

## Arterial calcification: Finger-pointing at resident and circulating stem cells

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### Abstract

The term "Stammzelle" (stem cells) originally appeared in 1868 in the works of Ernst Haeckel who used it to describe the ancestor unicellular organism from which he presumed all multicellular organisms evolved. Since then stem cells have been studied in a wide spectrum of normal and pathological conditions; it is remarkable to note that ectopic arterial calcification was considered a passive deposit of calcium since its original discovering in 1877; in the last decades, resident and circulating stem cells were imaged to drive arterial calcification through chondro-osteogenic differentiation thus opening the idea that an active mechanism could be at the basis of the process that clinically shows a Janus effect: calcifications either lead to the stabilization or rupture of the atherosclerotic plaques. A review of the literature underlines that 130 years after stem cell discovery, antigenic markers of stem cells are still debated and the identification of the osteoprogenitor phenotype is even more elusive due to tissue degradation occurring at processing and manipulation. It is necessary to find a consensus to perform comparable studies that implies phenotypic recognition of stem cells antigens. A hypoth-

esis is based on the singular morphology and amitotic mechanism of division of osteoclasts: it constitutes the opening to a new approach on osteoprogenitors markers and recognition. Our aim was to highlight all the present evidences of the active calcification process, summarize the different cellular types involved, and discuss a novel approach to discover osteoprogenitor phenotypes in arterial wall.

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**Key words:** Osteocalcin; Osteoprogenitor; Stem cells markers; Arterial calcification; Resident and circulating

**Core tip:** We review state of art on active arterial calcification, introduce new insight in arterial osteoprogenitors (OPs) phenotypes and the concept of amitosis. Analysis of literature of all markers used to define mesenchymal stem cells and OPs revealed the evident incongruity between the actual studies: each research has its own panel of antigen markers. Still, osteocalcin resulted the most promising marker of resident and circulating OPs. A new technique allows maintaining DNA/RNA integrity in highly calcified or ectopic bone formation: new studies should consider this technique and the particular division of OPs to identify them.

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### INTRODUCTION

**Physiological and pathological mechanisms of vascular calcification**

Previously considered passive and degenerative, vascular

calcification is now recognized as a pathobiological process sharing many features with embryonic bone formation<sup>[1]</sup>. Vascular cell differentiation responds to microenvironmental and mechanical cues, since substrates of great stiffness, such as fibronectin, promote osteochondrogenic differentiation, whereas distensible substrates, such as laminin, promote smooth muscle or adipogenic differentiation<sup>[2]</sup>. The biomineralization process begins from the so-called crystallization nucleators, which trigger the formation of a primary crystal nucleus, together with the removal of the mineralization inhibitors [ankylosis protein, nucleotide pyrophosphatase, matrix glutamyl protein (MGP)]. The extracellular matrix vesicles contain deposits of calcium and alkaline phosphatase (ALP), pyrophosphatase, *etc.*, which increase the inorganic phosphates in the vesicles<sup>[3]</sup>. They also stimulate the production of osteopontin, another nucleation inhibitor<sup>[4]</sup>. During the vessel calcification there are active processes similar to those in the bone biomineralization. In depositions in both tunica interna and media of the vessel wall, matrix vesicles have been identified<sup>[5]</sup>. Post-mortem studies have shown that vessel wall may contain a typical bone, cartilage or adipose tissue, with bone as the predominating type of metaplasia (10%-15% of samples), appearing in various morphological forms, from amorphous calcium deposits to mature bone tissue<sup>[6]</sup>.

The increasing interest in vascular calcifications derives from the fact that in the atheromatous disease they were considered a form of plaque regression, while more recently the extent of calcification was associated with a worse prognosis, albeit the real impact of calcification within a specific lesion is unclear<sup>[7]</sup>. Moreover, vascular calcification is commonly seen during other systemic disease, such as diabetes, end-stage renal disease and calciphylaxis, and it is generally considered as a bad outcome predictor<sup>[8]</sup>. In the coronary arteries the extent and dimensions of the calcification seem to play a key role, since small depositions increase the probability of atherosclerotic plaque rupture, especially on their edges, while with individual, large calcification foci such risk is even likely to decrease<sup>[8,9]</sup>. In a study on 10 stabiles and 10 ruptured coronary artery *post-mortem* specimens, calcifications did not significantly affect the stability of the atheroma, in contrast with the significant reduction in stability associated with the lipid content. Removing the calcification led to a statistically insignificant change in stress<sup>[7]</sup>. Anyway, vascular calcification is considered a worsening factor, probably due to the association with the general risk factors: a study by Iribarren *et al.*<sup>[10]</sup> found that aortic arch calcification was associated with coronary heart disease risk both in men and women. Thus aortic arch calcification may reflect the general burden of disease or be a marker of a more aggressive disease.

### **Histological patterns of vascular calcification**

Histologically, arterial calcifications can be classified in calcifications of the tunica intima, principally related to atherosclerosis and, calcifications of the tunica media,

unrelated to atherosclerosis (Monckeberg's type)<sup>[6,11]</sup>.

