaling proteins and transcription factors support the temporal expression/suppression of genes that characterize the stages of osteoblast differentiation.

Many growth factors affecting the osteoblast differentiation include fibroblast growth factors (FGFs) and their receptors, parathyroid hormone (PTH) and PTH-related protein (PTHrP) receptor, Indian hedgehog, members of the bone morphogenetic protein (BMP)/TGFβ superfamily, the Wnt pathway, and members of the growth hormone (GH)/insulin-like growth factor (IGF-1) system.

Osteoblast differentiation is also controlled by the transcription factors RUNX2 and Osterix. Alterations in function of various other non-bone specific transcription factors have been also demonstrated to affect osteoblastic differentiation and function. These include AP-1 and its related molecules (Sabatakos et al. 2000), homeobox proteins Msx1 (Satokata and Maas 1994), Msx2(Liu et al. 1999b; Satokata et al. 2000), Dlx3 and Dlx5 (Acampora et al. 1999), CBFβ1, Twist, TGFβ/BMP responsive SMAD proteins. Recently, an important role in osteoblast development for β-catenin has been shown. β-catenin is the downstream mediator of canonical Wnt signaling and it is necessary for gene transcription activation.

Also cell-cell interactions play an important role in influencing osteoblast phenotype and function. Both adherent and communicative junctions can regulate the abundance of signaling molecules or transcriptional factors, which in turn modulate gene expression.

## 1.2.6 Wnt signaling and β-catenin

Tissue differentiation requires the coordination between gene expression and interaction among neighboring cells. An important problem is to identify the signaling pathways that coordinate changes in gene expression with changes in cell adhesion and migration. Several growth factors have been identified to affect both gene expression and cell adhesion (Thiery 2002), for example Wnts and their signaling pathway involve proteins that directly participate in both gene transcription and cell adhesion.

Wnts are secreted lipid-modified proteins (Willert et al. 2003) which signaling pathway is highly conserved (Cadigan and Nusse 1997) and controls numerous developmental processes in evolution distant animals. Their function mutations lead to developmental abnormalities from stem cell loss to kidney and reproductive defects.

Wnts were discovered as Wingless (wg) in Drosophila and as MMTV proto oncogene (int-1) in mammalian cells (Rijsewijk et al. 1987). There are nineteen Wnts known in mammalian, which are classified in two classes: classical Wnts (Wnts-1,-3A,-8 and -8B) activate the canonical pathway involving β-catenin, non-classical Wnts (Wnts-a,-5A and -11) activate the calcium pathway. Also other Wnts have been shown to signal through other downstream signaling pathways.

Wnt ligands bind to Frizzled seven-transmembrane receptors which may be coupled to heterotrimeric G proteins; these receptors also contain an extracellular cysteine rich domain (CRD) and an intracellular carboxy domain (Hsieh et al. 1999). Ten different frizzled receptors have been identified in mammalian cells. With 19 Wnts and 10 receptors has been difficult to understand which Wnts bind to which frizzled receptors, because Wnts proteins are difficult to purify; the first

Wnts protein has been isolated only recently (Willert et al., 2003) and probably the response to frizzled receptors is ligand dependent. Signaling through frizzled receptors requires the low density lipoprotein receptor-related proteins LPR-5/6 which act as a co-receptor of Wnt ligands.

There are also various secreted factors, such as Cerberus (Cer) and FrzB, that bind to Wnts and block the interaction with frizzled proteins. In addition Dickkopf (Dkk) antagonizes Wnt action by blocking access to the LRP co-receptor and induction of LRP endocytosis in cooperation with kremen.

Soluble frizzled related proteins are present, which have the CRD of the frizzled receptors, but not transmembrane domains. They compete for binding with frizzled receptors for Wnts and can reduce the response to Wnt signaling.

In the canonical pathway, Wnt signaling leads to stabilization of cytosolic β-catenin, the key effector of Wnt signaling.

The β-catenin protein was initially discovered for its role in cell adhesion (Aberle et al. 1997), and as component of adherens junctions, it promotes cell adhesion by binding to the intracellular domain of the transmembrane protein cadherin, a $Ca^{2+}$-depended homotypic molecule, and linking cadherin to the actin cytoskeleton through the protein α-catenin. This adhesion function is conferred by a subcellular pool of β-catenin that is membrane-associate.

A soluble cytosolic pool of β-catenin is involved in the signaling and is strongly unstable in absence of Wnt, due to multiple phosphorylations in the protein's amino terminus, that make it available for the proteasome degradation. In absence of Wnt, the level of β-catenin is kept low through degradation of cytosolic excess; β-catenin is phosphorilated by the serin kinases casein kinase I (CKI) at Ser 45 and this enables glycogen synthase kinase 3β (GSK3β) to phosphorilate serine/threonine residues 41, 37 and 33. Phosphorylation of these last two residues targets β-catenin for the ubiquitination by βTrCP and degradation in the 26S proteosome.

In presence of Wnt stimulation, dishevelled (Dsh) blocks GSK-3β phosphorilation of β-catenin (Kengaku et al. 1998; Papkoff 1997) resulting in accumulation of cytoplasmic β-catenin, which

becomes available to bind the TCF/LEF family of transcription factors and to induce target gene expression.

LEFs/TCFs are co-transcription factors which in absence of β-catenin are associated with transcriptional repressors (groucho, CBP, HBP1), if β-catenin is present, it binds to TCFs/LEFs and displaces the inhibitors.

Seventy-five genes have been found to be activated by β-catenin-LEF/TCF complex. Some of these genes are involved in cell cycle (c-myc, cyclin D), some in developmental axis formation (siamois, twin), some are in tissue remodeling (matrilysin).

## 1.2.7 Wnt signaling in bone

Several works demonstrate that Wnts are involved in embryonic skeletal patterning, fetal skeletal development, and adult skeletal remodeling (Abe et al. 2003; Yamaguchi et al. 1999; Yang et al. 2003). Mice with mutations in Wnts, LPR5 and intracellular mediators such as β-catenin show a number of skeletal defects (Akhter et al. 2004; Boyden et al. 2002; Yamaguchi et al. 1999; Yang et al. 2003), and *in vitro* experiments also implicate Wnt signaling in osteogenesis and chondrogenesis (Daumer et al. 2004).

Anyway the literature is controversial and the functions of Wnt signaling in bone biology remain unclear. For example, two studies show that Wnt proteins inhibit the ability of human mesenchymal stem cells to differentiate into osteoblasts (Boland et al. 2004; de Boer et al. 2004), while three other studies show that Wnt signaling, through β-catenin, contributes to osteoblast differentiation (Hu et al. 2005; Luo et al. 2004; Westendorf et al. 2004). Besides, Wnt/β-catenin signaling seems to control osteoblast and chondrocyte formation when they differentiate from mesenchymal progenitors (Day et al. 2005), and in particular it is essential for skeletal lineage differentiation preventing transdifferentiation of osteoblastic cells into chondrocyte (Hill et al. 2005). Since timing

is critically important in the regulation of cell differentiation, this may explain the discrepancies in the understanding of Wnt signaling modulation.

## 1.2.8 β-catenin and bone

Several works in the past years demonstrate that β-catenin is essential for skeletal development, conditional Wnt1 Cre-mediated depletion of β-catenin blocked prechondrogenic condensation and craniofacial development (Brault et al. 2001); overexpression of a constitutively active β-catenin allele from the type I collagen promoter increased bone mass and rescued the skeletal defects in Lrp-5 deficient animals (Glass et al. 2005). In addition, β-catenin seems to promote osteoblast survival and differentiation: stable expression of activated protein altered the morphology and increased the density of C3H10T1/2 cultures (Bain et al. 2003).

Some studies indicate a role for Wnt/β-catenin signaling also in early osteochondroprogenitors and provide evidence that this signaling represents a mechanism in mesenchymal precursor cells for selecting between chondrocyte and osteoblast commitment. Conditional deletion of β-catenin in limb and head during the early embryonic development results in arrest of osteoblastic differentiation and lack of mature osteoblasts in membranous bones (Day TF et al., 2005; Hill TP et al., 2005). In the absence of β-catenin, progenitors differentiated into chondrocytes instead of osteoblasts. Interference with β-catenin function at an early stage, also block osteoblast precursors differentiation that instead develop in chondrocytes (Hill TP et al., 2005). These data are also confirmed by the finding of high levels of β-catenin and activation of canonical Wnt signaling in osteoblastic precursors in developing skull and limb bones (Day TF et al., 2005). If β-catenin regulates positively osteoblast differentiation, on the other side has been seen to be a negative regulator of osteoclast differentiation. Mice lacking Tcf-1, one of the potential targets of β-catenin signaling, have low bone mass with increased osteoclast number; Tcf-1 can both bind to a site in the OPG promoter and transactivate an OPG reporter in cells (Glass et al., 2005).

## 1.3 Mesenchymal stem cells

The non-hematopoietic Mesenchymal Stem Cells (MSCs) of bone marrow were discovered by Friedenstein (Friedenstein et al. 1974a; Friedenstein et al. 1974b), who described clonal, plastic adherent cells from bone marrow capable of differentiating into osteoblasts, adipocytes, chondrocytes (Friedenstein et al. 1974b; Pittenger et al. 1999; Sekiya et al. 2002), neuronal cells (Dezawa et al. 2004) and hepatocytes (Luk et al. 2005). These cells also support *ex vivo* culture of hematopoietic cells by providing extracellular matrix components, cytokines, and growth factors. Many investigators have demonstrated that multi-potent MSCs also can be recovered from several adult tissues and can differentiate into a variety of tissue lineages including condrocytes, myoblasts, hepatocytes and possibly even neural tissues (Reyes M et al., 2001; Jang Y et al., 2003).

Their ability to generate almost all the mesenchymal lineages of connective tissues has supported the idea that they represent, or contain, a population of mesenchymal stem cells from which all mesenchymal lineages originate under the influence of different microenvironments (Caplan 1991; Owen and Karsenty 1998).

In fact, plated at low density, single precursor cells are capable to form colony-forming units-fibroblastic (CFU-Fs) and give rise to distinct and heterogeneous colonies.

These colonies have been shown, under specific stimuli, to undergo to osteogenic, chondrogenic and adipogenic differentiation.

However, more studies remain to be done to understand whether marrow stroma contains a classically defined stem cell, that is a cell with unlimited self-renewal capacity to repopulate all the differentiated lineages (Morrison et al. 1997). It is estimated that only a low percentage (15%) of all CFU-Fs have stem cell-like proprieties, and only a portion of CFU-Fs are bone colony –forming cells (Aubin 1999; Wu et al. 2000). Immunoselection and flow cytometry procedures have been applied for enrichment of osteoprogenitor lineage cells from whole marrow (Aubin and Turksen 1996; Van Vlasselaer et al. 1994). Historically, the STRO-1 antibody identified the CFU population

in human bone marrow (Gronthos et al. 1994; Simmons and Torok-Storb 1991). Other antibodies include SP-10, a monoclonal antibody generated from human MSCs, which detects marrow stromal cells and osteoprogenitors, but not mature osteoblast (Bruder et al. 1997). The SH2 antibody, which immunoprecipitates CD105 and the HOP-26 antibody, which recognizes CD-63, also detect the more primitive cells of the osteogenic lineage, but are also present on nonosseus phenotypes. MSCs commitment to tissue-specific cell types is orchestrated by transcriptional regulators that serve as a master switches. The BMP2/4/7 proteins induce transcription factors that mediate commitment of early progenitors (stem-cell like) toward the osteogenic lineage. Potency of these factors is reflected by their ability to transdifferentiate to other phenotypes through forced expression of a transcriptional regulator, for example, by expressing either peroxisome proliferation-activated receptor γ2 (PPAR γ2) or RUNX2, in osteoblasts and adipocytes respectively, their phenotype can be changed (Jeon et al. 2003; Skillington et al. 2002). Committed cells from MSCs may dedifferentiate during proliferation and post-mitotically assume a different phenotype dependent on the local cellular environment (Park et al. 1999). Among MSC progeny, precursor cells have been identified as a bipotential adipocytes-osteoblast precursor (Aubin and Turksen 1996; Nuttall and Gimble 2004). It has been suggested that the inverse relationship sometimes seen between expression of the osteoblast and adipocytic phenotypes in marrow stroma (e.g., in osteoporosis) may reflect the ability of single or combinations of agents to alter the commitment or at least the differentiation pathway these bipotential cells will transit (Gimble et al. 1996; Nuttall and Gimble 2000). In some cases, individual colonies are seen in which both osteoblast and adipocytes markers are present simultaneously (Rickard et al. 1996). However, whether a clearly distinguishable bipotential adipo-osteoprogenitor exists or other questions about transdifferentiation in these two and other mesenchymal lineage need to be further analyzed. Factors maintaining the undifferentiated state of osteoprogenitors are being characterized and allow expanding a potential mesenchymal stem cell and osteoprogenitor population. Among these factors there is leukemia inhibitory factor (LIF), Sca-1/LY-6A, required for self renewal of mesenchymal progenitor cells

(Bonyadi et al. 2003) and interleukin-18, characterized as a mitogen for both osteogenic and chondrogenic cells (Cornish et al. 2003). Finally, transcriptional regulators, such as members of helix-loop-helix (HLH) family, have been proposed as mediators in maintaining the osteoprogenitor population (Lee et al. 1999). In summary the bone microenvironment supports continued growth and differentiation of osteoblast lineage cells by extracellular matrix (ECM) accumulation of circulating and osteoblast synthesized cytokines, growth factors, non-collagenous proteins, and other molecules that cooperate to recruit progenitor cells and to induce their maturation.

## 1.4 Cell-cell and cell-ECM interactions

Cell adhesion molecules (CAMs) are essential for a number of important cellular activities including proliferation and differentiation. Cell interactions with the ECM or neighboring cells are mediated through different types of receptors. These receptors can be divided into five major classes of CAMs: cadherins, the immunoglobulin (Ig) superfamily, selectins, integrins and mucins.

### 1.4.1 Cadherins

Cells in tissue are connected to one another and to the extracellular matrix by a diverse range of cellular junctions. A subset of these junctions is particularly important for the physical adhesion of cells to their surrounding; hence the name adherens junctions. These junctions play fundamental roles in determining and maintaining tissue organization. By the morphological criteria, the most prominent adherens junctions include focal adhesions, desmosomes, hemidesmosomes and the zonula adherens (ZA) junctions.

Cadherins constitute a major adhesion molecules class of adherens junctions that support calcium dependent, homophilic cell-cell adhesion in all solid tissues of the body. The cadherins mediate cell-cell recognition events and, in association with the actin cytoskeleton, bring about

morphological transitions that underlie tissue formation and maintain tissue architecture in the adult organism.

Cadherins are single chain transmembrane glycoproteins (Figure 1.2), with a single pass transmembrane domain, that mediate calcium-dependent cell–cell adhesion and interfere with intracellular signaling, in particular the Wnt/b-catenin pathway (MacDonald et al. 2009; Nelson and Nusse 2004). The molecular mass is about 120 kD, and they are composed of a long extracellular domain (EC), a single transmembrane domain (TM) and a small cytoplasmic C-terminus tail (CT).

Cadherin superfamily is classified in four subtypes characterized by structural differences: Classical cadherins, Atypical cadherins, Desmosomal cadherins and Proto-cadherins. Classical cadherins are also divided in type I, which includes Ncad, Ecad, Mcad, Rcad and more; and type II including, among others, VE-cadherin, Cadherin 6, and OB-cadherin. Atypical cadherins, whose function is obscure, include cadherins lacking the intracellular tail, Tcad and LIcad. Other classes of cadherins are Desmosomal cadherins, which are exclusively expressed in the desmosomes of epithelial and cardiac muscular cells (Garrod et al. 2002; He et al. 2003); and Proto-cadherins, which are only expressed in vertebrates (Nollet et al. 1999; Yagi and Takeichi 2000). Cadherin-related signaling proteins do not fall into a distinct subfamily (Nollet et al. 1999; Yagi and Takeichi 2000).

Classical cadherins interact with a number of different cytoplasmic proteins to carry out their functions, which include cell-cell adhesion, cytoskeleton anchoring and signaling (Gumbiner 2000; Takeichi 1995; Yagi and Takeichi 2000). Classical cadherins form a core protein complex that consists of a parallel (cis) cadherin dimer and the catenin polypeptides. α-Catenin interacts, through β-catenin, with the distal part of the Cadherin cytoplasmic domain, and P120 catenin interacts with a more proximal region of the cytoplasmic domain (Reynolds et al. 1994; Yap et al. 1998). α-Catenin can mediate physical links to the actin cytoskeleton, either by directly binding actin filaments (Rimm et al. 1995) or indirectly through other actin–binding proteins such as vinculin, α-actinin, and ZO1. P120 catenin also seems to influence cadherins function in several ways.

The extracellular domain of the classical cadherin molecule is a calcium binding site and consists of five cadherin-type repeats, called EC1-5 domains that are bound together by $Ca^{2+}$ in a rod-like structure (Boggon et al. 2002; Pokutta et al. 1994) and confer the ability to bind to the same cadherin.

Cadherin-mediated cell-cell adhesion occurs via the formation of cis-homodimers as extracellular $Ca^{2+}$ concentration increase to > 1mM. Upon binding and dimerization, the two cadherins of the dimer form an X complex through their EC1 and EC2 domains. This steric rearrangement allows docking of opposing cadherin dimmers through specific domains of EC1, forming a trans-homodimer and generating a "zipper" structure of multiple cis dimmers on opposing membranes (Troyanovsky et al. 1999). The cytoplasmic tail, highly conserved among cadherins, binds to β-catenin and plakoglobin, which link cadherins to the actin cytoskeleton via α-catenin; the assembly of cadherins and these cytoskeletal proteins form the adherens junctions. Both β-catenin and plakoglobin are targets of tyrosin kinases and phosphatases, which regulating their phosphorilation state modulate the strength of the adherens junction (reviewed in (Nelson and Nusse 2004)). Regulation of adhesion is also controlled by cadherin binding to other proteins as P 120 which stabilizes cadherin-β-catenin binding (Hatzfeld 1999; Reynolds et al. 1994).

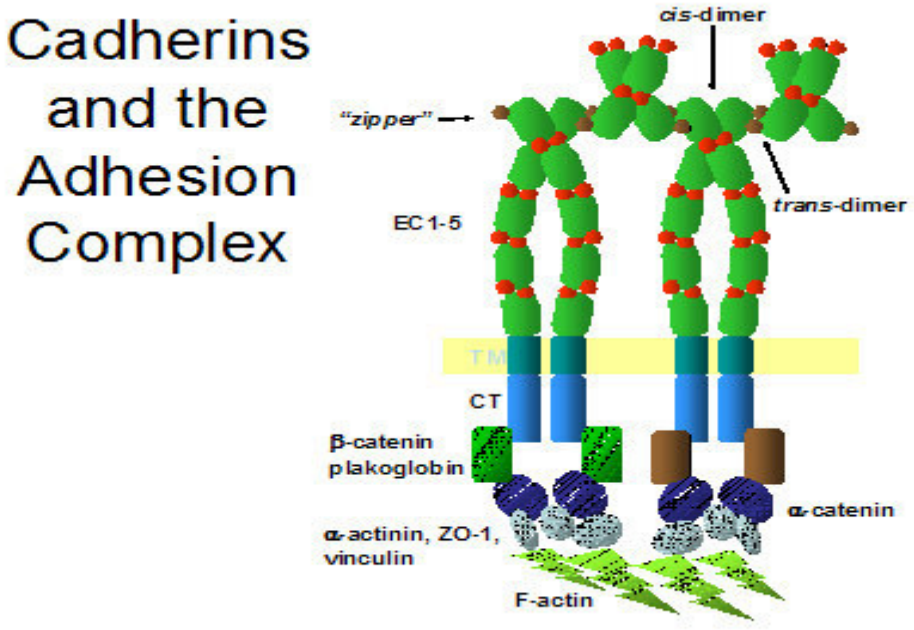


**Figure 1.2. The cadherin adhesion complex.** Cadherins are composed of five repeated extracellular domains (EC1-5), shown in green, a transmembrane domain (TM) and a short cytoplasmic domain (CT). Cadherins on the surface of the cell homodimerize with the cadherins on a proximal cell forming a $Ca^{2+}$ (in red) dependent zipper-like interaction that comprises the adherens junction. The CT is anchored to the actin cytoskeleton by accessory proteins. Some of these proteins (β-catenin) have been shown to also play an important role in signal transduction. (Stains and Civitelli 2005).