The intimal atherosclerotic calcifications are the most common form of arterial calcification. Calcium accumulation is initiated by an increase in the plaque of modified lipids, pro-inflammatory cytokines, phosphate and lipoprotein complexes, as well as foci of necrosis<sup>[1,6]</sup>. *In vitro* studies have shown that pro-inflammatory cytokines, oxidized low-density lipoprotein (LDL) or other macrophage release products promote the osteogenesis and the calcium accumulation<sup>[12-14]</sup>, while some studies correlated the vascular calcification with the duration of the hypercholesterolemia<sup>[15]</sup> and with inflammation *in vivo*<sup>[16]</sup>. The so-called punctate deposits start in the deeper intimal regions, adjacent to the media, but very large deposits, involving the whole intima, can be seen<sup>[11]</sup>. In this tissue, hematopoietic, osteoblast-like and osteoclast-like cells were described<sup>[17,18]</sup>. A finest and more diffuse pattern of calcification, involving the whole intima was recently described due to processing techniques that do not require decalcification<sup>[19]</sup>.

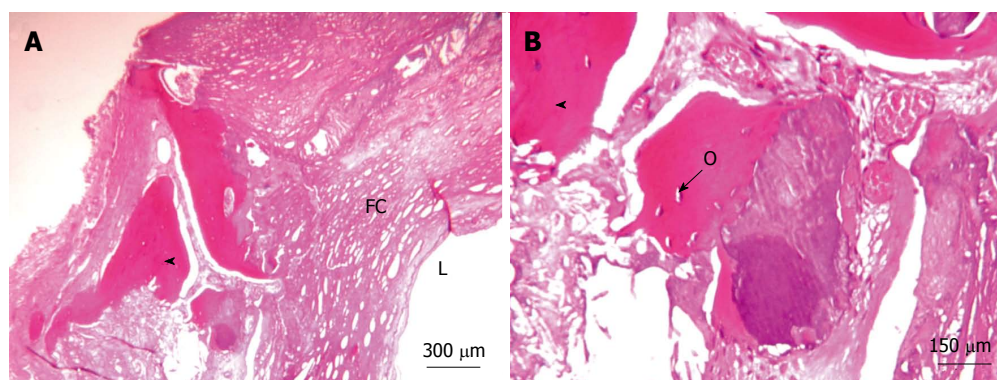
The medial calcification was firstly described by Monckeberg more than a century ago<sup>[20]</sup>. Since then, the "rail-road track" medial calcification was observed in patients with diabetes and chronic renal disease<sup>[21,22]</sup>, as well as in young patients without substitue patent with evident patent metabolic disorders<sup>[23,24]</sup>. In aging, medial calcification may develop by unknown etiology, or result from associated conditions such as chronic renal failure, diabetes, neuropathies and denervations<sup>[11,25]</sup>. In any case, these calcifications are likely to occur in not-atherosclerotic arterial segments<sup>[26]</sup>.

### **Premises for a stem cell origin of vascular calcification**

Classically, the heterotopic calcifications that can be found in the atheromatous plaques, in not-atheromatous arteries, as well as in many tissue, have been subdivided in active and passive<sup>[11]</sup>. The active calcifications follow different (and still unclear) mechanisms that can lead to a true ossification of the vessel wall<sup>[27,28]</sup>.

While very rare in veins<sup>[29]</sup>, ectopic calcification in arteries has been noted for many decades. In 1877, Howse found bone-formation in the wall of a ruptured axillary artery<sup>[30]</sup>. Until recently, however, this phenomenon was simply viewed as a passive consequence of aging<sup>[31]</sup>. However, as already observed in the 1900s, this condition was reported in the aorta of a girl eight years of age, aorta of adults between the ages of sixteen and twenty-four years, in an infant of fifteen months old and in an ossified aorta in a child of three years<sup>[30]</sup>.

Regardless to the arterial layer, calcifications are found in different vessels as coronaries, distal arteries and aorta. As stated above, clinical outcome depends mainly on the degree and the location of calcification, additionally to the underlying disorder<sup>[32]</sup>. Several models postulating mechanisms for the formation and inhibition of calcification have now been proposed<sup>[33]</sup>. These are the active model; the passive physicochemical model; and the arterial osteoclast-like cells model. One model doesn't exclude



**Figure 1** Carotid artery calcifications, hematoxylin and eosin staining. A: Sheet-like calcifications; B: Osteocytes are visible within the bone lacunae-like mature structure in development with lamellar bone. L: Lumen; FC: Fibrous cap; Arrowhead: Ossification; O: Osteocytes.

the other.

In some cases arteries can evolve into mature bone tissue histomorphologically indistinguishable from skeletal bone<sup>[11]</sup>. In our practice, this evolution occurs in at least 5% of diseased arteries. In Figure 1, a Haematoxylin-Eosin staining of a section of carotid atherosclerotic plaques revealed the presence of osteocyte cells within bone lacunae-like mature structure in development; lamellar bone is also visible.

One of the most recent mechanism proposed in order to shine a light on active vascular calcification is the possible role of stem/progenitor cells, either resident in the vessel wall or circulating cells deriving from the bone marrow. In addition, chondrocyte-like cells, typically not expressed in normal arteries, osteoblast-like cells and multinucleated osteoclast-like cells (OLCs) are found in calcified arteries<sup>[17,33]</sup>. These cells are recognizable thanks to their peculiar morphology and positivity to specific histological markers; osteoprotegerin, osteopontin (OPN), osteocalcin (OCN), MGP and bone matrix protein (BMP)<sup>[34]</sup>.

The present review focuses on the current and most recent knowledge on the mechanisms of active vascular calcification ascribable to resident and circulating cells that acquire the plasticity of the stem/progenitor cells and that trigger or participate to the vascular calcification processes.

## CIRCULATING STEM CELLS

The passive model of vascular calcification has been progressively abandoned, since evidence of a genetic and active process has been observed.

Bone marrow (BM)-derived mesenchymal stem cells (MSC) have the ability to differentiate into many stromal cell types, as myocytes, fibroblasts, astrocytes, adipocytes, chondrocytes and osteocytes; the last two are referred as osteo-progenitors<sup>[34]</sup>.

Progenitors are proliferative cells with a limited capacity for self-renewal and are often unipotent. Accumulating evidence indicates that the mobilization and recruitment of circulating or tissue-resident progenitor cells that give rise to endothelial cells (ECs) and smooth muscle

cells (SMCs) can participate in atherosclerosis, neointima hyperplasia after arterial injury, and transplant arteriosclerosis<sup>[35]</sup>. Specifically progenitor cells can contribute to calcification: BM contains both osteoblast and osteoclast precursors termed as osteoprogenitors (OPs) associated with bone remodeling<sup>[36]</sup>. This novel mechanism was named “circulating cell theory”: the bone marrow derived cell population may seed the arteries and contribute to disease or repair<sup>[37]</sup>. The mobilization is the process under the regulation of cytokines in which immature cells from the BM are recruited to the blood<sup>[38]</sup>.

Another common mechanism that can explain the recruitment of circulating OPs in arteries is homing<sup>[39]</sup>; in response to stress signal, injury, inflammation, repair or abnormal cytokine signalling, circulating cells cross the endothelium and invade the target tissue<sup>[40]</sup>. The endothelial phenotype selectively modulates bone marrow-derived stem cells homing: indeed different endothelial phenotypes hold functional differences. As an example, coronary artery endothelium enables the fastest bone marrow stromal cells integration. Transmigration requires the interaction of vascular cell adhesion molecule-1, very late antigen-4,  $\beta 1$  integrins, metalloproteinases (MMP) secretion and cytokines<sup>[40]</sup>.

Recently, a primitive CD14-positive cell population was defined and named monocyte-derived multipotent cells (MOMCs). These cells show a fibroblast-like morphology and the expression of several stem cell markers such as CD14, CD45, CD105, CD34 and type I collagen, but lack expression of CD117 (c-kit) or CD133. These characteristics are quite peculiar<sup>[41]</sup>. Due to this hybrid phenotype, a subpopulation of these cells is likely to overlap the endothelial progenitor cells (EPC) originally described by Asahara *et al.*<sup>[42]</sup>, characterized by the co-expression of CD14 and CD34. Conversely, the so-called monocyte-derived endothelial progenitor cells are described as a MOMC subpopulation positive for CD14 but with low expression for CD34. These cells have the ability to differentiate also into osteoblasts, adipocytes, or neuronal cells<sup>[43]</sup>.

Another subset of these cells showed bone resorption capacity on dentine slices and expression of genes for ca-



thepsin K and calcitonin receptor, characteristic of functional osteoclasts<sup>[41]</sup>. MOMCs express receptor activator of nuclear factor- $\kappa$ B ligand (RANKL), which is required for osteoclast formation from mononuclear precursors. These results indicate that human MOMCs can express RANKL and differentiate into functional osteoclasts without RANKL-expressing accessory cells.

Under specific stimulations (PDGF: Platelet-Derived Growth Factor, interleukin IL-4, IL-13) CD14<sup>+</sup> monocyte precursors can also differentiate into fibrocytes<sup>[44]</sup>. Discovered in 1994, fibrocytes are bone marrow-derived mesenchymal progenitors that co-express hematopoietic stem cell genes, markers of the monocyte lineage, and fibroblast products. Fibrocytes constitute another source of circulating cells able to differentiate in fibroblasts, myofibroblasts and adipocytes<sup>[45]</sup>.

In valve and arteries, myofibroblasts contribute to cardiovascular ossification; Vattikuti observed that adventitial activated myofibroblasts cells are diverted to the osteoblasts lineage: the hypothesis is that myofibroblasts, responding to vascular smooth muscle cell osteopontin production contributes to calcification in diabetes. Moreover pericytic myofibroblasts expressed BMP-2, a powerful bone morphogen<sup>[46]</sup>.

## RESIDENT STEM CELLS

### Mesenchymal stem cells

Bone marrow-derived MSC which reside in the vessel wall can differentiate in several cell types, including osteoblasts, chondrocytes and endothelial cells<sup>[47-51]</sup>.