### 1.4.2 Cadherins and MSCs

Cadherins control proliferation, differentiation and survival of MSCs, which express low levels of multiple cadherins and their pattern changes as stem cells are committed to a differentiated cell lineage. Several *in vivo* and *in vitro* studies have established a main role of cadherins, in particular N-cadherin and cadherin-11, in osteoblastogenesis and bone formation either by controlling cell-cell adhesion or interacting with Wnt intracellular signaling (Bienz 2005; Brembeck et al. 2006; Schambony et al. 2004).

## 1.4.3 Integrins

Integrins are heterodimeric transmembrane receptors which mediate cell-matrix interactions whereas the other types of Cell Adhesion Molecules (CAMs) are responsible for cell-cell adhesion.

Taken together, these interactions allow cells to adhere to each other and give tissues their strength and resistance to shear forces. In addition, adhesion mediated signaling is a fundamental requirement for physiological processes such as cell survival or wound healing, and pathological conditions, such as tumorigenesis (Albelda and Buck 1990; Berrier and Yamada 2007; Lodish et al. 1995).

### 1.4.4 Cell adhesion receptors of the integrin superfamily

Integrins are a class of heterodimeric cell surface proteins consisting of non-covalently associated α and β subunits, each consisting of a large ectodomain, a single-spanning transmembrane domain and a generally short cytoplasmic tail (Arnaout et al. 2005; Luo and Springer 2006). The extracellular part of an integrin can bind a specific repertoire of ECM proteins, among them there are fibronectin, laminin, collagens, tenascin, vitronectin, osteopontin, bone sialoprotein, and dentin matrix protein-1 (Docheva et al. 2007). Besides, integrins transmit crucial signals inside the cells: the cytoplasmic tail interacts with numerous proteins which can eventually connect the integrin to the cytoskeleton. The α and β subunits show no homology to each other, but the different subunits have similarities among themselves. In mammals, different combinations among 18 α- and eight β-subunits have been identified resulting in 24 heterodimers. It is possible to classify the integrins on the basis of ligand-binding properties or their subunit composition (Figure 1.3).

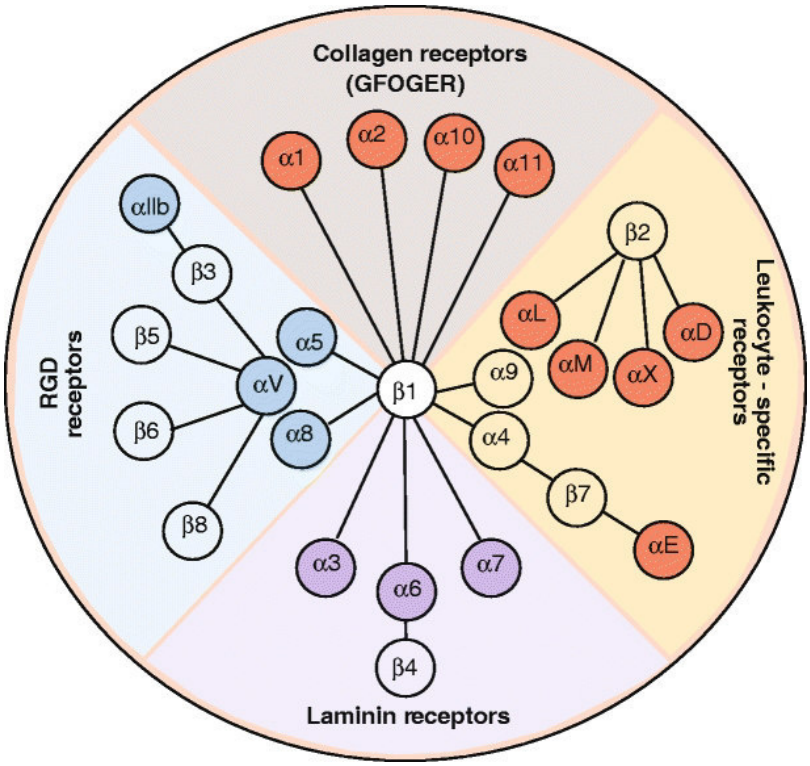


**Figure 1.3. The integrin receptor superfamily.** Integrins are heterodimers consisting of α and β subunits. In vertebrates 18 α-subunits can assort with 8 β-subunits to form 24 distinct integrins. Adapted from (Barczyk et al. 2010).

Since integrins form a direct link between the ECM and the actin cytoskeleton of the respective cells, this diversity in integrin subunit composition contributes to diversity in ligand recognition, binding to cytoskeletal components and coupling to downstream signaling pathways (Barczyk et al. 2010; Humphries et al. 2006; Hynes 2002).

Several experiments in knockout mice have shown that each integrin has specific and nonredundant functions. The knockout of some essential integrins leads to early embryonic lethality, while the lack of other integrins results in nearly normal phenotypes with only minor tissue-specific abnormalities (Bouvard et al. 2013; Hynes 2002).

### 1.4.5 Integrin activation and integrin-mediated cell adhesion

Cells respond to the mechanical and biochemical changes in ECM through the crosstalk between integrins and the actin cytoskeleton. In order to function correctly it is absolutely pivotal that integrin activity is strictly controlled and regulated.

The receptors can be activated either by ECM protein binding to the receptors' extracellular domain (= outside-in signaling) or by binding of integrin activating proteins such as talins or kindlins to the intracellular domain of integrins (= inside-out signaling). Therefore, integrins posses the unique ability to transmit signals bidirectionally across the plasma membrane and regulate many biological functions (Luo and Springer 2006; Shattil et al. 2010).

Upon activation, integrins cluster and recruit integrin-associated proteins in specialized adhesion structures that differ from each other in their morphology, subcellular localization, lifespan and protein composition.

At the leading edge of motile cells, a network of branched actin drives lamellipodium protrusion. In this part of the cell the first adhesion structures become visible by microscopy as nascent adhesions. This type of adhesion complex is highly transient and either disassembles or matures into the larger focal complexes (FXs). FXs are short-lived structures which can be found in the transition zone between the lamellum and the lamellipodium (Figure 1.4).

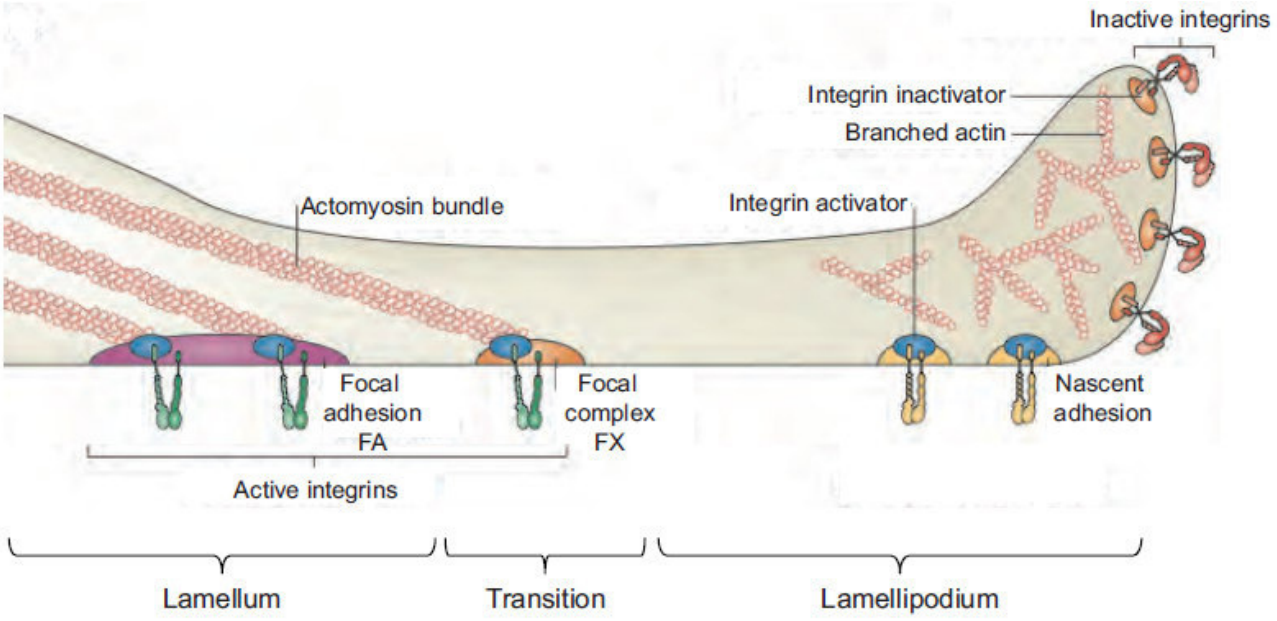


**Figure 1.4. Integrin-mediated adhesion maturation.** At the leading edge of motile cells nascent adhesions are the first adhesion structures that can be observed. They are highly transient and either disassemble or mature into larger focal complexes (FX), which can be found at the transition zone between the lamellum and lamellipodium. FX are already linked to the actin cytoskeleton. Adhesion is further strengthened during the subsequent maturation into focal adhesions (FAs). Finally FAs can develop into fibrillar adhesions, which are involved in matrix remodeling. Adapted from (Bouvard et al. 2013).

They typically contain, amongst others, $\beta_3$ integrin, talin and vinculin and are already linked to the actin cytoskeleton. The subsequent maturation of nascent adhesions and FXs into focal adhesions (FAs) is force dependent and induces stronger association between integrin and actin. Additional proteins are recruited to the structures that contain such proteins as $\alpha_v\beta_3$, paxillin and zyxin. FAs play an important role in cell motility: they provide robust anchor points to the ECM, therefore allowing to pull the cell body forward but may also restrain the migration process.

Finally, FAs can develop into fibrillar adhesions (FBs) that are located at more central positions of the cell. FBs are found in association with fibronectin fibrils, containing $\alpha_5\beta_1$ integrin and tensin and being involved in matrix remodelling. Adhesion maturation is a very dynamic process and all adhesion types can be turned over to facilitate cell movement (Bouvard et al. 2013; Geiger and Bershadsky 2001; Geiger et al. 2001; Sun et al. 2014; Zaidel-Bar et al. 2003; Zaidel-Bar et al. 2004).

### 1.4.6 Role of cell adhesion to the ECM in regulating osteogenic differentiation

Integrins are crucial in ECM assembly and cell fate control since they interact with multiple extracellular and intracellular ligands. Several studies suggest that cues from the ECM regulate not only cell adhesion, proliferation and migration but also differentiation by activating specific integrin subunits and heterodimers (Cheng et al. 2001; El-Amin et al. 2002; Hamidouche et al. 2009; Schneider et al. 2001). In detail, the binding of integrins to their ligands induces the recruitment and phosphorylation of focal adhesion kinase (FAK) and subsequent activation of key signaling proteins including phosphatidylinositol 3-kinase (PI3K), mitogenactivated protein kinase (MAPK) extracellular signal-regulated protein kinases ERK1/2, protein kinase C (PKC), and GTPases of the Rho family (Kim et al. 2011). Integrins, by using these signaling proteins, are able to modulate both focal adhesion dynamics and cellular functions (Kim and Kim 2008).

| Integrin | Ligand | Function |
|---|---|---|
| $\alpha_1\beta_1$ | Collagen 1 | Cell adhesion, differentiation |
| $\alpha_2\beta_1$ | Collagen 1 | Cell adhesion, differentiation, survival |
| $\alpha_4\beta_1$ | Fibronectin | Cell adhesion, differentiation, bone formation |
| $\alpha_5\beta_1$ | Fibronectin | Cell adhesion, differentiation, survival, bone formation, mechanotransduction |
| $\alpha_v\beta_3$ | Fibronectin<br>Vitronectin<br>Osteopontin<br>Bone Sialoprotein | Cell adhesion, differentiation |
| $\alpha_{11}\beta_1$ | Collagen 1 | Cell adhesion, survival |

**Table 1:** Roles of the main integrins involved in osteoblastogenesis. Adapted from (Marie 2013).

It has been shown that $\alpha_5\beta_1$ integrin and its interaction with fibronectin is necessary for preosteoblast adhesion to the ECM and their subsequent differentiation into mature osteoblasts (Damsky 1999). Experiments in growing mice showed that disruption of $\beta_1$ leads to skeletal defects and impairment of $\beta_1$ integrin signaling in mature osteoblasts results in decrease of osteoblast activity, bone formation and bone mass (Globus et al. 2005; Iwaniec et al. 2005; Zimmerman et al.

2000). Interestingly, Hamidouche et al. (Hamidouche et al. 2009) showed that the expression of $\alpha_5$ integrin is upregulated in MSCs under osteogenic conditions and that activation of this subunit is sufficient to induce osteoblastic differentiation. These observations also implicate $\alpha_5\beta_1$ integrin in the control of osteoblastogenesis.

In addition, Schneider et al. (Schneider et al. 2001) suggested that adhesion and signaling via $\alpha_v\beta_3$-integrin might be required for osteoblastic differentiation. Although these reports show evidence that integrin-mediated adhesion influences and regulates osteoblastic differentiation, some of the results are still a matter of debate (Cheng et al. 2001). In particular, the specific role of different integrins in MSC differentiation remains to be elucidated. Moreover, it is still unknown whether initial attachment and spreading of cells due to integrin binding to RGD motifs of different ECM glycoproteins may affect cell commitment.

## 1.5 Vitamin D

Vitamin D (Vit D) is crucial for many biological processes, i.e. the bone mineralization of vertebrates, the maintenance of calcium homeostasis, cell proliferation and differentiation.

The actions of Vit D are carried out by its active metabolite, 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$ or calcitriol] that is produced through a series of enzymatic steps starting from cholecalciferol or Vitamin $D_3$.

The ergocalciferol, or Vitamin $D_2$, follows the same metabolic stages, so both Vitamin $D_2$ and Vitamin $D_3$ introduced with the diet are biologically active in humans; the main portion of Vitamin $D_3$ is derived from the conversion of 7-dehydrocholesterol (Provitamin D) after the exposure of the skin to ultraviolet radiation. Age, skin surface exposed to the sun and thickness, irradiation time are all factors that control the Vit D synthesis (Holick 2006b).

Foods rich in Vitamin $D_3$ are marine fish (especially salmon, sardines, herring and cod liver oil), liver, egg yolks and mushrooms; milk, butter, oil, orange juice and other foods are sometimes supplemented with Vit D.

Foods provide only few units of Vit D compared to the amount produced by the skin in response to sunlight.

Vit D is fat-soluble and is absorbed in the duodenum and jejunum and subsequently distributed through the lymphatic circulation almost to the adipose tissue from which it is then released in small amounts. So a higher fat mass decreases the concentration of Vit D and this would explain why the Vit D deficiency is more elevated in obese people (Holick 2006b).

Vit D is first hydroxylated in the liver by the cytochrome P450 25-hydroxylase to 25-hydroxyvitamin D [25(OH)D] also called calcidiol (DeLuca 2004). The 25(OH)D is the fundamental circulating metabolite of Vit D, binds to alpha-globulin in plasma and its concentrations in serum are the most reliable biochemical index of the state of vitamin repletion (Reichel et al. 1989). Subsequently the 25(OH)D is transformed, in the renal tubules, either in 1,25-dihydroxyvitamin D, also called calcitriol [$1,25(OH)_2D$], (which regulates calcium, phosphorus, and bone metabolism) by the 1-alpha hydroxylase enzyme (CYP27B1), or in 24,25-dihydroxyvitamin D by the enzyme 24-hydroxylase (CYP24A1).

From the metabolic standpoint, Vit D is regulated by calcium, phosphorus, parathormone (PTH) and calcitonin (DeLuca 2004). PTH stimulates hydroxylation of $25(OH)D_3$ to $1,25(OH)_2D_3$, which acts at intestinal level by increasing the absorption of calcium and phosphorus.

Calcitonin stimulates the 1-alpha hydroxylase enzyme in the kidney to ensure the presence of circulating Vit D.

Vit D deficiency during fetal life and infancy, can cause rickets and an increase in bone fractures, while the hypovitaminosis D in adult life can exacerbate a situation of osteopenia or osteoporosis, osteomalacia, can cause muscle weakness and increasing risks of fracture.

One of the most common causes of hypovitaminosis is obesity. It has been showed, in fact, an inverse correlation between levels of 25-hydroxyvitamin D and BMI (Body Mass Index), due to a seizure of it in the adipose tissue (Kremer et al. 2009; Wortsman et al. 2000).

$1,25(OH)_2D_3$ exerts its action by binding to a specific nuclear receptor, i.e. the Vitamin D receptor (VDR). It is a member of the class II steroid hormones and, as such, it possesses the C-domain, a DNA-binding domain, the E-domain, a ligand-binding domain, and an F-domain, which is one of the activating domains (DeLuca 2004).

$1,25(OH)_2D_3$ interacts also with a putative cell membrane receptor (VDR(mem1,25)) creating non genomic effects. This interaction is linked to signal transduction systems that can mediate the opening of calcium and chloride voltage gated channels as well as the activation of MAP-kinase (Norman et al. 2002).

Mice with targeted ablation of the nuclear VDR (conventional-VDR knockout mice) represent an important model to study the actions of the system $1,25(OH)_2D_3$/VDR. The KO mice showed, after weaning, alopecia, an early development of hypocalcemia, which in turn induces a state of secondary hyperparathyroidism, infertility and severely impaired bone formation: these are typical features recurrent in humans with vitamin D-dependent rickets type II (Erben et al. 2002; Li et al. 1997; Takeyama et al. 2007; Yoshizawa et al. 1997).

The VDR, like other nuclear receptors, is expressed in different cell types including Bone Marrow Stromal Cells (BMSCs), osteoblasts and adipocytes (Gruber et al. 1999; Kong and Li 2006; Langub et al. 2000; Schneider et al. 2005).

### 1.5.1 Vitamin D and osteoblasts

The VDR KO mice have an increase in the size and number of adipocytes in the cultures of BMSCs, compared to those made from wild-type mice. This increase of adipogenesis in VDR knockout cultures is associated to an increase of the expression of inhibitors of Wnt, Dkk1 and SFRP2 (Cianferotti and Demay 2007). The Wnt pathway is an essential stage for osteoblastogenesis

and it has been shown to promote osteogenesis and inhibit adipogenesis (Ross et al. 2000). This effect is at least partly due to a ligand-dependent action of the VDR, as $1,25(OH)_2D_3$ suppresses the expression of Dkk1 and SFRP2 in wild-type BMSCs cultures. Therefore, vitamin D3, by binding VDR, suppresses adipogenesis of BMSCs. This is, at least in part, due to a suppression of the expression of inhibitors of the canonical Wnt signaling pathway (Cianferotti and Demay 2007).

Pluripotent precursors exist, among BMSCs, capable of giving rise to multiple cell lines, including osteoblasts and adipocytes, whose differentiation is often mutually inhibited. The canonical Wnt pathway plays a key role in the early stages of differentiation of BMSCs, promoting bone formation and inhibiting adipogenesis. The ablation of the VDR does not alter the osteoblastic differentiation of BMSCs; however, if the cells from VDR KO mice are grown in adipogenic conditions, they express higher levels of mRNA for PPAR-γ, the master gene in the regulation of adipocyte differentiation, compared to those obtained from wild-type mice (Cianferotti and Demay 2007).
Recently, it was also observed that the fatty component of the bone marrow significantly increases with age. This could increase the commitment of mesenchymal precursors towards the adipogenic line rather than the osteoblastic line, with a further reduction of neo-formative activity of bone tissue (Wehrli et al. 2000).

Osteoblasts are among the cells expressing VDR, therefore they represent a functional target of $1,25(OH)_2D_3$ action.
$1,25(OH)_2D_3$ can affect human osteoblast growth and differentiation stimulating bone formation and mineralization (van de Peppel and van Leeuwen 2014). Moreover $1,25(OH)_2D_3$ modulates bone deposition process preventing excessive and pathological mineralization (Woeckel et al. 2013).
The effects of $1,25(OH)_2D_3$ on bone matrix protein expression have been also studied in cultures of calvaria cells, comparing young cells with more differentiated ones for different time periods.

Following treatment with $1,25(OH)_2D_3$, mature osteoblasts were more inhibited, favoring those which were in an earlier state of differentiation (Bellows et al. 1999).