Previous results from our group showed that it is possible to isolate and culture spindle-shaped resident cells with the characteristics of MSC directly from the vessel wall of thoracic aortas harvested from multiorgan and tissue donors. These vessel-wall MSC (vw-MSC) are CD45<sup>+</sup> and show low expression for CD34, but most co-express CD44, CD90 and CD105, like the bone marrow-derived MSC<sup>[52]</sup>. Moreover, at reverse transcription polymerase chain reaction these cells express transcripts of embryonic stem cell (OCT4, IL6 and BCRP-1) and hematopoietic stem cell (c-Kit, BMI-1)<sup>[52]</sup>. Years after we confirmed that vw-MSC expressed the stemness markers Stro-1, Notch-1 and OCT4, and that they were able to differentiate into adipogenic, chondrogenic and leiomyogenic lineages, when cultured in induction media<sup>[53]</sup>. Recently, Klein *et al.*<sup>[54]</sup> described a CD44<sup>+</sup> population of “vascular wall-resident multipotent stem cells”, expressing also CD90 and CD73, and negative for CD34 and CD45. Moreover, vw-MSC were also isolated and cultured from arterial specimens frozen up to 5 years, and showed positivity for HLA-G, Stro-1, Oct-4 and Notch-1, in addition to the above mentioned<sup>[55]</sup>.

Recently it was hypothesized that MSC might play a role in the pathogenesis of atherosclerosis, and it was demonstrated that, under particular conditions, MSC in culture acquire an osteoblastic phenotype *via* the activation of the Wnt pathway<sup>[56]</sup>. In hyperlipidemic rats treated

with angioplasty to have a vascular damage, MSC started the vessel wall remodelling and triggered calcification, mediated by paracrine BMP-2<sup>[57]</sup>, which is considered one of the main mediators in the differentiation of MSC (and others) along the osteoblastic lineage<sup>[58,59]</sup>. Interestingly, MSC cultured in a uremic serum for one month (mimicking partly the renal failure stimuli) hyperexpressed alkaline phosphatase, osteopontin, Runx2 and showed an up-regulation of BMP-2<sup>[60]</sup>.

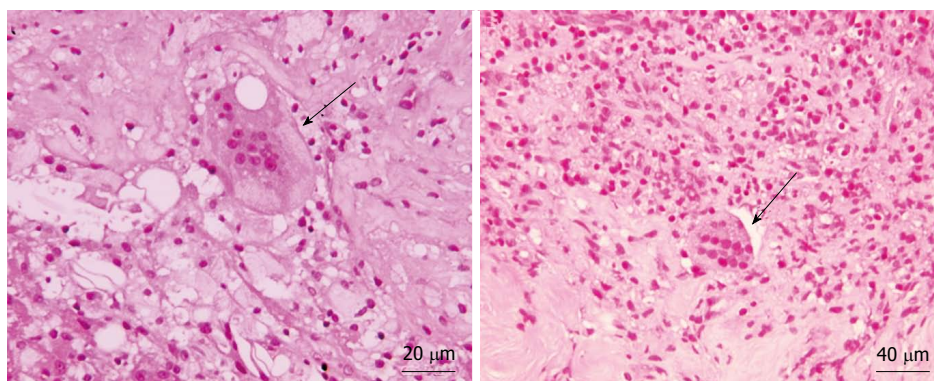
### SMC

SMC of the human arterial wall show a great phenotypic plasticity, since it was demonstrated that in culture they can differentiate in almost all mesenchymal lineages (except adipocytic), and in particular conditions they can calcify<sup>[61,62]</sup>. These cells were originally described as calcifying vascular cells (CVC), *i.e.*, SMC that under cAMP stimulus undergo osteoblast differentiation (with expression of alkaline phosphatase, type I collagen and matrix glutamyl protein), aggregate and form mineralized nodules<sup>[12]</sup>. The matrix carboxyglutamic acid protein (MGP)-deficient mice are a well-known animal model characterized by a progressive calcification of not-atherosclerotic arteries: in these mice vascular SMC were replaced by mineralizing chondrocyte-like cells<sup>[63]</sup>. The possibility of a phenotypic transition by the cells of the arterial wall opened new possibilities in the theories of the active calcification model.

Steitz *et al.*<sup>[64]</sup> demonstrated the phenotypic transition of cultured bovine aortic smooth muscle cells into mineralizing cells: after 10 d from the administration of  $\beta$ -glycerophosphate, the smooth muscle cells lost their contractile properties (and the smooth muscle  $\alpha$ -actin expression) and acquired an osteocalcin- and osteopontin-positive phenotype. Years later, researchers from the same group demonstrated that vascular SMC from MGP-knock-out mice expressed *Runx2/Cbfa1* and gave rise to osteogenic precursors<sup>[65]</sup>. In SMC from human arteries, an increased expression of osteo- and chondrogenic transcription factors (Cbfa1, Msx2, Sox9) was observed concomitantly with a decreased expression of muscle markers<sup>[66]</sup>. SMC cultured in 2D scaffolds and treated 2 wk with lyso-phosphatidylcholine (LPC) underwent trans-differentiation to CVCs by up-regulation of the *Runx-2* gene<sup>[67]</sup>, while more recently the same authors demonstrated that using 3D cultures (a more reliable model of *in vivo* conditions) the growth and mineralization of cultured SMC is even more efficient, and adjustable by external factors such as LPC (enhancer) and Schnurri-3 (inhibitor)<sup>[68]</sup>.