Vit D effects on osteoblast differentiation may be dissimilar according to the animal species considered; a discrepant responsiveness has been shown between two categories: human/rat osteoblasts and murine osteoblasts. Thus, in contrast to the stimulatory effect of Vit D on human and rat osteoblasts, Vit D has been demonstrated to determine an inhibitory effect on murine osteoblasts (van Driel and van Leeuwen 2014).

Vit D has been reported to induce Alkaline Phosphatase activity and to increase Collagen Type I expression in the course of proliferation and differentiation of human osteoblasts; this effect is less evident when lower concentrations are used (van Driel et al. 2004).

Kveiborg M et al. have shown that $1,25(OH)_2D_3$ is able to offset, in aging osteoblasts *in vitro*, the reduction of gene expression which is normally required for osteoblast functionality (Kveiborg et al. 2001).

Osteoblasts precursors are present on periosteal surface and in bone marrow, moreover they can differentiate from Mesenchymal Stem Cells (MSCs).

Adult stem cells are pluripotent cells capable of regenerating different tissues. Bone marrow contains cells with a high clonogenic capacity, the Colony-Forming Units Fibroblasts (CFU-Fs), which give rise to the cells of bone tissue, cartilage tissue, adipose and fibrous tissues (Friedenstein 1990). These CFU-Fs cells are called MSCs.

MSCs, can be induced to differentiate into osteocytes, adipocytes or chondrocytes (Pittenger et al. 1999), and also neuronal cells (Dezawa et al. 2004) or hepatocytes (Luk et al. 2005).

It is estimated that about 15% have a "stem" power, and among them there are those who can give origin to osteoblasts (Friedenstein 1990). The commitment of MSCs towards differentiated cell lines is regulated by transcriptional mechanisms called master switches.

The signal induced by Wnt/β-catenin is the initial step in order to determine the commitment of MSCs towards the osteoblastic line (Logan and Nusse 2004). Wnt can stimulate the differentiation

of MSCs towards osteoblastogenesis rather than towards adipogenesis, inhibiting the transcription of PPAR-γ and inducing that of RUNX2 (Gaur et al. 2005).

RUNX2 is a transcription factor with a key role in the control of osteoblast differentiation and function.

In the early embryonic stages RUNX2 can be identified already after 9.5 days in the notochord, after 10.5 in the skeleton in growth and allows osteoblast differentiation of multipotent stem cells (Ducy et al. 1999). RUNX2 binds to OSE2, which is located in the promoter region of many genes that control osteoblastogenesis. In fact, both endochondral and membranous ossification are completely absent in Murine RUNX2 knock-out (Ducy et al. 1999).

The proteins whose genes are regulated by RUNX2 during osteoblastogenesis are osteocalcin, osteopontin, bone sialoprotein, galectin, I receptor of TGFβ, dentin, sialofosfoprotein-1, collagenase 3, osteoprotegerin and RANKL (Yin and Pacifici 2001).

$1,25(OH)_2D_3$ is an important regulator of the RUNX2, with which it cooperates in inducing the expression of Osteocalcin, that is the key protein regulating bone matrix mineralization. (Li and Xiao 2007).

However there are still many doubts about the existence of a direct interaction of $1,25(OH)_2$ vitamin $D_3$ on the promoter of RUNX2 (Li and Xiao 2007).

### 1.5.2 Vitamin D and MSCs

It has been demonstrated that $1,25(OH)_2D_3$ influences Human MSCs (hMSCs) inhibiting cell proliferation and stimulating their differentiation into osteoblasts (Geng et al. 2011; Liu et al. 1999a). It has also been shown, in hMSCs pre-implantation cultures, that Vit D is able to determine the formation of mature osteoblasts, resulting in an increase of mineralized matrix formation, but after implantation of these cells the same Vit D is not sufficient in enhancing bone formation (De Kok et al. 2006). That leads to assume a different *in vivo* effect of Vit D.

In order to overcome some negative aspects connected with the use of MSCs from bone marrow, i.e. morbidity and pain, other sources of MSCs have been examined.

MSCs can be isolated from tissues different from the bone marrow such as adipose tissue, brain, skin, liver, several fetal tissues and so on; little is known about Vit D influence in osteogenic differentiation of MSCs from these alternative sources. Anyway, with MSCs from adipose tissue, the capacity of Vit D in osteoblast formation has been confirmed (Logovskaya et al. 2013).

According to the evidence based research dental tissues represent an alternative and promising source of post-natal MSCs (Ballini et al. 2007a; Di Benedetto et al. 2014; Gronthos et al. 2000; Miura et al. 2003; Mori et al. 2013).

Deciduous teeth, or the wisdom tooth, that is a tooth with a limited chewing function and that often creates problems of overcrowding, can be used to isolate MSCs. In the case of immature teeth, a productive source of MSC is the Dental Bud (DB) that consists in non calcified tissues; in the case of the mature tooth the sources are the Periodontal Ligament (Ballini et al. 2007b), the Dental Pulp (DP) (Ballini et al. 2007b; Giorgini et al. 2011; Mori et al. 2011; Mori et al. 2010), and the Apical Papilla (Sonoyama et al. 2008).

Dental Pulp Stem Cells (DPSCs) are obtained from wisdom teeth pulp of adult donors, while Dental Bud Stem Cells (DBSCs) come from the DB. The DB, which is the immature form, and therefore not yet fully calcified of the tooth, is an excellent source of stem cells, since it is considerably larger, it is constituted of cells more undifferentiated than the ones composing the pulp and can be removed in children of age between 8 and 12 in case of expected overcrowding with a safe technique called piezo-surgery.

The advantage of the tooth bud, compared to the pulp, is first of all dimensional, it contains a greater number of cells, moreover almost all the tissue is made from stem cells that have a high proliferative capacity and an excellent degree of stemness (Di Benedetto et al. 2015; Di Benedetto et al. 2014; Mori et al. 2012).

The tissues constituting the mature tooth: cement and periodontal ligament, enamel, dentin, pulp, the central part of the bud, corresponding to the dental papilla, are all derived from DBSCs, obtained from the DB, thus containing MSCs which can successfully differentiate into osteoblast-like cells.

DBSCs are more undifferentiated MSCs, if compared to the ones isolated from the bone marrow, for this reason they can be considered an ideal model for studying the early stages of the osteoblastic differentiation process.

The differentiation into osteogenic lineage has already been demonstrated in DPSCs (Ballini et al. 2007b; Ballini et al. 2014; Mori et al. 2011; Mori et al. 2010) and a considerable mineral matrix deposition has been observed using innovative scaffolds for their culturing (Marrelli et al. 2015; Paduano et al. 2016); moreover these cells, if cultured on β-tricalcium phosphate/poly (l-lactic acid/caprolactone) three-dimensional scaffolds, showed an increased osteogenesis in the presence of Vit D compared to Dexamethasone (Khanna-Jain et al. 2012).

No data were present in literature about Dental Stem Cells and Vit D interaction, before our paper (Posa et al. 2016).

## 1.6 Aim of the study

The main general idea of this work is that the active metabolite of Vit D, $1,25(OH)_2D_3$, can influence DBSCs differentiation directing the process into the formation of osteoblast like cells.

The idea comes from the observation that osteoblasts, the bone forming cells, treated with Vit D, are influenced in their growth and differentiation being stimulated in bone formation and mineralization (van de Peppel and van Leeuwen 2014). In particular several studies have shown that Vit D acts on MSCs from bone marrow inducing their osteoblastic differentiation (Geng et al. 2011; Liu et al. 1999a).

The most characterized source for MSCs is still the bone marrow; however, in the past decade, populations of stem cells have been isolated from different dental tissues (Gronthos et al. 2000; Miura et al. 2003).

Dental Bud Stem Cells (DBSCs) could represent a useful model to understand the metabolic activity of the bone cells, starting from very undifferentiated osteoblasts precursors.

To confirm the hypothesis of the harvested cells stemness, we first studied the expression of MSC surface markers in our cell model.

Since MSCs adhesion to Extra Cellular Matrix (ECM) is still poorly characterized, as well as protein matrix role in regulating osteogenic differentiation, we specified our cell model of DBSCs for the expression of four different integrin subunits and receptor heterodimers. Then we analyzed the effect of the adhesion on three ECM glycoproteins containing the integrin binding sequence RGD, namely Fibronectin (FN), Vitronectin (VTN), and Osteopontin (OPN) and adhesion receptors changes in the course of the DBSCs osteogenic differentiation process.

Therefore we stimulated DBSCs using physiological concentrations of Vit D to mimic the condition of a proper intake of $1,25(OH)_2D_3$.

We analyzed the expression levels of Alkaline Phosphatase in DBSCs treated with Vit D and cells capacity to form calcium-rich deposits using Alizarin Red, a histochemical assay. Then we evaluated the main osteoblastic markers in DBSCs during the different steps of their osteogenic differentiation.

Little is still known about the influence of matrix molecules on integrin mediated structures during the osteogenic differentiation process and there are no data in literature regarding a possible action of Vit D in this process.

With the purpose of analyzing how Vit D may affect cell adhesion, we focused on the organization of integrin $\alpha_V\beta_3$ in cells treated with Vit D on surfaces coated or not with FN.

Several studies suggest that cues from the ECM regulate not only cell adhesion and migration but also differentiation by activating specific integrin subunits and heterodimers (Cheng et al. 2001; El-Amin et al. 2002; Schneider et al. 2001). Especially the integrins $\alpha_5$ and $\alpha_v\beta_3$ are suggested to be required for osteoblastic differentiation (Hamidouche et al. 2009; Schneider et al. 2001). However, it remains to be elucidated whether initial attachment and spreading of cells due to integrin binding to RGD motifs of different ECM glycoproteins may affect cell commitment.

An approach to better define, engineer, and analyze cell behavior is the fabrication of micropatterned adhesive substrates, which present spatially defined ECM proteins surrounded by non-adhesive molecules to backfill the area in between (Folch and Toner 2000; Jackman et al. 1999; Whitesides et al. 2001; Zheng et al. 2013).

Considering these assumptions, we tried to inquire if Vit D may modulate the early stages of the cell adhesion process, with our cell model, using gold nanostructured surfaces.

# *2.Materials and methods*

## Patients, materials and methods

### *2.1 Materials*

1α,25-Dihydroxyvitamin $D_3$, Ascorbic Acid, β-Glycerophosphate, Dexamethasone, Alizarin Red powder, Alkaline Phosphatase kit, Poly-L-Lysine (PLL), Fibronectin (FN), Osteopontin (OPN), and Vitronectin (VTN) were from Sigma Aldrich, Milan, Italy.

Antibody anti RUNX2 was from Abnova, anti Coll I, anti OPN, anti BSP II were from Abcam, Cambridge, UK.

Antibody anti N-Cadherin, E-Cadherin, P-Cadherin, Integrin $\alpha_5$, $\alpha_V$, $\beta_1$, and $\beta_3$ were from BD Bioscience; antibody anti Cadherin-11 was from Zymed; Antibody anti-$\alpha_V\beta_3$ clone LM609 and antibody anti-$\alpha_5\beta_1$ clone JBS5 were from Millipore.

### 2.2 Patients and Cell cultures

Normal human third molar buds were collected from tooth buds of 10 healthy pediatric patients, 8-12 years of age, that underwent extractions for orthodontic reasons, mainly overcrowding, after informed consent from both patient parents.

The study was approved by the Institutional Review Board of the Department of Clinical and Experimental Medicine, University of Foggia and patients' parents gave written informed consent.

The central part of DBs, corresponding to the dental papilla, was placed in sterile culture dish and cut in small pieces in sterile conditions under laminar flow hood with a sterile scalpel, digested with agitation for 1 hour at 37°C in a solution of 3 mg/ml type I collagenase plus 4 mg/ml dispase (Gibco Ltd., Uxbridge, UK). Single-cell suspension was obtained by passing the cells through a 70 μm BD Falcon strainer (Falcon) (Becton & Dickinson, Sunnyvale, CA).

After filtration, single cell suspension was centrifugated at 1300 rpm for 5 min; the pellet was resuspended and cultured in Mesenchymal Stem Cell Culture medium supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin-G, 100 μg/ml streptomycin (Gibco Limited, Uxbridge, UK). Cells were seeded at a density of $5x10^3$ cells/$cm^2$.

Flasks were incubated at 37°C and 5% $CO_2$ and the medium was changed every 3 days.

In order to induce osteoblastic differentiation, 1500 cells/$cm^2$ were seeded and cultured in osteogenic medium consisting of α-MEM supplemented with 5% FBS, $10^{-8}$ M dexamethasone and 50 μg/ml ascorbic acid (Sigma Aldrich, Milan, Italy).

For the evaluation of DBSCs ability to form mineralized matrix nodules *in vitro*, cells were cultured in the osteogenic medium supplemented with 10 mM β-glycerophosphate (Sigma Aldrich, Milan, Italy).

$1,25(OH)_2D_3$ (Sigma Aldrich, Milan, Italy) was reconstituted at $10^{-4}$ M in 95% ethanol and stored at -20°C.

For all cell cultures, a 95% ethanol (vehicle) control was included at a concentration equivalent to that of Vit D. Thus cells were cultured in replicates with $1,25(OH)_2D_3$ and an equivalent dilution of 95% ethanol.

**2.3 Immunophenotypic analysis**

The following fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated mAbs were used for immunofluorescent staining of DBSCs: PE-CD73, PE-CD90, sotype control (all BD-Pharmingen, Milano, Italy), as well as FITC-CD105, and FITC-CD45 (Beckman Coulter, Inc., Milano, Italy). Cells from each donor were washed in FACS buffer (phosphate-buffered saline pH 7.2, 0.2% bovine serum albumin, and 0.02% sodium azide) containing 3% human serum, incubated with fluorochrome-conjugated mAbs for 30 min and then washed with the same buffer before flow cytometric analysis.

Data were acquired using a MACSQuant® Analyzer 10 (Miltenyi Biotec, Inc., Milan, Italy) flow cytometer. The area of positivity was determined using an isotype-matched mAb.

### 2.4 Alizarin Red Staining (ARS)

The ability of DBSCs to generate mineralized matrix nodules *in vitro*, a feature normally attributed to osteoblasts, was assessed by performing Alizarin Red Staining on cells cultured for 21 days in osteogenic medium.

Thus, cells were gently rinsed with PBS and fixed with 10% formaldehyde at room temperature for 10 min.

Then they were washed twice with deionized water and incubated with 1% ARS solution for 10 min at room temperature. Cells were afterwards washed twice with deionized water, to remove excess staining, and air dried. The monolayer appeared red stained.

To quantify the ARS, cells were incubated with 10% acetic acid at room temperature for 30 min with shaking. The cell layer was scraped, vortexed and then the solution was incubated for 10 min at 85°C. After 5 min on wet ice, the suspension was centrifuged at 20,000*g* for 15 min and 500 µl of the supernatant were treated with 10% ammonium hydroxide to neutralize the acid. The Optical Density (OD) was read in triplicate at 405 nm.

### 2.5 Alkaline phosphatase (ALP)

The expression of alkaline phosphatase, which is a marker of osteoblast differentiation, was assessed with the Leukocyte Alkaline Phosphatase kit (Sigma Aldrich).

Cell media was removed from all wells and cells were fixed by adding 0.5 ml/well of a fixative solution for 5 min at room temperature, according to manufacturer's instructions.

Subsequently, the wells were washed with deionized water and cells were stained with 0.3 ml ALP solution (a mixture of FRV-Alkaline Solution, Naphthol AS-BI Alkaline Solution, Sodium Nitrite Solution) in each well. Following a 15 min incubation in the dark, the wells were washed again with deionized water, air dried and cells were then inspected under the microscope. ALP positive cells are stained in purple.

## 2.6 Western Blot

Detection of osteoblastic markers as protein levels was performed by SDS-PAGE gel electrophoresis and western blot analysis. DBSCs were lysed after 7, 14 and 21 days of osteogenic differentiation. Plates were washed twice with cold PBS and cells were lysed with lysis buffer (50 mmol/l TRIS-HCl (pH 8.0), 150 mmol/l NaCl, 5 mmol/l EDTA, 1% NP40 e 1 mmol/l PMSF) containing proteinase inhibitors. The supernatant was collected and protein concentrations were determined according to the method of BCA assay (Pierce). The cell lysates were cleared with a centrifugation at 13000 rpm for 15 min at 4°C. The total protein concentration of the supernatant was determined using a protein assay (BIORAD).

Equal amounts of protein for each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham, UK) with a Trans-Blot (Biorad, USA).

The unspecific sites were blocked by incubation of the nitrocellulose membranes in a solution of dry milk (BioRad) at 5% in TBS-T for 1 hour at room temperature.

The membranes were probed with primary antibodies overnight at 4°C, then samples were incubated for 90 min with secondary antibodies conjugated to horseradish peroxidase at room temperature. The reaction was analyzed with the Odyssey Infrared Imaging System of LI-COR (LI-COR Biotechnology Lincoln, Nebraska, USA).

## 2.7 Immunoprecipitation

Cells were lysed and the lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was recovered and the protein concentration was assessed by protein assay (BIORAD). The primary antibodies, LM609 and JBS5, which recognize respectively only the complex $\alpha_V\beta_3$ and $\alpha_5\beta_1$ were added to the cell lysates. They were incubated with gentle rocking (put on ice into agitation) for 2 hours at 4°C.

Protein G was added and incubated with a gentle rocking at 4°C overnight. The next day the samples were centrifuged, washed, and finally western blotted with $\alpha_V$ and $\beta_3$ antibodies, $\alpha_5$ and $\beta_1$ antibodies.

### 2.8 ECM glycoproteins and coating procedure

Tissue culture treated polystyrene surfaces were coated with Vitronectin (VN, human plasma, 0.5 μg/cm$^2$, Sigma, Steinheim, Germany), Fibronectin (FN, human plasma, 4 μg/cm$^2$, Sigma) and Osteopontin (OPN, human recombinant, 0.5 μg/cm$^2$, Sigma) diluted in 1 × phosphate buffered saline (PBS, pH 7.2, PAA, Coelbe, Germany) or water according to the manufacturers' recommendations. Poly-L-lysine (PLL, 2 μg/cm$^2$, Sigma) was used as control. The different amounts ensured the complete coating of the surface for each type of ECM protein. Surfaces were coated with the ECM protein solution for 30 min at 37°C, washed twice with PBS, blocked with 1% bovine serum albumin (BSA, Sigma) in PBS for 10 min at room temperature and sterilized with UV light for 30 min. To confirm the coating adsorption, the protein content in the coating solution was measured using a micro BCA assay. The amount of adsorbed protein was determined by calculating the difference in the coating solutions pre- and post-coating. Additionally, the coatings were validated by fluorescence microscopy using fluorescently labeled proteins.

### 2.9 Preparation of gold nanostructured surfaces

In order to control the surface density of immobilized protein gold nanoparticle arrays produced by block copolymer micellar nanolithography (BCMN) instead of homogenous gold layers can be used.

BCMN is a tool for the fabrication of nanostructured surfaces with an accessible length scale below 100 nm, which is hardly achievable with other lithographic methods (Bhusan 2010).

In this work an amphiphilic diblock copolymer, consisting of a non-polar polystyrene (PS) block and a polar poly(2-vinyl pyridine) (P2VP) block, is used to fabricate gold nanoparticle arrays on glass surfaces (Figure 2.1).

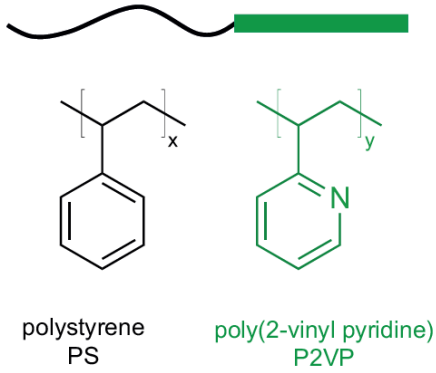


**Figure 2.1.** Diblock copolymer, consisting of a non polar polystyrene block (PS, black) and a polar poly(2-vinyl pyridine) block (P2VP, green).

### 2.9.1 Cleaning and activation of glass substrates

Glass coverslips and containers were cleaned in piranha solution, a 3:1 mixture of concentrated sulfuric acid (95%, AppliChem) and hydrogen peroxide (30%, p.a., AppliChem). In an exothermic equilibrium reaction peroxymonosulfuric acid ($H_2SO_5$, Caro's acid) is produced. It is one of the strongest oxidants known, removes organic contaminations and activates the glass surface. Substrates were sonicated for 5 min, thoroughly rinsed with MilliQ water and dried off under nitrogen flow. Glass coverslips were processed immediately.