### Neoangiogenesis and endothelial cells

According to several observations, neoangiogenesis and vascular calcification are closely correlated: first of all, neovessels can simply be considered as means of transportation for progenitor cells in the tissue, but endothelial cells are able to produce cytokines that can stimulate osteoprogenitor cells, *in vitro* and *in vivo*. Moreover, many



**Figure 2** Osteoclasts-like giant cells admixed with inflammatory infiltrate. Arrows point osteoblast cells.

growth factor (such as FGF-2 and VEGF) can stimulate both neoangiogenesis and the activation of osteoblasts and osteoclasts<sup>[8]</sup>. Endothelial cells cultured under pro-atherogenic stimuli produce pro-osteogenic factor, such as BMP-2<sup>[69]</sup>. This is particularly interesting, considering that most of plaque neoangiogenesis derive from adventitial *vasa vasorum*, and can drive many progenitor cells, pericytes, and inflammatory stimuli, including cytokines, in the media and intima layers<sup>[70,71]</sup>.

The potentiality of endothelial cells to become directly a source of stem cells was demonstrated in a diabetic mouse model by Yao *et al.*<sup>[72]</sup>, who found that the stimulation with BMP-4 induced endothelial-to-mesenchymal transition and expression of osteogenic markers in aortic endothelial cells. In cultured human aortic endothelial cells, high glucose concentrations cause the acquisition of a “chondrocyte-like” phenotype, with the expression of *STRO-1*, *CD44* and *SOX9*<sup>[73]</sup>. Previous *in vivo* data from our group have demonstrated that quiescent *vasa vasorum* in normal arteries from healthy subjects express markers of progenitor cells, namely Nestin and WT1, thus showing proliferative potential<sup>[74]</sup>. The same phenotype is expressed by intraplaque neoangiogenesis, and particularly Nestin is correlated with complicated plaques<sup>[75]</sup>.

### Osteoclast-like giant cells

Like in the normal bone tissue, the calcification of the vessel wall and/or atheromatous plaque is likely to depend on a balance between pro-osteogenic and anti-osteogenic stimuli. In this setting the so-called osteoclast-like giant cells (Figure 2) play a role in calcium reabsorption, as it was demonstrated decades ago by the findings that apoE-knockout mice lacking also the gene for macrophage colony stimulating (*M-CSF*, a cytokine involved in osteoclast survival) developed massive arterial calcifications<sup>[76]</sup>. The origin of the OLC in the vessel wall are not clear yet, and whether they derive from resident stem cells, from circulating hematopoietic precursors, from a differentiation of mononuclear cells or from other cells not yet established is still to be clarified. The mononuclear cells commonly found in atheromatous plaques share many phenotypic and genetic features with osteoclasts and they have a hematopoietic origin, while many circulating precursor cells express receptors for RANK

and M-CSF, both essential for the osteoclast activity<sup>[11]</sup>.

### Pericytes and macrophage progenitor cells

Pericytes share several phenotypic markers with CVCs, including  $\alpha$ -actin,  $\beta$ -actin, and the 3G5 epitope of monoclonal antibody-defined ganglioside antigen<sup>[8]</sup>. The putative role of pericytes as a “reservoir” of progenitor cells, and their potential to differentiate into several cell types, including osteoblasts, is well known<sup>[66,77,78]</sup>. In the last three decades, using different models, a lot of evidence have been adduced that pericytes can undergo chondro- and osteogenic differentiation<sup>[50,79,80]</sup>. After 8 wk of culture, pericytes have been shown to proliferate and form multicellular clumps with a mineralized matrix containing type I collagen, Gla protein, osteocalcin and osteopontin<sup>[81,82]</sup>. Furthermore, culturing these cells in a chondrogenic media (TGF- $\beta$ 3: Transforming growth factor  $\beta$ 3) pericytes undergo chondrogenic differentiation<sup>[50]</sup>. Other authors hypothesize that adventitial pericytes (expressing activating *Mx2* and other osteoblastic transcription factors) might also be able to stimulate the production of alkaline phosphate, the Wnt pathway activation and the  $\beta$ -catenin nuclear activation in medial cells (SMC)<sup>[83]</sup>. This represents an interesting example of indirect stimulus towards calcification mediated by the synergic cross-talk between different cells of the vessel wall. Indeed, arterial adventitia contain different progenitor cells, as it was demonstrated in murine aorta, where a population of Sca-1+/CD45+ macrophage progenitor cells has been recently described, which represents a *reservoir* of non-circulating precursors cells<sup>[84]</sup>.