### 2.9.2 Preparation of micellar solutions

Nanopatterning of glass substrates was performed according to Arnold et al., 2004. Polystyrene-block-poly (2-vinylpyridine), PS-b-P2VP diblock copolymer (Polymer Source Inc., Canada) (Tab. 4.1) was weighed inside a sealable and clean glass container and the appropriate volume of toluene (p.a., Merck, Germany) was added. The solution was stirred at RT for at least 16 h.

Depending on the desired loading parameter (L), which describes the molar ratio of PVP-units and the metal salt $HAuCl_4$, a stoichiometric amount of $HAuCl_4$ (Sigma-Aldrich, Schnellendorf) was added to the prepared solution. The amount of $HAuCl_4$ needed is given by equation:

$$m(HAuCl_4) = \frac{m(Polymer) \cdot M(HAuCl_4 \cdot 3H_2O) \cdot P2VP_{units} \cdot L}{M(Polymer)}$$

The molecular weight of $HAuCl_4$ depends on the content of crystal water, in this work $HAuCl_4$ trihydrate ($MHAuCl_4$) = 393.83 $gmol^{-1}$ was used. The mixture was stirred for at least another 16 h until all $HAuCl_4$ was dissolved. To remove polymer aggregates and undissolved metal salt residues, the micellar solution was purified through a polytetrauoroethylene (PTFE) filter with a pore size of 0.22 µm (Millipore, Eschborn) before the first usage. Micellar gold solutions were stored air-sealed under nitrogen and light protected. The different solutions used in this work with corresponding polymer concentration and loading parameter are listed in table 2.

| Polymer | M [$gmol^{-1}$] | c [$mgml^{-1}$] | L |
|---|---|---|---|
| PS(30000)-b-P2VP(12500) | 42500 | 5 | 0.5 |
| PS(110000)-b-P2VP(52000) | 165000 | 7 | 0.5 |
| PS(190000)-b-P2VP(55000) | 245000 | 2 | 0.5 |

**Table 2:** Polymers used for the production of nanopatterns. The amount of polymer units is indicated in brackets. MW: molecular weight of the diblock copolymer, c: concentration of the polymer, L: loading or molar ratio between $HAuCl_4$ and P2VP.

### 2.9.3 Dip coating

Glass coverslips were cleaned with precision wipes and in freshly prepared piranha solution as described before. A custom-made dip-coating device was used to retract glass coverslips from gold polymer solutions with constant velocity to retrieve a micellar monolayer. After evaporation of the solvent, substrates were exposed for 45 min to an isotropic microwave-induced hydrogen plasma (PVA TePla AG, Germany) to reduce the gold salt to metallic gold and remove the polymer. Here, either a TePla100-E (pH2 = 0.4 mbar, 150 W) or TePla PS210 (pH2 = 0.3 mbar, 600 W) was used. Both machines led to comparable gold nanostructures. One sample of each batch was analyzed by scanning electron microscopy (SEM) to ensure a comparable quality of the nanopatterned substrates.

### 2.9.4 Spin coating

Spin coating offered another possibility to decorate glass substrates with a hexagonally ordered micellar monolayer. The glass substrate was fixed on a rotary plate by applying vacuum. Then approximately 20 - 25 µl (depending on the size of the substrate: 20 x 20 $mm^2$: 20 µl; 24 x 24 $mm^2$: 25 µl) of the gold polymer solution were deposited onto the center of the spinning substrate (8000 rpm) in order to spread the fluid by centrifugal force. After evaporation of toluene substrates were plasma treated as described in subsection "Dip Coating". The advantage of this method is the homogeneous coverage of the substrate with gold nanoparticles without a dipping or dripping edge.

### 2.9.5 Covalent immobilization of PEG2000

To prevent non-specific protein and cell interactions with glass, the space between the gold nanoparticles was passivated with a protein repellent PEG2000 monolayer (Fig. 2.2).

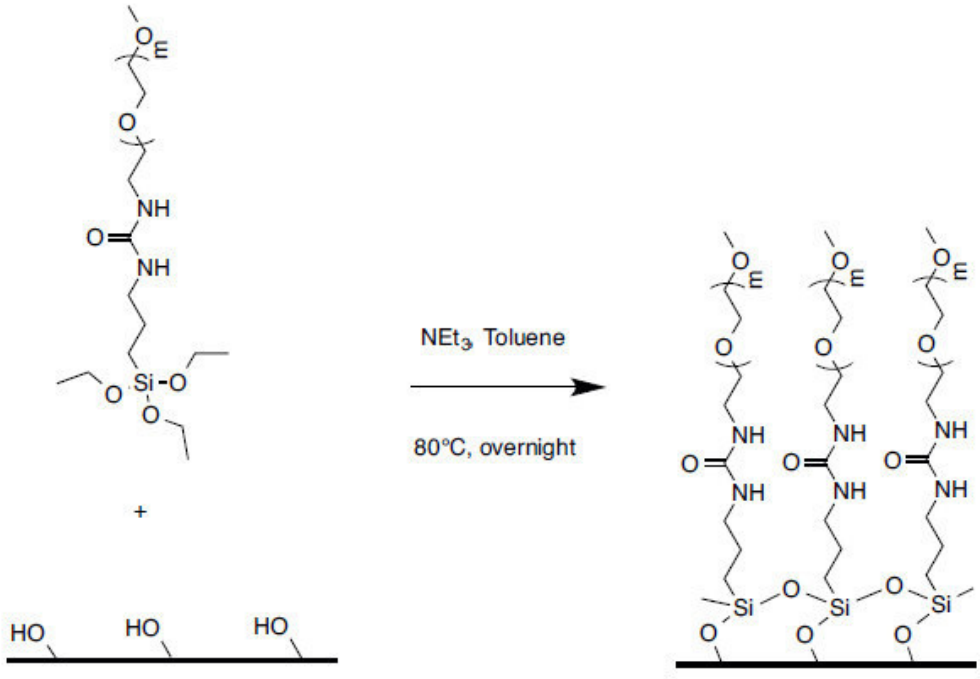


**Figure 2.2. PEG passivation of glass on nanopatterned substrates.**
Substrates were activated in hydrogen plasma prior to PEG passivation. PEG2000 was coupled via its triethoxysilane anchor groups to the hydroxy-terminated glass surface.

During this one-step reaction the triethoxysilane anchor groups of PEG2000 react with the activated, hydrophilic glass, resulting in its covalent binding to the silicon dioxide surface.

Nanopatterned surfaces were activated in reactive hydrogen plasma (pH2 = 0.4 mbar, 600 W) for 10 minutes and eventually immersed in a solution of 250 µM PEG2000 in dry toluene (p.a., Merck) and 2.5 µM of triethylamine (≥99.5%, Sigma) which acts as a catalyst for at least 12 h at 80°C . The reaction was kept under nitrogen atmosphere. After removal of the toluene, samples were first sonicated in ethylacetate (p.a.), then in methanol (p.a.) for 5 min each. After rinsing with methanol the surfaces were dried with a stream of nitrogen and immediately processed.

Briefly (figure 2.3), micelles of copolymers containing gold nanoparticles are deposited on glass coverslips; after treatment with hydrogen gas plasma, only the polymers are removed from the surface, leaving just gold nanoparticles of 8 nm diameter (Glass et al. 2003; Spatz et al. 2000). These particles form a hexagonal lattice and are separated by 28–110 nm, depending on the size of the copolymer used. The space between the gold nanoparticles is covered with polyethylene glycol ((PEG) molecular weight 2000) to prevent cell adhesion and protein adsorption. Each gold particle is biofunctionalized by binding adhesion molecules via a thiol group.

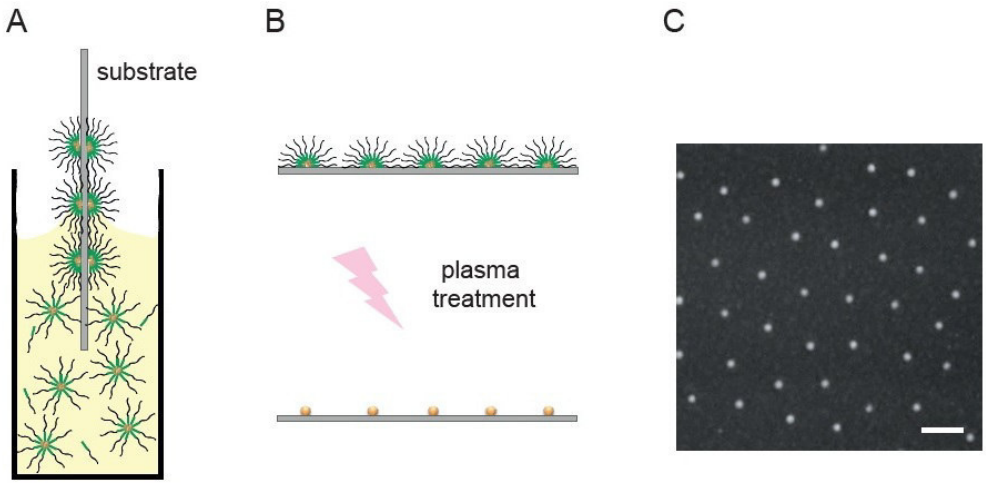


**Figure 2.3. Preparation of nanostructured surfaces by BCMN.**
Substrates are immersed into the micellar solution and retracted at constant speed (Dip Coating). Upon evaporation of the solvent the micelles self-assemble into a quasi-hexagonal packed pattern (A). Substrates are plasma treated to remove the polymer matrix and to reduce the metal precursor salt, $HAuCl_4$, to elemental gold (B). Gold nanoparticle arrays can be visualized and analyzed by scannning electron microscopy (SEM, C), scale bar = 100 nm.

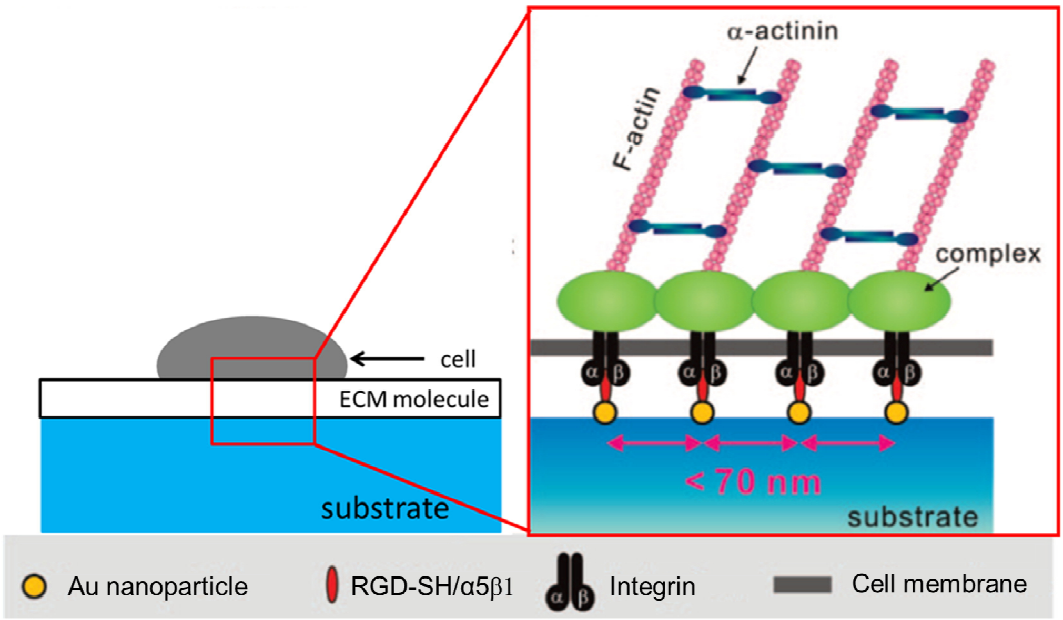


**Figure 2.4. Experimental setup.** Cells were cultured on gold nanoparticles on glass with and without adhesion molecules. The focal adhesions and contractile actin cytoskeletal stress fibers allow cell spreading, when the ligand distance is <70 nm (Wei et al. 2015).

### 2.9.6 Immunochemical reagents and indirect immunofluorescence of BCMN

Primary antibodies that were used in this study include mouse anti-human vinculin monoclonal (Sigma-Aldrich Chemie GmbH, Munich, Germany), TRITC-conjugated phalloidin (Sigma). Cultured cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized for 1 min in PBS containing 0.1% Triton X-100 (Sigma). The cells were incubated for 1 h at room temperature with the primary antibody diluted in PBS, washed, and further incubated with the secondary antibodies for 1 h. After extensive washing with PBS, coverslips were mounted samples were mounted with mounting medium (Mowiol+Dabco). Cell nuclei were stained with DAPI (Sigma) which was added to the mounting medium to a final concentration of 1 µg/ml.

Fluorescence images were acquired with a Delta Vision (DV) system (Applied Precision Inc., Canada; data processing controlled by Resolve 3D (Applied Precision Inc., USA)).

### 2.9.7 Immunofluorescence of coated surfaces

Cells were counted and seeded on glass coverslips, grown in the appropriate culture medium, and then fixed in 4% (PFA)/PBS. The fixed cells, were washed with PBS, and blocked in 1% BSA, 5% normal goat serum in PBS for 20 min. The samples were incubated with the following antibodies: $\alpha_V$, $\beta_3$, $\alpha_5$, $\beta_1$, and $\alpha_V\beta_3$ (clone LM609 antibody). After washing, bound antibodies were detected using 2 μg/ml of fluorescently labeled goat anti-mouse or anti-rabbit secondary antibody (Alexa Fluor 488, Alexa Fluor 568, Invitrogen); cytoskeleton was counterstained with Phalloidin (Invitrogen). The cells were then visualized and photographed using a multispectrum confocal microscope Leica TCS SP5.

### 2.9.10 Statistical analyses

Statistical analyses were performed by Student's t-test with the Statistical Package for the Social Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). The results were considered statistically significant for $p<0.05$.

# *3.Results*

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### *3.1. Immunophenotype of DBSCs*

In the first step DBSCs from 20 donors were tested for the criteria to be called MSCs (Dominici et al. 2006), as was done for DFSCs (Mori et al. 2012), in order to ensure that these cells had actually stem and mesenchymal feature.

Flow cytometric analysis of research accepted MSC surface markers was performed and all populations exhibited ≥95% expression of CD73, CD90, and CD105 while were negative for CD45, a common leukocyte antigen. DBSC samples from three donors were selected to perform the experiments, since they showed higher and similar percentage values of stemness marker expression. Figure 3.1 shows flow cytometry histograms from one representative DBSC culture.

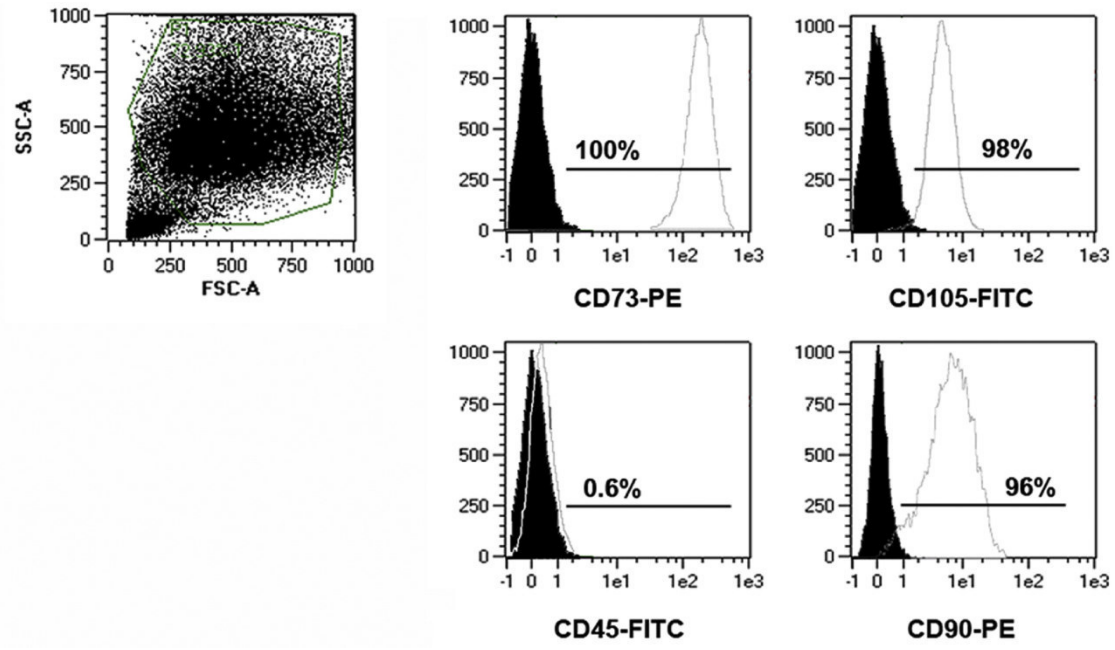


**Figure 3.1. Immunophenotype of DBSCs.** The expression of the indicated mesenchymal stem cell markers on DBSCs was analyzed using flow cytometry. Results from one representative DBSCs culture are shown. The black histograms signify staining with isotype controls, and the white histograms represent staining with the specified surface marker antibody.

### *3.2. Cadherin expression profile in DBSCs reflected their mesenchymal origin*

Expression of "classical" cadherins was investigated in undifferentiated DBSCs and during their osteogenic differentiation. The cells were kept in favoring conditions for MSC maintenance and were characterized for cadherin expression. Subcellular localization showed a high expression of N-cadherin, as well as for cadherin-11 (Fig. 3.2 A–B). Ecadherin was expressed to a lesser extent and P-cadherin was poorly expressed (Fig. 3.2 C–D). Notably, only N-cadherin was markedly localized at the cell–cell contact, perhaps contributing to maintain the stem cells niche in the dental bud.

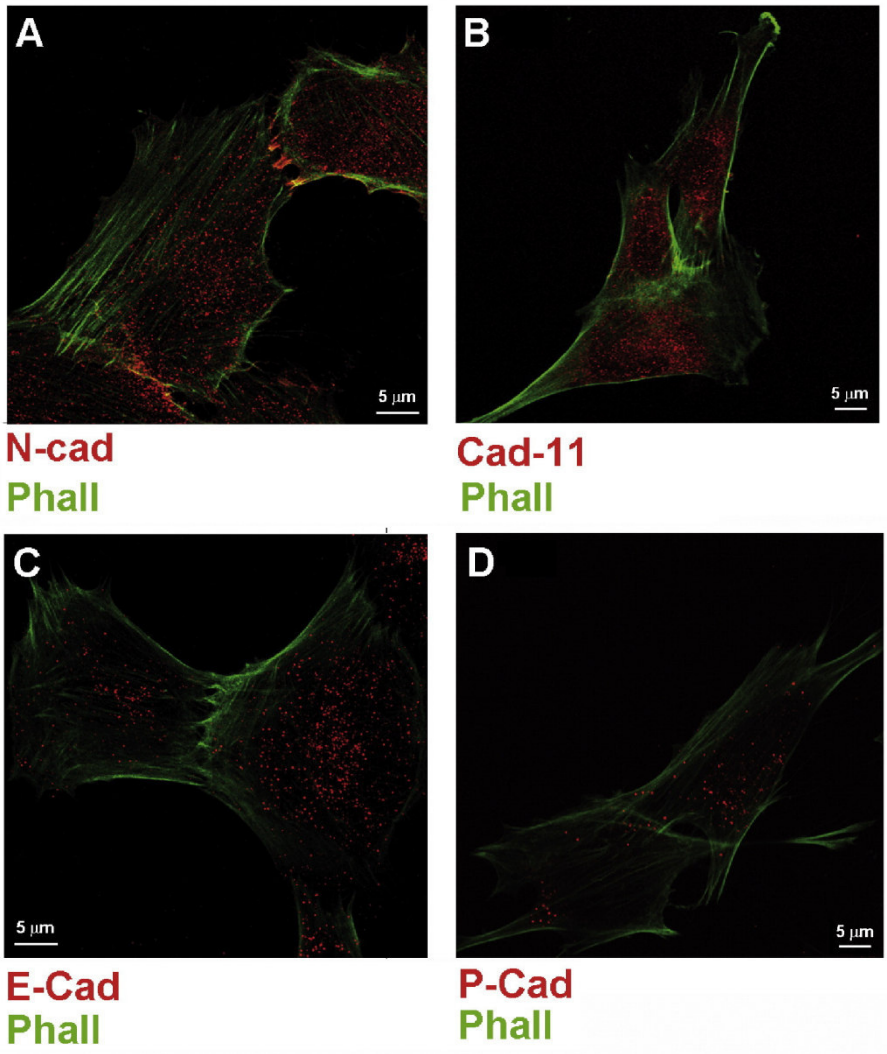


**Figure 3.2. Cadherin expression profile in DBSCs.** Midsection confocal microscopy images of DBSCs cultured in basal conditions, counterstained for different type of Cadherins (red) and Phalloindin (green). Subcellular localization shows high expression of N-Cadherin (markedly localized at the cell–cell contact) and Cadherin-11 (A–B). E-Cadherin and P-Cadherin are expressed to a lesser extent (C–D). Results are depicted for one donor but are representative of three different donors.

Thus cadherin expression profile was evaluated during osteogenic differentiation. As already demonstrated for DFSCs, (Mori et al. 2012) DBSCs cultured under osteogenic conditions differentiated into osteoblast-like cells producing mineralized matrix nodules and expressed the typical osteoblastic markers RUNX2, alkaline phosphatase (ALP), and Collagen I (Coll I) (data not shown). Cadherin expression profile was analyzed by Western blotting during the different steps of osteogenic differentiation. As shown in Fig. 3.2.1 panel E, N-cadherin was expressed in undifferentiated DBSCs, thus confirming the subcellular observation; its expression remained constant after 7 days of osteogenic differentiation, but a slight decrease initiated after 14 days keeping a low expression of differentiation at 21 and 28 days. Similarly, cadherin-11 was expressed in undifferentiated DBSCs and its expression did not change after 7 days of differentiation, however after 2 weeks cadherin-11 strongly increased and remained highly expressed also at the later stages. Thus, while N-cadherin and cadherin-11 were present in undifferentiated DBSCs and their expression underwent changes during the osteogenic process, expression of E-cadherin and P-cadherin was low in DBSCs and did not change during the differentiation steps.

Such expression profile of cadherins in DBSCs, and more precisely the changes undergoing cadherin-11 and N-cadherin (increasing and decreasing respectively), confirmed that these stem cells can acquire an osteoblastic phenotype under appropriate osteogenic conditions. The subcellular localization of cadherins was observed only during the early phases of osteogenic differentiation (3–7 days), since these cells usually reach the confluence in few days and grow in multilayers, thus preventing their microscopic observation. E-cadherin and P-cadherin were poorly expressed and homogenously distributed in DBSCs and their localization did not change after 3–7 days of osteogenic trigger (data not shown) as their expression until 28 days of differentiation (Fig. 3.2.1 panel E). Conversely N-cadherin and cadherin-11 changed their localization in response to osteogenic stimulation. In Fig. 3.2.2 F on time zero (T0) N-cadherin was localized at the cell–cell adhesion sites, being probably involved in the formation of adherens junction complexes as revealed by the “red bridges” merging with actin cytoskeleton marked by Phalloidin and resulting in

an intense yellow staining. After 3 days N-cadherin remained localized in the adherens junction complex, while after 7 days N-cadherin lost the characteristic bridge-like appearance although in part remaining at the cell–cell boundaries not changing its expression. On the contrary cadherin-11 seemed to have an opposite trend. Indeed on T0, DBSCs expressed Cadherin-11, which was not localized at the cell–cell adhesion sites, but homogenously distributed in multiple sites. After 7 days of osteogenic differentiation, cadherin-11 accumulated at the cell periphery and localized at adherens junction sites acquiring the bridge-like trend observed for N-cadherin in less differentiated cells.

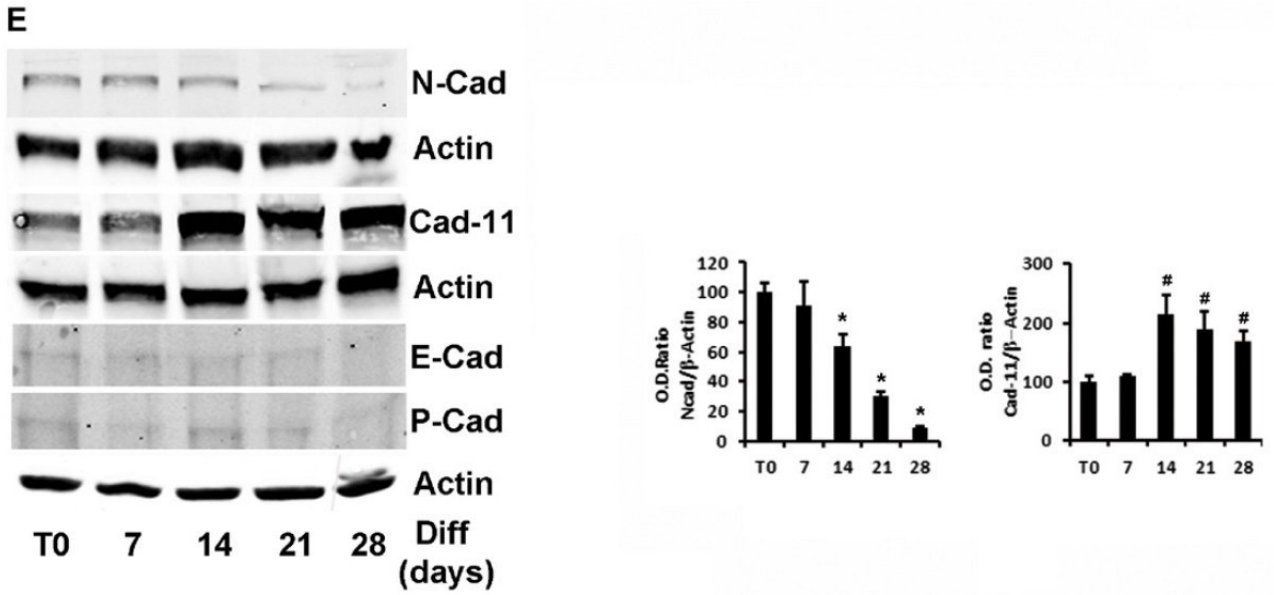


**Figure 3.2.1. Immunoblots showing the cadherin expression profile during the osteogenic differentiation process (0–28 days).** Data are presented as mean ± SEM of 3 independent donors *P < 0.01 and #P < 0.05 compared to T0. Student's t-test was used for single comparison.

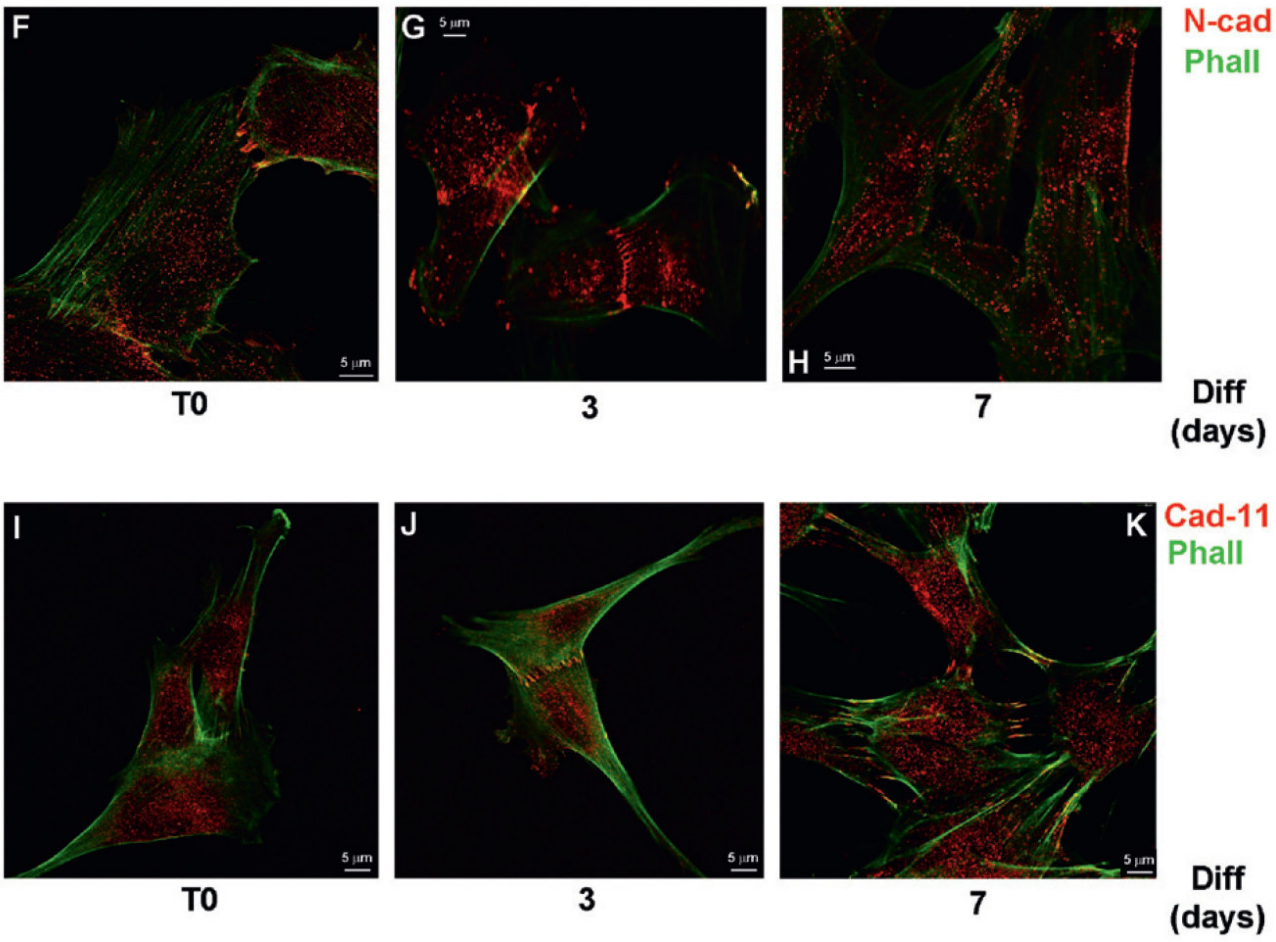


**Figure 3.2.2. Cadherin expression in DBSCs osteogenic differentiation.** Confocal images show that N-Cadherin and Cadherin-11 change their localization in response to osteogenic stimulation (F–K). On time zero (F) and after 3 days (G), N-Cadherin is localized at the cell–cell adhesion sites forming "red bridges", while after 7 days N-Cadherin loses the characteristic bridge-like appearance (H). Cadherin-11 accumulates and localizes at adherens junction sites after 3–7 days of differentiation (J–K).

### *3.3. $\alpha_V$ and $\beta_3$ integrin subunits increase during DBSCs osteogenic differentiation and form the functional integrin*

Integrins trigger tissues morphogenesis, thus we investigated the subunits expressed in mesenchymal tissues (Schwab et al. 2013; Shin et al. 2004; Takada et al. 2007).

Again, due to the rapid propensity of the cells to form a multilayer, the presence of single integrin subunits $\alpha_V$ and $\beta_3$ was analyzed by confocal microscopy in DBSCs undifferentiated and after 7 days of osteogenic differentiation. As shown in Fig. 3.3 A–C both subunits were localized on the surface of undifferentiated cells, distributed in multiple sites and after 7 days of differentiation, each

subunit seemed to reorganize and localize at the focal adhesion sites (Fig. 3.3 B–D). The expression profile of $\alpha_V$ and $\beta_3$ was analyzed by Western blotting during 28 days of osteogenic differentiation (Fig. 3.3, panel E) confirming that both subunits were expressed at T0, increased after 7 days and progressively raised.

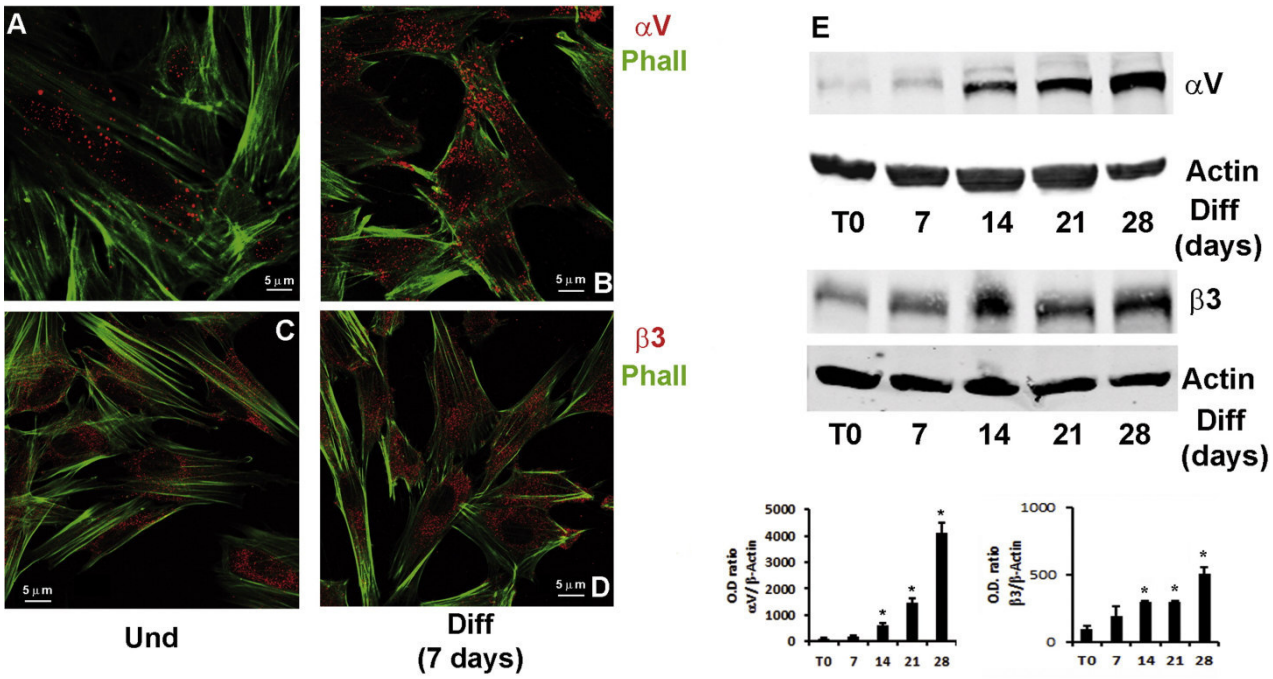


**Figure 3.3. Expression of $\alpha_V$ and $\beta_3$ subunits and formation of the functional Integrin in DBSCs.** Midsection confocal microscopy images show the expression of single integrin subunits $\alpha_V$ and $\beta_3$ (red) in basal and differentiated conditions, cytoskeleton was counterstained with green Phalloidin. A–C show that both single subunits were distributed in multiple sites in undifferentiated cells. After 7 days of differentiation, each subunit seems to localize on the periphery of the cell where are the focal adhesion sites (B–D). (E) Immunoblots showing the trend expression of $\alpha_V$ and $\beta_3$ during the osteogenic differentiation process (0–28 days) of DBSCs. Each graph represents means + SE of 3 independent donors. *P < 0.01 and #P < 0.05 compared to T0. Student's t-test was used for single comparison.

Since integrin receptors are heterodimeric molecules, and alpha subunits form complexes with various beta subunits, we verified if $\alpha_V$ and $\beta_3$ were associated in DBSCs and formed the integrin complex. DBSCs co-stained for $\alpha_V$ (red) and $\beta_3$ (green) (Fig. 3.3.1 F–G), showed some dots of colocalization (yellow staining) in osteogenic condition (7 days) (Fig. 3.3.1 G), suggesting that the subunits could be associated.

To assess whether the subunits $\alpha_V$ and $\beta_3$ were associated to form the integrin receptor, whole cell lysates of DBSCs in basal and osteogenic condition for 7–14–21–28 days were immunoprecipitated with LM609 antibody (which recognizes only the complex) and western blotted with $\alpha_V$ and $\beta_3$ antibodies (Fig. 3.3.1 H). This experiment failed to reveal the association of $\alpha_V$ and $\beta_3$ in undifferentiated DBSCs, while showed that the presence of the receptor dimer was strictly time depending during the differentiation process, reaching the higher expression after 21 days in DBSCs that turned in osteoblast-like cells. The subcellular distribution of integrin $\alpha_V\beta_3$, observed by confocal microscopy, revealed a scarce presence of the receptor in undifferentiated DBSCs (possibly not detected by the biochemical assay), however its localization appeared strongly augmented on the focal contacts in differentiated cells. (Fig. 3.3.1 I–J; K–L).

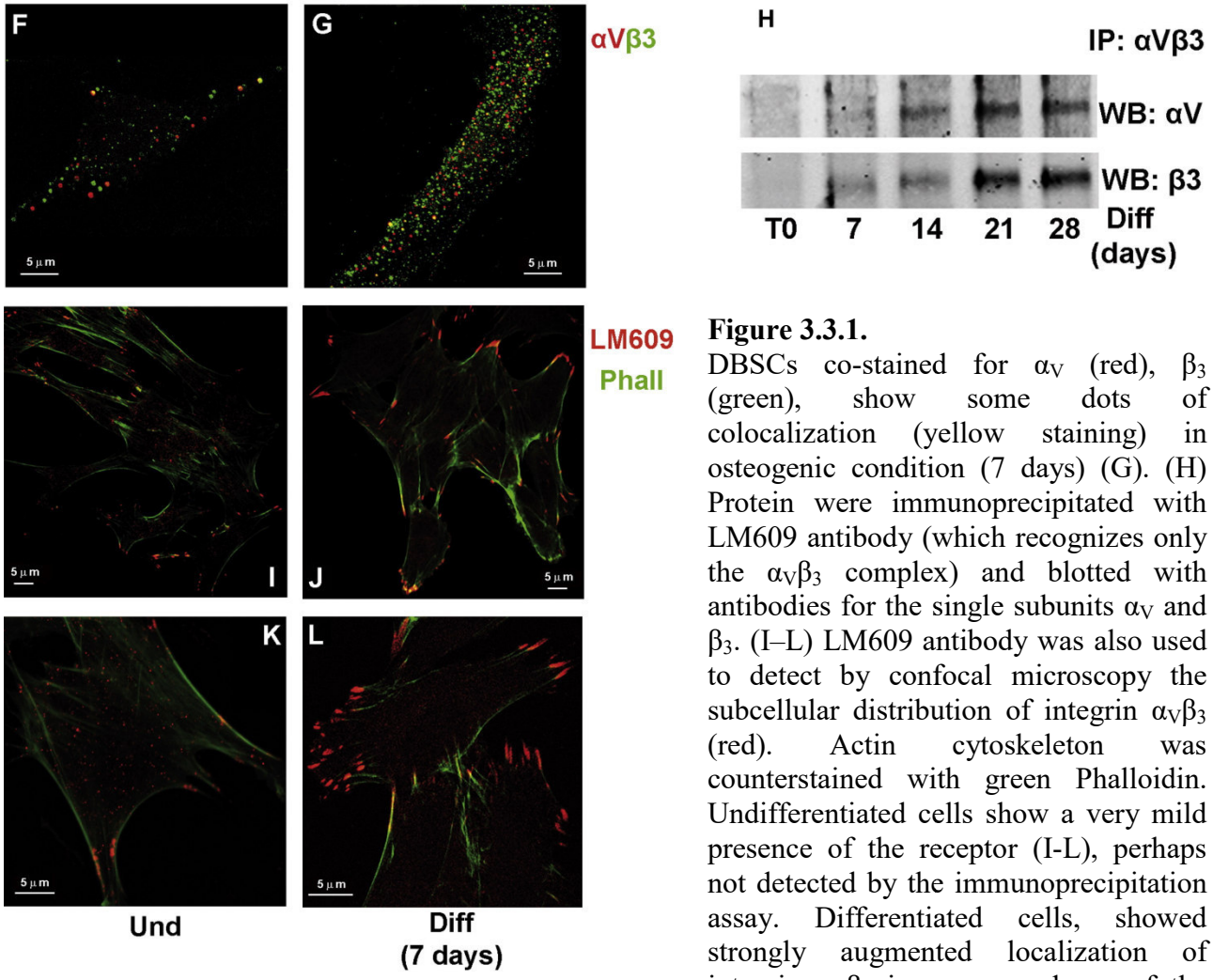


**Figure 3.3.1.**
DBSCs co-stained for $\alpha_V$ (red), $\beta_3$ (green), show some dots of colocalization (yellow staining) in osteogenic condition (7 days) (G). (H) Protein were immunoprecipitated with LM609 antibody (which recognizes only the $\alpha_V\beta_3$ complex) and blotted with antibodies for the single subunits $\alpha_V$ and $\beta_3$. (I–L) LM609 antibody was also used to detect by confocal microscopy the subcellular distribution of integrin $\alpha_V\beta_3$ (red). Actin cytoskeleton was counterstained with green Phalloidin. Undifferentiated cells show a very mild presence of the receptor (I-L), perhaps not detected by the immunoprecipitation assay. Differentiated cells, showed strongly augmented localization of integrin $\alpha_V\beta_3$ in correspondence of the focal contacts (J–L). Results are depicted for one donor but are representative of three different donors.