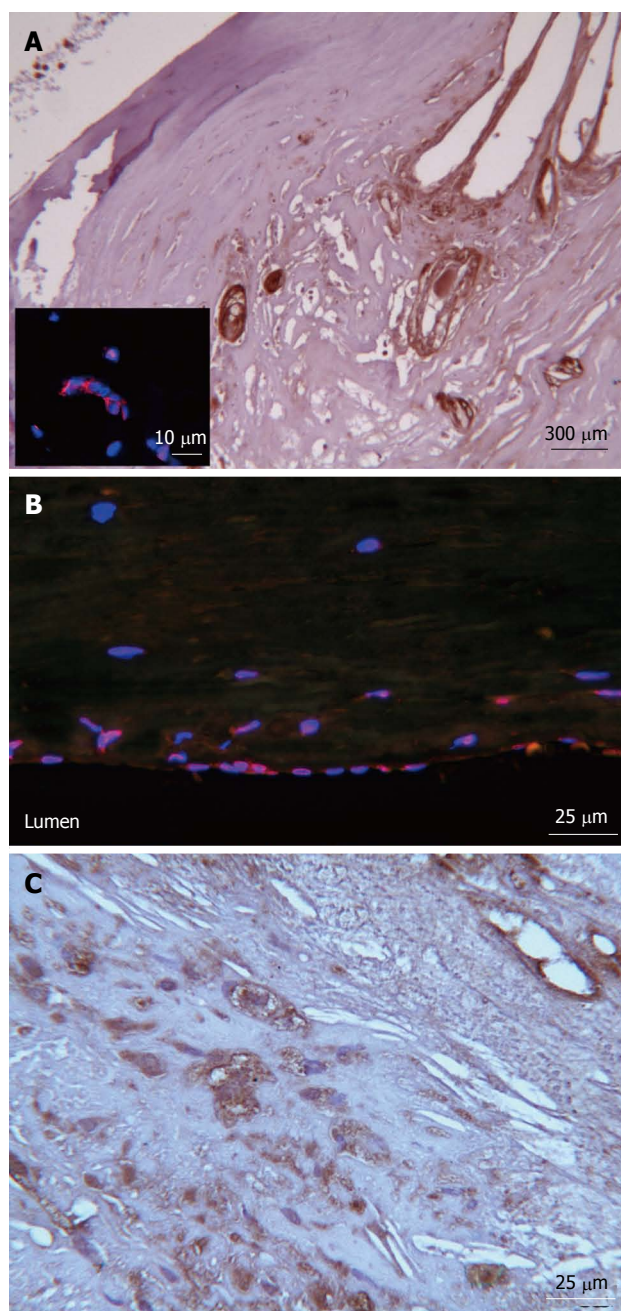
The role of the adventitial cells in the regulation of the functions of the vessel wall, both physiologically and in pathological conditions including calcifications, surely deserves future in-depth analyses.

## DEFINITIONAL CRITERIA OF OSTEOGENIC LINEAGES

### Osteoblastic “profile” and mechanisms

As shown above, several *in vitro* and animal models have demonstrated that a main mechanism of vascular calcification is represented by BMP-2 and 4. BMP-2 upregu-





**Figure 3 Osterix and osteocalcin expression in carotid plaques.** A: Osterix immunohistochemistry (IHC) positivity in vessels single-label immunofluorescence micrographs representing Osterix (red) is also seen in the nucleus of endothelial cells of a single vessel; B: In the carotid endothelium layer, nuclei (blue) were counterstained with DAPI (4',6-diamidino-2-phenylindole); C: Cytoplasmic and extracellular matrix osteocalcin IHC pattern.

lates *Runx2*, which induces the production of type I collagen and alkaline phosphatase<sup>[85,86]</sup>. As demonstrated in murine models, MGP is the principal inhibitor of BMP-2, and a loss of MGP leads to tissue calcification<sup>[63]</sup>. One of the master genes essential for driving differentiation of mesenchymal cells into terminally differentiated osteoblasts is Osterix<sup>[11]</sup>, that can be also found expressed in endothelial cells of the diseased arterial wall (Figure 3).

Recently, the receptor activator of NF- $\kappa$ B ligand (RANKL) was identified as another key molecule in the

differentiation of osteoblasts and osteoclasts: in apoE<sup>-/-</sup> mice, the immunostaining for RANKL was diffusely positive in activated chondrocytes involved in the vascular ossification process<sup>[87]</sup>, and its serum level seems to increase with ageing proportionally to the risk of cardiovascular events<sup>[88]</sup>.

Osteopontin is a normal component of the bone and plays a role in the regulation of the mineralization. In calcified human plaques, OPN is expressed in SMC, endothelial cells and macrophages<sup>[89,90]</sup>.

Osteocalcin is one of the most studied markers of osteoblast lineage. OCN is synthesized by osteoblasts and is the major component of the bone matrix (1%-2%). OCN is capable of binding hydroxyapatite (HA) thanks to his glutamyl (GLA) residues. Five Ca<sup>2+</sup> ions are bound by 3 GLA residues carboxylated by vitamin K1<sup>[91]</sup>, thus the OCN can dock on the HA and add calcium and growth crystal leading to the grow of bone. Transcription of OCN is regulated by Vitamin D3. In addition to binding to hydroxyapatite, OCN functions in cell signaling and the recruitment of osteoclasts and osteoblasts<sup>[92]</sup>.