### *3.4. Increased expression of $\alpha_5$ integrin subunits in DBSCs osteogenic differentiation and association with $\beta_1$ subunit*

The expression of $\alpha_5$ and $\beta_1$ subunits was detected by confocal microscopy in undifferentiated DBSCs showing that both were present and homogenously localized on the cell surface (Fig. 3.4 A–C). After 7 days in osteogenic conditions, expression of $\alpha_5$ seemed to increase and accumulate in large peripheral clusters apparently localized on the base of the cell. The same trend was observed for β1 suggesting that following the osteogenic trigger the subunits were recruited to form the integrin receptor (Fig. 3.4 B–D). Furthermore, the expression trend of both subunits was analyzed by Western blotting during 28 days of osteogenic differentiation. $\alpha_5$was upregulated mainly at 7–14–28 days of differentiation (Fig. 3.4 panel E), while $\beta_1$ did not exhibit significant expression changes. As shown in Fig. 3.4 F–G, where α5 was stained in green and $\beta_1$ in red, the two subunits appeared mainly separated in undifferentiated cells, with only few merged yellow dots, whereas they co-localized in differentiated cells and accumulated on the focal contacts. Immunoprecipitation experiments confirmed that $\alpha_5$ and $\beta_1$ were physically associated essentially in differentiated cells.

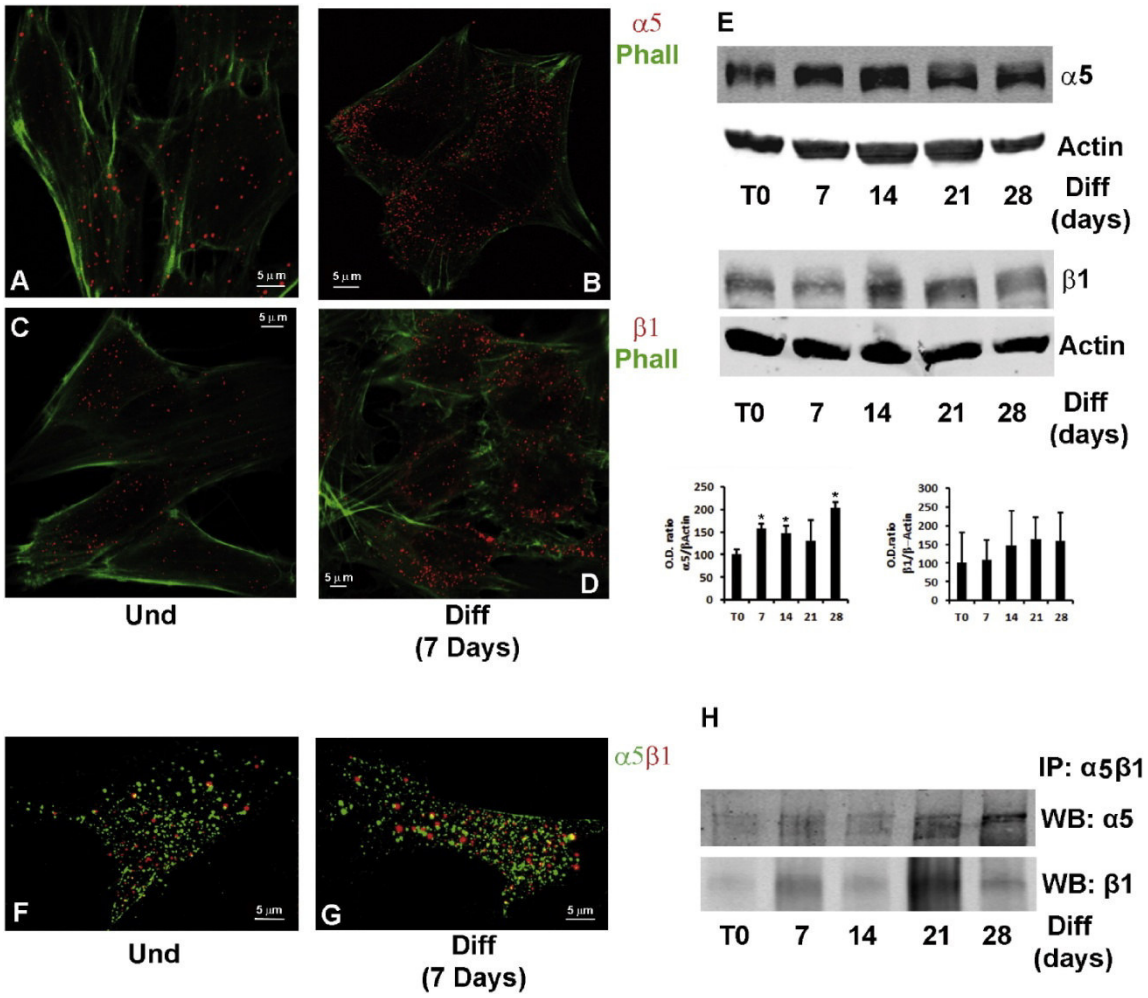


**Figure 3.4. Expression of $\alpha_5$ and $\beta_1$ subunits and formation of the functional integrin in DBSCs.** Midsection confocal microscopy images show the expression of single integrin subunits $\alpha_V$ and $\beta_3$ (red) in basal and differentiated conditions (7 days), Phalloidin is green. A–C show that both subunits were homogenously localized on the cell surface. After 7 days in osteogenic conditions, expression of both subunits accumulates in large peripheral clusters (B–D). (E) Immunoblots showing the trend expression of $\alpha_V$ and $\beta_3$ during the osteogenic differentiation process (0–28 days). Each graph represents means + SE of 3 independent donors. *P < 0.01 compared to T0. Student's t-test was used for single comparison. (F–G) DBSCs co-stained for $\alpha_5$ (green) and $\beta_1$ (red), show some dots of colocalization (yellow staining) in osteogenic condition (7 days) (G).
(H) Immunoblots of protein immunoprecipitated with antibody JBS5 (which recognizes only the $\alpha_5\beta_1$ complex) and blotted with $\alpha_5$ and $\beta_1$ antibodies, show that subunits are physically associated essentially in differentiated cells. Results are depicted for one donor but are representative of the three different donors.

### *3.5. Interaction of integrins with correspondent extracellular matrix proteins enhances osteogenic differentiation and mineralization of DBSCs*

We found that DBSCs expressed integrin subunits $\alpha_V$, $\beta_3$,$\alpha_5$, and $\beta_1$ and that osteogenic trigger enhanced the expression of $\alpha_V$, $\beta_3$, and $\alpha_5$.

Additionally, the osteogenic differentiation induced the association of single subunits to form the integrin heterodimers $\alpha_V\beta_3$ and $\alpha_5\beta_1$, that localized at the focal adhesions. Integrin binding to ECM proteins affects intracellular signaling cascades involved in cell adhesion and migration, as well as in stem cell commitment and differentiation. Thus we investigated DBSCs osteogenic differentiation in the presence of three different ECM proteins, FN (which binds $\alpha_5\beta_1$ and to a lesser extent $\alpha_V\beta_3$), VTN (which binds only $\alpha_V\beta_3$), and OPN (which binds $\alpha_V\beta_3$ and to a lesser extent $\alpha_5\beta_1$). The cells were seeded on the three protein coatings and on poly-L-lysine (PLL) as control (CTR), and cultured in osteogenic conditions. Western blot analysis was performed to investigate the expression of two early markers of osteogenic differentiation, RUNX2 and Collagen I (Coll I), that were found to increase during osteogenic differentiation of DFSCs (Mori et al. 2012). Moreover, using histochemical assays, we evaluated the expression of the osteoblast marker ALP after 7 days and the mineralization capacity with ARS after 21 days.

The differentiation pattern of DBSCs on all 3 surfaces was enhanced in comparison to the control (Fig. 3.5). Indeed we detected higher abundance of RUNX2 and Coll I protein in DBSCs differentiated for 7 days on FN, VTN, and OPN, relative to CTR conditions (Fig. 3.5 A). In particular cells grown on FN showed a 18% and 30% increase of RUNX2 and Coll I respectively, while the increase of both markerswasmore accentuated on VTN and OPN (VTN: 45% for RUNX2, 73% for Coll I; OPN: 46% for RUNX2, 52% for Coll I). This suggests that integrin interaction with ECM proteins promoted intracellular responses essential for the commitment of DBSCs to osteogenic phenotype. Therefore, we applied a histochemical assay to assess the ability to differentiate in osteoblast-like cells evaluating ALP expression and found that after 7 day interaction with the all three ECM proteins highly increased ALP staining, as revealed by pixel quantification. Accordingly, mineral matrix nodule deposition capacity assayed after 21 days by ARS staining, was significantly higher in cells cultured on all three coating surfaces compared to control coating (Fig. 3.5. B). Differentiation on FN was apparently less accentuated as revealed especially by RUNX2 and Coll I expression and mineralization, although the differences observed

among the different ECM proteins were not significant. This might be due to the observation that both integrins, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ had a similar expression trend during osteogenic differentiation of DBSCs and interaction with their ECM partners activated signaling cascades that gave positive cues to osteogenic differentiation.

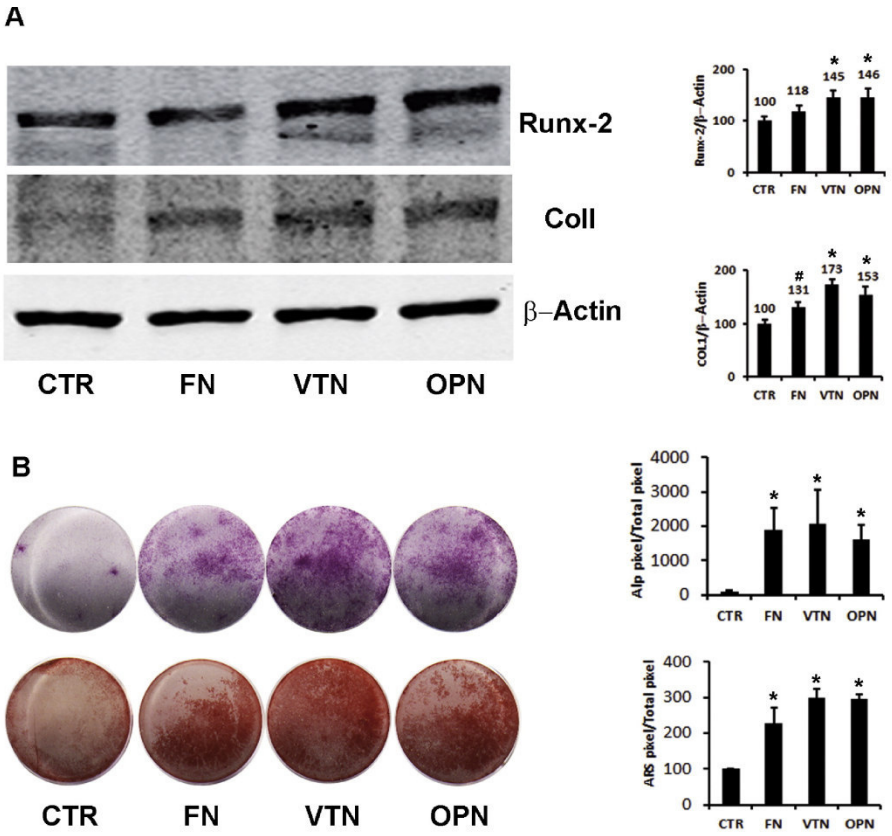


**Figure 3.5. Integrin interaction with ECM proteins enhances osteogenic differentiation and mineralization of DBSCs.**
(A) immunoblots show the expression of two early markers of osteogenic differentiation, RUNX2 and Collagen I (Coll I), in DBSCs seeded and differentiated for 7 days on FN, VTN, OPN coatings and on PLL as control (CTR). Higher levels of both markers are detectable in cells cultured on ECM proteins compared to control. One among three representative experiments was chosen for Image J quantification of reactive bands.
(B) ALP histochemical assay (purple staining) performed on DBSCs seeded and differentiated for 7 days on all three surfaces and CTR. Mineral matrix deposition was assayed after 21 days by ARS (red staining).
The graphs show quantification of positive staining as percentage compared to CTR (*P < 0.001) and are representative for 3 independent donors. Data are presented as mean ± SEM. Student's t-test was used for single comparisons.

To establish if such interactions were essential for the commitment of DBSCs to osteogenic phenotype, we performed the same culture conditions in the presence of neutralizing antibodies

directed against integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$. More precisely the antibody against $\alpha_5\beta_1$ was added to the FN coating cultures, while the antibody against $\alpha_V\beta_3$ to VTN and OPN coating cultures. We showed that the treatment with anti-$\alpha_V\beta_3$ completely reversed the increase of ALP and ARS staining in cultures of DBSCs differentiated on VTN and OPN, while, the inhibition of interaction between $\alpha_5\beta_1$ and FN failed to reach similar results (Fig. 3.5.1 C). This finding demonstrated that enhanced differentiation of DBSCs to osteoblast phenotype achieved by culturing these cells on VTN and OPN is strictly dependent by the adhesion via $\alpha_V\beta_3$. By contrast, the increased differentiation on FN coating dispensed from interaction with integrin $\alpha_5\beta_1$.

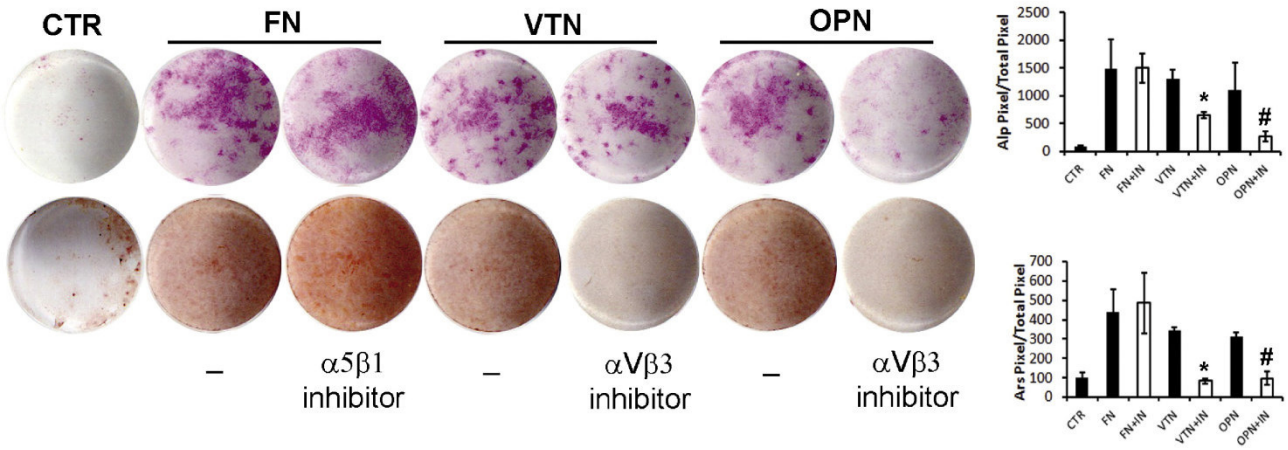


**Figure 3.5.1. Osteogenic differentiation and mineralization of DBSCs cultured with integrin blocking antibodies.**
ALP staining performed after 7 days and ARS staining after 14 days on DBSCs seeded and differentiated on FN + $\alpha_5\beta_1$ blocking antibody, VTN + $\alpha_V\beta_3$ blocking antibody, OCN + $\alpha_V\beta_3$ blocking antibody and CTR (*P < 0.001 vs control culture without antibody). Data are representative for 3 independent donors, and are presented as mean ± SEM. Student's t-test was used for single comparisons.

### *3.6 Vitamin D effects on osteoblastic differentiation of DBSCs*

In order to investigate Vit D ability to induce the differentiation of DBSCs into osteoblasts, cells were cultured in osteogenic medium and stimulated with $1,25(OH)_2D_3$ $10^{-8}$ M. This concentration has been observed as the most efficacious among Vit D physiological concentrations analyzed in previous experiments.

DBSCs cultures were stopped at 7, 14 and 21 days after continuous Vit D treatment in differentiating conditions.

### *3.6.1 ALP positivity and calcium-rich deposits in DBSCs*

We used an histochemical assay to evaluate the expression of Alkaline Phosphatase, a marker of osteoblastic differentiation, a key enzyme in the process of osteodeposition.

We found that DBSCs cultured in an osteogenic medium and treated with $1,25(OH)_2D_3$ expressed higher levels of ALP after 7 days, compared to those of the control (figure 3.6.1 A). There was a considerable increase of ALP levels after 14 days, with slight differences between vehicle and treatment (data not shown).

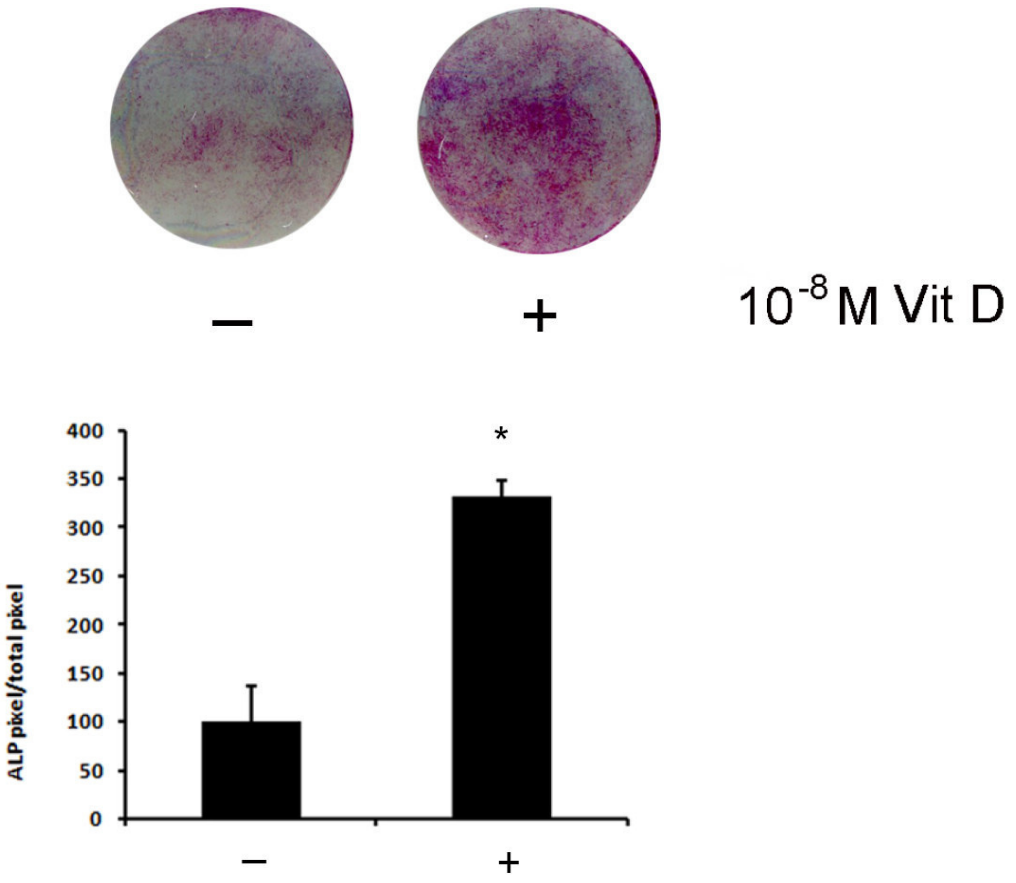


**Figure 3.6.1 A. ALP positivity.** ALP histochemical assay (purple staining) performed on DBSCs seeded and differentiated for 7 days with vehicle (-) and $1,25(OH)_2D_3$ (+). The graph shows quantification of positive staining as percentage compared to untreated (-) (*$P < 0.05$) and is representative for 3 independent donors. Data are presented as mean ± SEM. Student's t-test was used for single comparisons.

Moreover, long-term cultures of DBSCs demonstrated the capacity to form calcium-rich deposits; this activity, representing the osteoblast final step matrix secretion, was assessed with ARS quantification.

Interestingly DBSCs deposition of mineral matrix nodules was significantly higher in cells cultured with Vit D, compared to the control (figure 3.6.1 B).

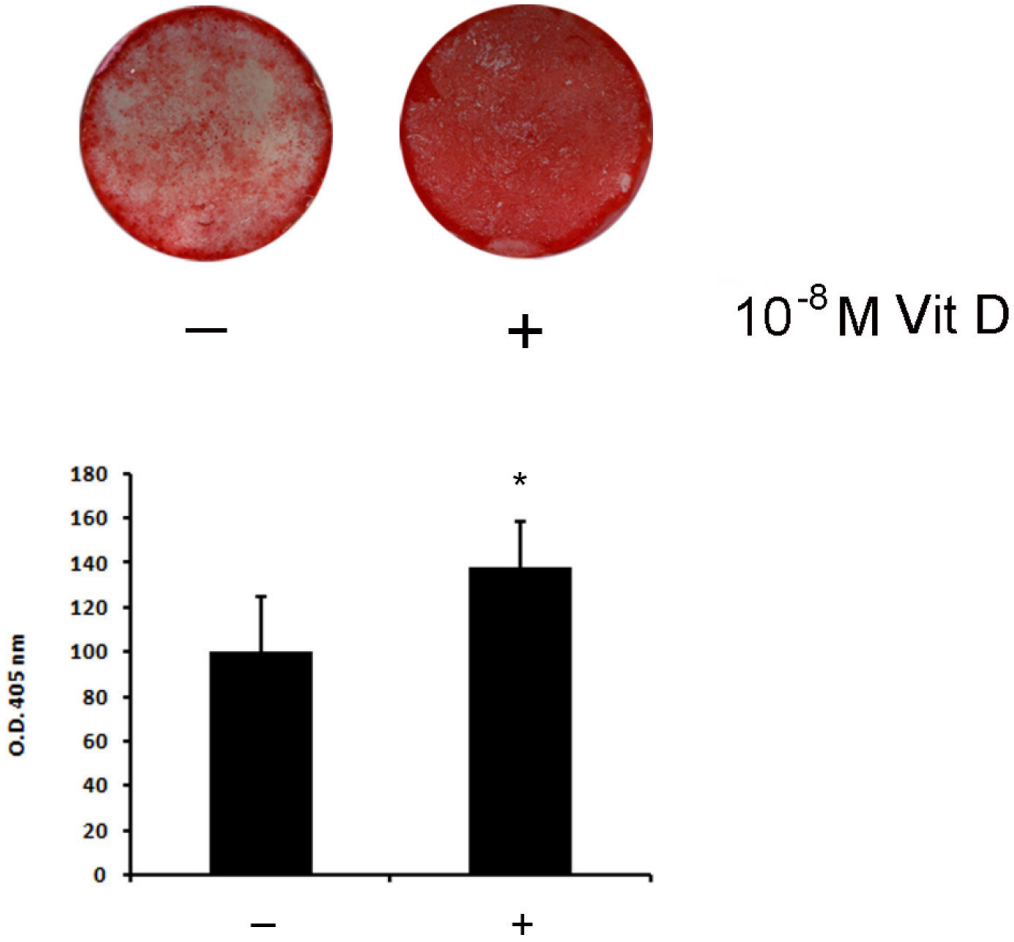


**Figure 3.6.1 B. Mineralized nodules formation.** Alizarin Red (red staining) shows mineralized nodules formation by DBSCs incubated for 21 days in osteogenic medium with vehicle (-) and $1,25(OH)_2D_3$ (+). The graph shows quantification of ARS with the Optical Density (OD) at 405 nm (B), * indicate statistical significance ($P < 0.001$). Data are representative for 3 independent donors and are presented as mean ± SEM. Student's t-test was used for single comparisons.

### *3.6.2 Osteoblast markers expression in DBSCs*

The main osteoblastic markers, such as Collagen I (Coll I), its precursor Pro-Collagen I (Pro-Coll I), RUNX2, Bone Sialoprotein (BSP) and Osteopontin (OPN), were analyzed in DBSCs during the different steps of their osteogenic differentiation.

RUNX2 is the master gene of osteogenic differentiation; it directs MSCs to an osteoblastic lineage and inhibits their differentiation into other lineages such as adipocytes and chondrocytes (Komori 2006).

As shown in Figure 3.6.2 A, RUNX2 expression raised in a quite constant way during DBSCs osteogenic differentiation, however the addiction of Vit D greatly increased its expression level after 7 and 14 days of treatment. At 21 days of culture, no difference between vehicle and Vit D was observed.

A trend similar to the one already described for RUNX2 was observed for Coll I (figure 3.6.2.B): Vit D upregulated its expression in the first 7 days of differentiation, the effect was still present, although attenuated , after 14 and 21 days of treatment.

Interestingly Pro-Coll I**,** the immature precursor form of Coll I, showed a reverse trend (Figure 3.6.2 C): Vit D reduced its expression at 7, 14 and 21 days of culture suggesting that this treatment promotes the conversion of Pro-Coll I in the mature Coll I.

It is remarkable how these data confirmed the typical trends of Coll I and its precursor during osteoblastogenesis, indicating that DBSCs are a reliable model of osteoblastic differentiation: moreover Vit D can sustain and increase this process.

Then we evaluated the expression of OPN and BSP which are considered the most important non-collagenous proteins produced by bone cells during the formation of bone matrix *in vitro* (Kasugai et al. 1991; Nagata et al. 1991a; Nagata et al. 1991b).

The expression of BSP was slightly upregulated after 7 days of Vit D treatment, while at 14 days there was a remarkable reduction; at 21 days BSP did not exhibit significant expression changes (Figure 3.6.2 D).

No significant effect was attributed to the Vit D treatment on the expression of OPN (Figure 3.6.2 E).

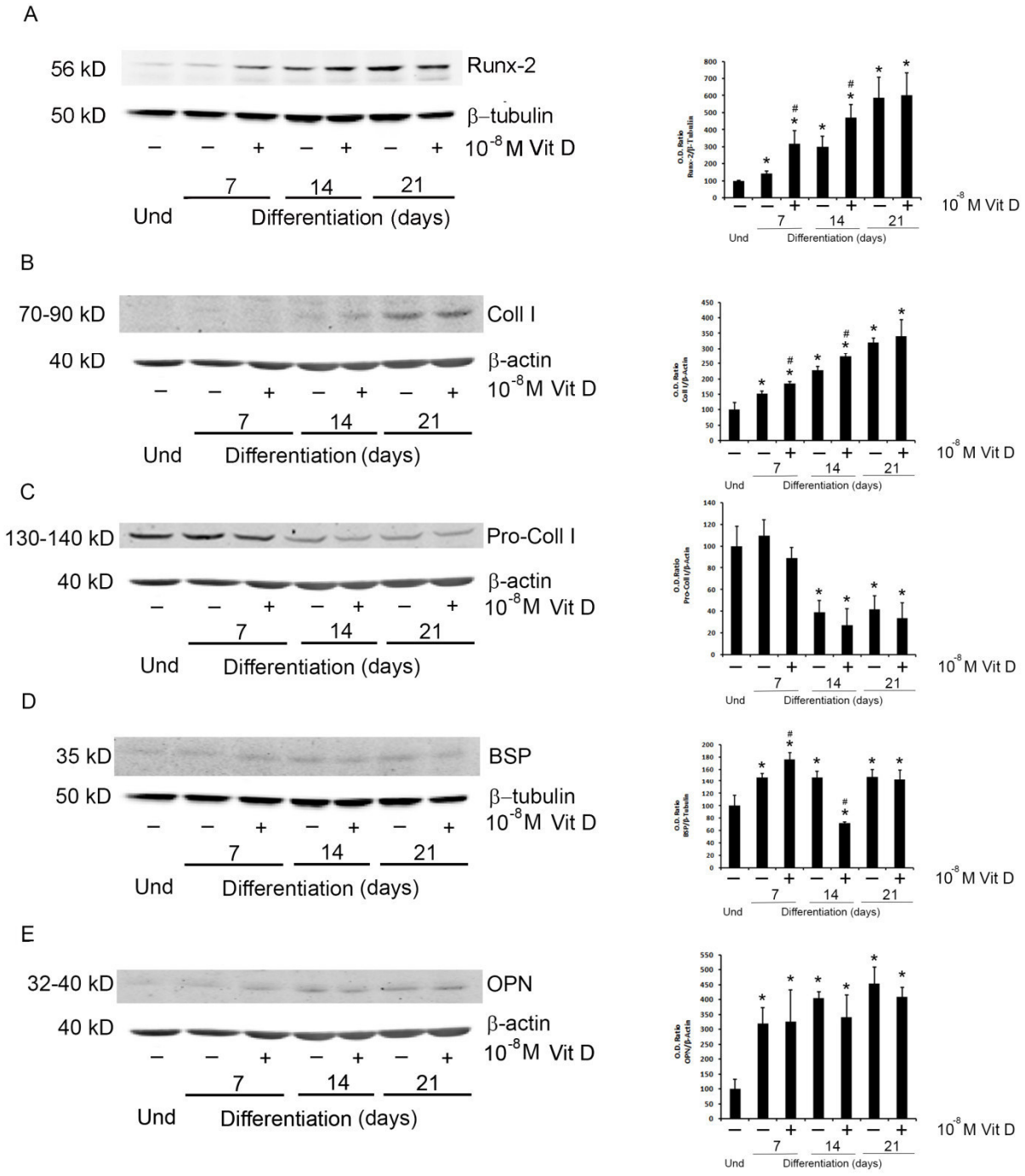


**Figure 3.6.2. Protein expression.** Immunoblots showing the trend expression of RUNX2 (A), Coll I (B), Pro-Coll I (C), BSP (D) and OPN (E) during the osteogenic differentiation process (0-21 days) of DBSCs cultured in osteogenic medium with vehicle (-) and 1,25$(OH)_2D_3$ (+). Each graph represents means ± SEM of 3 independent experiments. *P < 0.01 compared to undifferentiated (und) and #P < 0.05 compared to untreated (-). Student's t-test was used for single comparison.

### *3.7 Vitamin D treatment ad $\alpha_V\beta_3$ subcellular distribution in DBSCs*

We analyzed by immunofluorescence the subcellular distribution of $\alpha_V\beta_3$ integrin, which is the receptor for two bone matrix proteins: Vitronectin and Osteopontin. Integrins not only are important in the tissue architecture, but they mediate proliferation and differentiation signals.

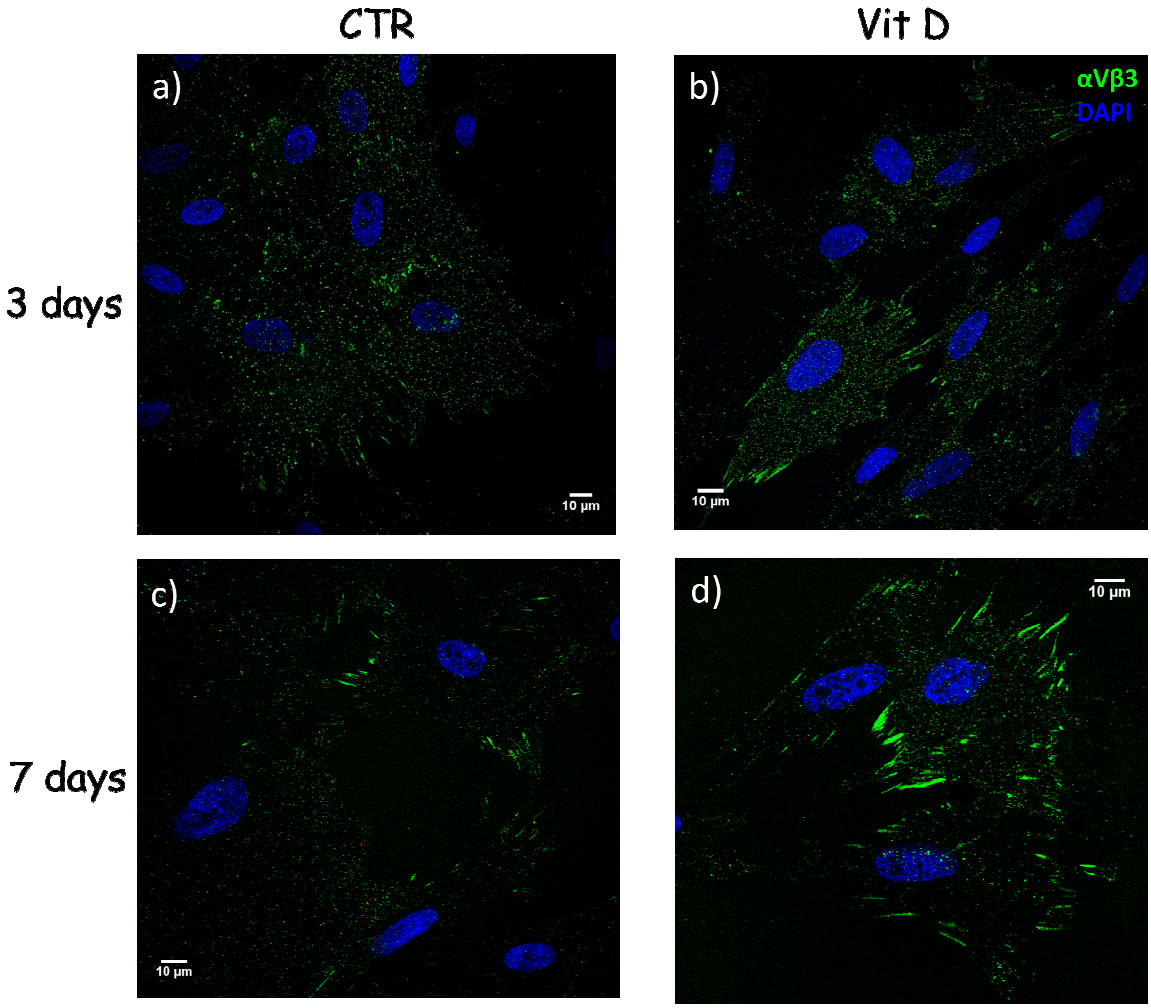


**Figure 3.7. Expression of $\alpha_V\beta_3$ in DBSCs.** Midsection confocal microscopy images show the expression of integrin $\alpha_V\beta_3$ (green) after 3 and 7 days of differentiation; a-b show that the integrin was distributed in multiple sites in cells. After 7 days of differentiation, $\alpha_V\beta_3$ seemed to localize on the periphery of the cell where there are the focal adhesion sites (c-d). Vit D treatment seemed to produce a different integrin organization. Blue for nuclei, green for $\alpha_V\beta_3$.

Vit D treatment induced a different integrin organization that appeared to be more clustered and localized in the basal part of the cell if compared to its distribution in the control. This effect was evident after 3 and 7 days of differentiation (Figure 3.7).

The receptor of undifferentiated cells without Vit D was distributed throughout the cell (Figure 3.7 a-c) while in differentiated cells with Vit D it was present in focal adhesions (Figure 3.7 b-d).

### *3.8 Vitamin D treatment and $\alpha_V\beta_3$ subcellular distribution on coated surfaces*

As described in the first part of the results section, DBSCs differentiation toward osteoblastic lineage was enhanced when cells were grown on Fibronectin (FN), Vitronectin (VTN), and Osteopontin (OPN), ECM glycoproteins which contain an integrin-binding sequence, the RGD motif.

In order to understand how Vitamin D treatment could affect cell adhesion in the presence of ECM glycoproteins, we prepared surfaces coated with PLL, as control, and FN respectively. Cells were seeded and cultured on that surfaces in osteogenic conditions for 7 days, in the presence or not of Vit D treatment, then focal adhesion formation was analyzed looking at $\alpha_V\beta_3$ subcellular distribution.

We detected a greater number of cells on FN compared to PLL, furthermore FN coating seemed to slightly induce $\alpha_V\beta_3$ organization into focal adhesions. Vit D appeared to be able to assist the effect of the FN coating in the formation of focal adhesion: in the treatment with Vit D, $\alpha_V\beta_3$ strings were highly visible (Figure 3.8).

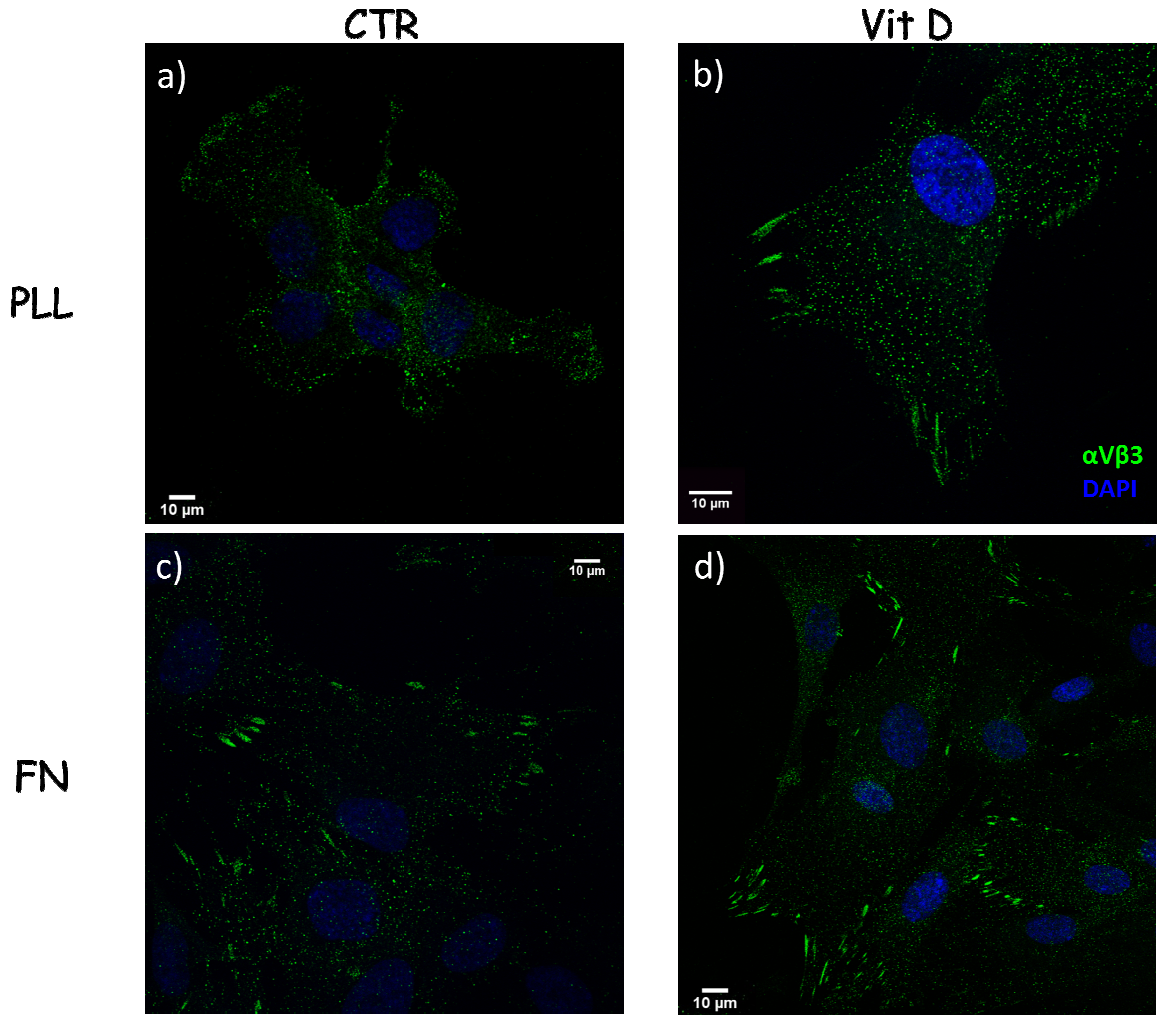


**Figure 3.8. Expression of $\alpha_V\beta_3$ in DBSCs on coated surfaces.** Confocal images showing the expression of integrin $\alpha_V\beta_3$ (green) after 7 days of osteogenic differentiation. (a-b) show the integrin distribution in cells cultured on PLL coating, in the case of the control, $\alpha_V\beta_3$ appeared to be present homogeneously in the whole cell; Vit D treatment induced an accumulation of the receptor in the focal contacts (b-d)., $\alpha_V\beta_3$ clustering was increased on FN coating (c-d), more evident in Vit D treatment (d) compared to the control (c). Blue for nuclei, green for $\alpha_V\beta_3$.