In patients with peripheral artery disease, the percentage of circulating bone marrow-derived OPs, positive to OCN, increased with the severity of aortic calcification<sup>[93]</sup>. Wang *et al*<sup>[94]</sup> demonstrated that in injured arteries the release of TGF $\beta$  mobilize MSC from the blood stream to the neointima. In a mouse model LDLR<sup>-/-</sup>, Nestin+/Sca+ cells were all recruited in the calcified arteries were OCN+ osteoblastic cells were seen: they observed that MSC generated OCN+ osteoblastic cells in the calcified lesions and that the migration of MSC to the lesions depends on TGF $\beta$  production from the lesions. Finally, when TGF $\beta$  receptor 1 was inhibited in mice there was a decrease of the number of MSC in the blood concomitant to their recruitment to the arterial lesions at the calcified lesions.

Different studies correlate the amount of circulating OCN-positive cells to the presence of coronary disease. Flammer *et al*<sup>[95]</sup> counted with flow cytometry the blood circulating population of cells positive to both immature EPC markers CD133+, CD34-, KDR+ and OCN. They observed that this fraction of cells, OCN+ EPC, increased in patients with cardiovascular risk factors compared to patients with a stable coronary artery disease history. Of note that the blood circulating cells expressing OCN have been shown to be able to calcify *in vitro* and *in vivo*<sup>[96]</sup>. In a similar study, Gössl *et al*<sup>[97]</sup> compared the fraction of EPC circulating cells CD34+/KDR+/OCN+ in 3 groups; the control group (normal coronary arteries/no endothelial dysfunction) versus two groups with coronary atherosclerosis: early coronary atherosclerosis (ECA: Normal coronary arteries but with endothelial dysfunction) and late coronary atherosclerosis (LCA: Severe, multi-vessel coronary artery disease). The number of CD34+/KDR+/OCN+ cells were increased by -2-fold in the ECA patients, with smaller increases in the LCA patients.

The prevalence and extent of calcification seems to

**Table 1** Phenotypic markers for circulating and resident progenitors cells

Name of the progenitors	Antigens	Notes (origin)	Ref.
MSC, EPC and CEC in human			
MSC	CD105+, CD73+, CD90+, CD14-, CD34-, CD45-, CD79-, and CD19-	Resident from various tissues	Dominici <i>et al</i> <sup>[103]</sup>
vw-MPSC	CD44+, CD90+, CD73+, CD34-, CD45-	Resident from arterial adventitia	Klein <i>et al</i> <sup>[54]</sup>
vw-MSC	CD44+, CD90+, CD73+, CD105+, CD29+, CD166+, Stro-1, Notch-1 and Oct-4	Resident from aortic arches, thoracic and femoral arteries	Pasquinelli <i>et al</i> <sup>[52,53]</sup>
Circulating MSC	CD105+, CD166+, CD54+, CD55+, CD13+, CD44+; CD34-, CD45-, CD14-, CD31-, CD133-	Circulating and resident from bone marrow; cartilage; synovial membrane; peripheral blood; umbilical cord blood; teeth	Qian <i>et al</i> <sup>[104]</sup>
Circulating EPC	CD34+, CD133+, VEGFR2+ (KDR)	Circulating and resident from bone marrow; peripheral blood; umbilical cord blood; hematopoietic stem cells; hemangioblast; fat tissues	
Circulating total PC	CD133+; CD34+; CD133+/CD34+	Circulating	Baker <i>et al</i> <sup>[105]</sup>
Circulating EC	CD146+/CD31+	Circulating	
Circulating EPC	CD34+ VEGFR+ CD133+	Circulating	
Circulating EPC	(CD34+/CD133-/KDR+/CD45-) expressing VDR+ or OCN+, and VDR+ and OCN+	Circulating (in chronic kidney disease)	Cianciolo <i>et al</i> <sup>[106]</sup>
Circulating EPC	CD133+, CD34+, KDR+	Circulating (in rheumatoid arthritis with coronary Calcification)	Yiu <i>et al</i> <sup>[107]</sup>
OPs in human			
OP	CD44+, CD63+, CD146+, Stro-1+	Resident bone marrow	Gronthos <i>et al</i> <sup>[108]</sup>
CVC	3G5+	Resident from arteries (Pericytes markers)	Bostrom <i>et al</i> <sup>[109]</sup>
Circulating osteocalcin-positive mononuclear cells	OCN+	Circulating bone marrow derived	Pal <i>et al</i> <sup>[93]</sup>
Circulating progenitor cells (Pro-calcific differentiation)	CD34+/OCN+; CD34+/BAP+; CD34+/OCN+/BAP+; OCN+/KDR+ ratio; BAP+/KDR+ ratio; OCN+/BAP+/KDR+ ratio	Circulating (in diabete mellitus)	Fadini <i>et al</i> <sup>[110]</sup>
Circulating EPC expressing osteocalcin	OCN+/CD133+/CD34-/KDR+	Circulating (in cardiovascular disease)	Flammer <i>et al</i> <sup>[95]</sup>
Circulating EPC expressing osteocalcin	OCN+/CD133+/CD34-/KDR+	Circulating (in coronary atherosclerosis, plaques instability)	Gössl <i>et al</i> <sup>[97]</sup>
Circulating T cells	CD28- CD8 +T cells	Circulating (in calcific aortic stenosis)	Winchester <i>et al</i> <sup>[111]</sup>
OPs in mouse models			
Mesenchymal OPs	CD45-/TER119-/ Sca-1+/PDGFRa+	Resident in the mouse bone marrow	Morikawa <i>et al</i> <sup>[112]</sup>
Bone marrow-derived calcifying cells	Sca-1+/PDGFRa- and Sca-1+/PDGFRa+	Resident from mice aorta	Cho <i>et al</i> <sup>[113]</sup>
Circulating osteogenic cells	Sca-1+, PDGFRa+, CD45-, CD44+, CXCR4+	Circulating in ectopic bone formation in a mouse model	Otsuru <i>et al</i> <sup>[114]</sup>