### *3.9 Vitamin D and nanostructured surfaces*

Quasi-hexagonally ordered nanoparticle arrays were produced using BCMN as described in Materials and Methods section. Unspecific protein adhesion was prevented by depositing a layer of polyethylene glycol (PEG2000) on the glass surface between the gold nanoparticles. Polyethylene glycol (PEG) based substrates are widely used as biologically inert interfaces (Elbert and Hubbell 2001; Koo et al. 2002; Maheshwari et al. 2000).

In this way molecularly well-defined adhesive spots are separated by nonadhesive regions so that individual integrins are positioned in the cell membrane by the interaction of a cell with such surfaces. RGD-SH and $\alpha_5\beta_1$ were then bound to the nanoparticles via the thiol group that selectively binds to gold. The peptide RGD-SH as well as the $\alpha_5\beta_1$ integrin peptidomimetic served as ligands for integrins, therefore promoting integrin-mediated cell attachment to modified surfaces. Here, the cell adhesive RGD-SH sequence was selectively recognized by different αv integrins such as $\alpha_V\beta_3$ integrin (Haubner et al. 1996) whereas the $\alpha_5\beta_1$ integrin peptidomimetic selectively mediated cell adhesion by this specific integrin heterodimer (Rechenmacher et al. 2013).

In order to investigate Vit D ability to induce Focal Adhesion formation in DBSCs during their osteogenic differentiation, cells were seeded on nanostructured surfaces with different adhesive molecules and cultured in an osteogenic medium with or without the stimulation with $1,25(OH)_2D_3$. Although preliminary experiments have produced no significant differences between Vit D treatment and control cells (data not shown), we took advantage of the opportunity provided by our collaboration with Heidelberg University to test and develop the nanostructured surfaces method for the purpose of optimizing it.

# *4.Discussion*

# 4. Discussion

***Chapter I: Role of integrins and cadherins in DBSCs osteogenic differentiation***

Dental tissues represent an alternative and promising source of postnatal MSCs for tissue engineering (Ballini et al. 2007b; Di Benedetto et al. 2014; Gronthos et al. 2000; Mori et al. 2013). Indeed, despite other sources of MSCs in the adult body (bone marrow, adipose tissue, skin, brain, liver, etc.), dental tissues are formed at a later stage and therefore with a high amount of stem cells, due to late completion of odontogenesis process and tooth eruption. Furthermore DPSCs and DBSCs can be obtained from the wisdom tooth (mature and at the stage of germ respectively) not essential for human masticatory function and frequently extracted for orthodontic reasons or dysodontiasis.

In addition, the tooth extraction, especially by piezo-surgery technique, is less invasive compared to bone marrow or other tissue biopsy. The challenge of new bone formation and graft integration is strictly dependent on recruitment and adhesion of stem cells on the scaffolds, to obtain a successful cell commitment and differentiation. The cell adhesion molecules cadherins and integrins, address tissue morphogenesis and architecture, but also act intracellularly by modulating crucial pathways of cell commitment and differentiation. A main role of some cadherins has been established in the control of mesenchymal cells survival, proliferation, and differentiation (Gumbiner 2005; Larue et al. 1996; Wheelock and Johnson 2003) and in osteoblastogenesis and bone formation (Cheng et al. 1998; Ferrari et al. 2000; Kawaguchi et al. 2001; Lemonnier et al. 2001). In this work, expression of "classical" cadherins was characterized in undifferentiated DBSCs and during their osteogenic differentiation. Our data indicated that N-cadherin and cadherin-11 were expressed in undifferentiated DBSCs and their expression underwent changes during the osteogenic process (decreasing and increasing respectively), while expression of E-cadherin and P-cadherin was low in DBSCs and did not change during the differentiation steps. Similar expression pattern was reported in various cell lines of the mesenchymal lineage (Cheng et al. 1998; Marie and Hay 2013; Marie et

al. 2014; Shin et al. 2000). Kawaguchi et al., 2001 found that N-Cad was expressed in different lines of mesenchymal cells, Cad-11 in preosteoblasts, preadipocytes, and in differentiated osteoblast while decreased in adipocyte cells, while P-Cad was only poor expressed in calvaria OBs. More recently, Alimperti et al. (2014) reported that human hair follicle derived MSCs (HF-MSCs) expressed both N-Cadherin and Cadherin-11, that localized at the cell–cell contact sites. In addition our data indicated different subcellular localization, with Ncadherin forming adherens junctions in undifferentiated cells, while Cadherin-11 only in differentiated cells. The cadherin results reflected the mesenchymal origin of DBSCs and confirmed their osteoblast-like features. Indeed literature data support that both N-Cadherin and Cadherin-11 prompt the osteogenic commitment of precursor cells, but to proceed in the osteogenic program and terminal differentiation N-Cadherin must be down regulated and Cadherin-11 highly expressed in fully differentiated osteoblasts (Di Benedetto et al. 2010; Greenbaum et al. 2012; Lai et al. 2006). Furthermore a recent review from Alimperti (Alimperti and Andreadis 2015) elegantly summarized the body of evidences that N-Cad and Cad-11 are present in all mesenchymal cells but their expression pattern may vary as they commit to different lineage, or during the transition from one cellular type to another.

For example it is accepted that the epithelial to mesenchymal transdifferentiation (EMT) is characterized by augmented expression of N-Cad and Cad-11 and diminished expression of E-Cad. The latter observation is also in agreement with our finding showing a very low expression E-Cadherin in DBSCs. This supported the issue to address a possible use of DBSCs as bone forming cells, of stomatognathic and other systems. The effectiveness of bone regeneration depends on cell adhesion on biomaterial scaffolds, and several studies suggested that cues from ECM regulated cell adhesion and migration but also differentiation by activating specific integrin subunits and heterodimers (Cheng et al. 2001; El-Amin et al. 2002; Hamidouche et al. 2009; Schneider et al. 2001). Our data indicated that osteogenic trigger in DBSCs, induced the upregulation of single subunits, $\alpha_V$, $\beta_3$, $\alpha_5$, and the high formation of integrin receptors $\alpha_5\beta_1$ and $\alpha_V\beta_3$, localized in the adhesion sites of the cells to the underlying ECM, well known as focal adhesions. MSCs adhere

mainly via $\alpha_5\beta_1$- integrin binding to FN (Ode et al. 2010) and via $\alpha_V\beta_3$-integrin to OPN and VTN (Shin et al. 2004; Takada et al. 2007) and when BMSCs were cultured under osteogenic conditions, their commitment to the osteoblastic lineage and their ability to form a mineralized matrix increased in the presence of FN and OPN (Schwab et al. 2013). We cultured DBSCs on FN, VTN, and OPN coated surfaces and studied the effects of these interactions during osteogenic differentiation. DBSCs uniformly colonized all the three coated surfaces after 2–3 days while in the control the cells maintained the typical colony-forming unit (CFU) appearance (not shown). This observation is in agreement with other data demonstrating a strong adhesive interaction between MSCs and RGD-containing glycoproteins (Gronthos et al. 2001; Klees et al. 2005a; Lavenus et al. 2011; Ode et al. 2010; Schwab et al. 2013). The CFU appearance in the control would suggest a major cell retention in the "niche" stem status compared to the cells on coated surfaces and thus a less committed state of the cells. This idea was supported by DBSC expression of two early markers of osteoblast commitment and differentiation, RUNX2 and Coll I, that were strongly increased in the cells cultured on VTN and OPN and to a lesser extent on FN. Accordingly, the higher expression of these genes was accompanied by increased expression of ALP and enhanced mineral deposition, with no significant difference among the three glycoproteins. So far these data strengthen the idea that MSCs from dental tissues could be a good promise for bone tissue regeneration. Indeed we demonstrated for the first time that DBSCs not only differentiated into osteoblast-like cells depositing mineral matrix (Mori et al. 2012) but also expressed a pattern of adhesion receptors as cadherins very similar to BM-MSCs, switching to the typical osteoblast phenotype as they were maintained in osteogenic conditions.

A main role for cadherins has been established in the control of mesenchymal cells survival and osteoblastogenesis, although still remaining a matter of debate, thus a deeper knowledge of cadherins behavior in these cells would help to promote their possible use in bone reconstruction.

Furthermore we found that the expression pattern of integrins would determine the optimal extracellular environment to achieve an efficacious osteogenic differentiation of these cells. Indeed

DBSCs differentiation toward osteoblastic lineage was enhanced when cells were grown on FN, VTN, and OPN. The interaction between $\alpha_V\beta_3$ and their ECM partners VTN and OPN appeared to be crucial in DBSC commitment.

Certainly the accentuated ALP expression and mineralized matrix formation obtained growing the cells on VTN and OPN was completely abolished when cultures were treated with $\alpha_V\beta_3$ neutralizing antibody.

Conversely, by using $\alpha_5\beta_1$ neutralizing antibody we did not have any inhibition of ALP and ARS staining in cultures performed on FN coating.

Although MSCs adhere to FN via $\alpha_5\beta_1$, it is known that multiple integrins can bind to a single ECM protein and multiple ECM proteins can bind a single integrin (Miranti and Brugge 2002), thus a possible explanation of these results is that some other integrin could compensate for inactivated $\alpha_5\beta_1$ in the binding to FN and give evenly positive cues to osteogenic differentiation. On the contrary, the results on $\alpha_V\beta_3$ inhibition demonstrated that accentuated differentiation gained on VTN and OPN is mostly exclusively mediated by integrin $\alpha_V\beta_3$. Few previous studies reported an involvement of $\alpha_V\beta_3$ in the regulation of osteoblastic differentiation, although most of them were conducted on committed osteoblast or osteoblast cell lines (Schneider et al. 2001; Weyts et al. 2002) and some of those demonstrated essentially a synergy with other signaling pathways (Kim et al. 2007; Lai and Cheng 2005; Su et al. 2010). However, little is known about the role of $\alpha_V\beta_3$ in MSCs commitment to osteoblast lineage. We demonstrated in DBSCs, which are a good cell model of MSCs, that integrin $\alpha_V\beta_3$ is indispensable for the commitment to osteoblast lineage and its interaction with ECM ligands VTN and OPN enhanced this process.

In conclusion the profile expression of adhesion receptors, and their expression changing during osteogenic differentiation, reflected the mesenchymal origin of DBSCs and confirmed their capacity for committing to osteoblast-like cells. Furthermore these data suggest that stratification of biomaterials with ECM protein as FN, VTN, and OPN would improve the bone reconstruction therapies starting from dental stem cells. In addition we established that Integrin $\alpha_V\beta_3$ plays a

crucial role during the commitment of MSCs to osteoblast lineage, while Integrin $\alpha_5\beta_1$ seems to be dispensable.

***Chapter II: Vitamin D effects in DBSCs osteogenic differentiation***

The main actions of Vit D are those concerning mineral and skeletal homeostasis. The protracted deficiency of Vit D has different skeletal consequences in humans determining decreased bone mass and mineralization, with the manifestation of diseases known as Rickets in children and Osteomalacia in adults (Holick 2003; Holick 2006a). Low levels of Vit D also result in osteopenia or osteoporosis mainly attributed to an increased osteoclast bone resorption. These effects depend mostly on the indirect actions of $1,25(OH)_2D_3$. Vit D may affect the bone indirectly, stimulating the intestinal calcium absorption, or directly, that is by acting on bone cells; which of the two actions is prevailing is still debated (Bikle 2012) and the mechanisms whereby Vit D affects osteoblasts are mostly unknown (St-Arnaud 2008).

Some studies have described $1,25(OH)_2D_3$ as able to increase human osteoblasts mineralization, leading to earlier and higher rate of mineral deposition (Woeckel et al. 2010), and able to accelerate hMSCs commitment with the subsequent osteoblast maturation (Piek et al. 2010; Zhou et al. 2012).

The bone marrow has been considered the major source of MSCs, however these cells can be obtained from other tissues and organs, which in some cases could represent an easier harvesting site being perceived as less invasive for donors. This is the case of stem cells isolated from the dental tissues.

Contrary to other sources used in adults to obtain MSCs, the dental tissues are formed at an older age, this is due to the late completion of odontogenesis and tooth eruption; consequently these tissues contain a higher amount of stem cells that have been discovered to be multipotent cells (Gronthos et al. 2000; Huang et al. 2009). DPSCs and DBSCs can be isolated from the third molar, in adult donors or in children respectively. The dental bud is an immature organ, which originates

several years after birth and is composed of undifferentiated cells with a higher proliferation rate than that observed for MSCs from bone marrow.

DBSCs fully satisfy the requirements to be considered MSCs, in fact it has been shown that they express more than 95% of mesenchymal stem markers (CD44, CD73, CD90, CD105, CD146, and HLA-I) and express the typical mesenchymal adhesion molecules (Di Benedetto et al. 2015). Therefore all these mesenchymal features of DBSCs, together with their easy accessibility, make MSCs from dental tissues an excellent substitute to the bone marrow cells.

We have already demonstrated that osteoblastogenesis can successfully, and in a very productive way, take place from dental follicle, which is the peripheral part of the dental bud (Mori et al. 2012) and from dental bud (Di Benedetto et al. 2015). These cells differentiate toward an osteoblastic phenotype, they express RUNX2, Coll I and ALP, characteristic osteoblast markers, and produce mineralized matrix nodules.

In this work we analyzed how Vit D could influence the osteogenic differentiation of DBSCs. To this purpose we studied the expression of typical osteoblastic markers and mineral matrix deposition during DBSCs osteogenic differentiation in the presence, or not, of $1,25(OH)_2D_3$. Our results confirmed the functional osteogenic differentiation of DBSCs (Di Benedetto et al. 2015), in addition in this work we demonstrated that Vit D treatment enhances the commitment of DBSCs toward osteoblastic lineage. Indeed we observed that DBSCs treated with $1,25(OH)_2D_3$, expressed increased levels of the main osteoblastic markers, RUNX2, Coll I and ALP. Furthermore our results showed that enhanced commitment of DBSCs in presence of Vit D was also accompanied with an augmented production of mineralized matrix.

The enhanced expression of RUNX2, which is an early marker of osteoblastic differentiation, indicated that Vit D acts on uncommitted cells, prompting them to differentiate toward osteogenic lineage and then to express the typical osteoblastic markers ALP and Coll I. These pro-osteogenic effects exerted by $1,25(OH)_2D_3$ on undifferentiated mesenchymal progenitor cells results

subsequently in an accelerated formation of mineralized matrix nodules *in vitro* after 21 days of differentiation.

Our results also showed that the expression of RUNX2 and Coll I was accentuated in cells treated with Vit D during the early phases of osteogenic differentiation (7-14 days), while their expression turned to the control levels around 21 days of culture, indicating that the effects of Vit D are predominant at the beginning of the culture.

These results suggest that Vit D probably acts on the first steps of MSCs differentiation toward the osteoblastic phenotype, becoming less efficacious on differentiated cells. Our data are in line with previous data on human osteoblasts that the effect of Vit D depends on the time and the cells differentiation phase seeming to vanish in ongoing mineralization (Woeckel et al. 2010).

Our observations demonstrated that also stem cells from dental tissues respond to Vit D signal and are consistent with the data in literature which attribute to Vit D a key role in inducing osteogenic differentiation of MSCs (Liu et al. 1999a; Piek et al. 2010; Zhou et al. 2010) from human bone marrow. These results confirm that MSCs from dental tissues share similar features with MSCs from bone marrow and suggest that cultures of DBSCs in presence of Vit D could be taken in consideration for bone regenerative therapies.

Thus these data reflect the mesenchymal origin of DBSCs and their osteogenic capacity, moreover this study shows that osteoblastic differentiation of DBSCs was stimulated by $1,25(OH)_2D_3$; our observations suggest that Vit D acts directly on these cells, that can be considered osteoblast precursors, directing them to an increased bone matrix deposition.

The point of force of this work is that DBSCs are post-natal stem cells more undifferentiated than those isolated from bone marrow, i.e. adult stem cells comparable to embryonic stem cells. The limit of our data is that all these conclusions have been already reported for MSCs from bone marrow, they are anyway a confirmation.

**Chapter III: Vitamin D modulation of focal adhesion formation in DBSCs osteogenic differentiation**

Since there are no data in literature about the influence of Vit D in cell adhesion, but it is well known that interactions between cells and surfaces are involved in the activation of a series of signals which are responsible for cell commitment and differentiation, we tried to figure out if Vit D might, or not, have a role in these mechanisms.

Here the specific contribution of $\alpha_V\beta_3$ integrin to early adhesion-mediated events of DBSCs, including attachment and proliferation, was analyzed.

We started culturing DBSCs on normal surfaces, under differentiating conditions, with or without Vit D stimulation with the purpose to investigate whether the Vitamin could, in some way, induce the cell adhesion process.

As detailed in the first chapter of this discussion, we determined that the $\alpha_V\beta_3$ integrin assumes a key role in DBSCs commitment. The neutralization of this receptor with a specific antibody had led to a reduction of both the ALP expression and the mineralization process. In light of this knowledge, we focused our attention on $\alpha_V\beta_3$ distribution in our cell model.

Our data indicated that $\alpha_V\beta_3$ was expressed in DBSCs, cultured under osteogenic conditions, after 3 days in culture, but its expression underwent changes not only with the advancement of the osteogenic differentiation, but also with the treatment with Vit D. Vit D induced $\alpha_V\beta_3$ localization in the adhesion sites represented by focal adhesions; this reorganization of the receptor appeared to increase in a time dependent way.

Although there are many different types of integrins with specificity to different ECM, a major portion of cellular and biophysical studies have focused on fibronectin-binding $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins. We cultured our cell model on FN coated surfaces and studied the effects of Vit D and these interactions during osteogenic differentiation. DBSCs uniformly colonized all the surfaces

after some days of culture, but we observed that, compared to PLL control, cells on FN exhibited a more flattened morphology and seemed to be more numerous.

Upon binding via their extracellular domains to the ECM, integrins cluster and their cytoplasmic tails associate with cytoskeletal and intracellular signal transduction molecules forming macromolecular aggregates known as FAs (Shekaran and Garcia 2011). We used $\alpha_v\beta_3$ immunofluorescence to label stable FAs. After 7 days in culture, DBSCs treated with Vit D on FN-coated surfaces showed large and discrete $\alpha_v\beta_3$ positive clusters while in control cells a lower number of $\alpha_v\beta_3$-containing complexes could be seen.

Our data demonstrate a strong adhesive interaction between DBSCs and FN, as also reported in other studies (Gronthos et al. 2001; Klees et al. 2005b; Lavenus et al. 2011; Ode et al. 2010).

Furthermore these results are consistent with a recent study in which it has been proven that FN coating can be considered able to induce $\alpha_V\beta_3$ integrin expression in MSCs (Hung et al. 2013). Our study goes forward identifying in Vit D a further support to the effect of FN by increasing focal adhesions formation.

This detail is highly significant because it is known that adhesion is also important in differentiation: osteogenesis requires larger numbers of FAs, while both adipogenesis and chondrogenesis are promoted by preventing the formation of robust FAs (Mathieu and Loboa 2012). This could support the results on DBSCs osteogenic differentiation induced by Vit D obtained with the experiments previously described.

Finally Vit D appears to promote DBSCs spreading, survival and proliferation on coated surfaces, a conclusion which is aided also by the observation of a greater number of cells in the samples treated with Vit D.

# 5. Conclusion

It is known that the efficacy of calcium intake on osteoporosis and fracture prevention is conditioned by the concomitant assumption of Vit D. Without adequate Vit D assumption only the 10-15% of the calcium can be utilized for building new bone (Holick and Garabedian 2006).

Our finding that Vit D stimulates osteoblastic differentiation of DBSCs with the subsequent increase of bone mineral matrix deposition, suggests in addition a possible use of Vit D as food aid in reconstructive therapies of bone with MSCs.

Widening the $1,25(OH)_2D_3$ intake in the population could be recommend for both preventive and therapeutic purposes in bone diseases and trauma.

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