MSC: Mesenchymal stromal cells; EPC: Endothelial progenitors cells; CEC: Circulating endothelial cells; vw-MPSC: Vascular wall-resident multipotent stem cells; vw-MSC: Vascular wall-resident multipotent mesenchymal stem cells; PC: Progenitors cells; CVC: Calcifying vascular cells; OPs: Osteoprogenitors; CD: Cluster of differentiation; Oct-4: Octamer-binding transcription factor 4; VEGFR: Vascular endothelial growth factor receptor; OCN: Osteocalcin; VEGFR2 (KDR): Kinase insert domain receptor; VDR: Vitamin D receptor; BAP: Bone alkaline phosphatase; Sca-1: Stem cells antigen-1; PDGFR: Platelet-derived growth factor receptor; CXCR-4: C-X-C chemokine receptor type 4.

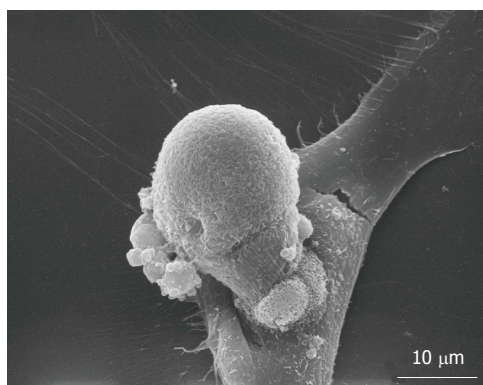
have a genetic component that appears to be partially independent of those involved in atherogenesis. Specific genes that have been linked to arterial calcification in humans are also involved in atherosclerosis and include angiotensin I-converting enzyme, apo E, E-selectin, MMP-3, MGP, CC chemokine receptor 2, and estrogen receptor  $\alpha$ <sup>[11]</sup>.

### New processing techniques of calcified tissue

Due to the tissue composition, morphological analysis of calcified or bone-like tissue is often incomplete: the decalcification procedure degrades the 3D structure of cells and hydrolyses the nucleic acid molecule<sup>[98]</sup>. Decalcification procedure with ethylenediaminetetraacetic acid or chloride acid put significant limitations to the study of

ectopic tissue calcification. Based on this consideration, we recently decided to apply a new technique to preserve nuclear morphology and nucleic acid content, whilst preserving the 3D cellular structure. This protocol was patented at the Massachusetts Institute of Technology of Cambridge (Patent number WO2006009860 A3)<sup>[99,100]</sup>. Thanks to this method, a new set of cells missed for almost 100 years<sup>[101]</sup> were discovered: the shape of the nucleus was difficult to spot because of the standard 2.5  $\mu$ m cut. Metakaryotic cells, also called bell-shaped cells, were identified first in developing fetus, then in adult cancerous tissue and finally in vascular tissue and represent the first possible evidence of stem cells lineages<sup>[53,100,102]</sup>.

Briefly, the spreading protocol<sup>[99,100]</sup> is based on the digestion of Carnoy fixed tissue with of a collagenase



**Figure 4** Scanning electronic microscopy. Budding process in vascular wall-resident multipotent stem cells *in vitro*.

type II enzyme that slowly disaggregate calcified tissue, after maceration in acetic acid, tissue are spread on a slide in a single monolayer of cells. At this point, standard immunohistochemical and molecular analyses could be performed: the result is that morphology of single cells from mineralized tissues is visible (Fittipaldi *et al* unpublished data).

## CONCLUSION

As it is evident from Table 1, the literature of the last 20 years concerning stem cells is characterized by a general incongruity about which marker panel defines the progenitor cells and the different progenitor lineages. This is reflected by the Babel-like terminology used to define the progenitor cells by different groups. This issue is even foggier when it comes to the OPs identification, which became crucial in the last years, with the acceptance of the active model of vascular calcification<sup>[11]</sup>.

It becomes necessary to uniform the phenotypic definition of osteoprogenitor cells. Paradoxically, the typical morphology of resident osteoblasts and osteoclasts could be of help, since these cells are easily recognizable at optical microscopy. Therefore, the morphology could constitute the basis of the future identification of those resident cells which deserve more attention for the identification of their phenotype. These observations will push towards the study of alternative methods of morphological analysis, including the spreading analysis on calcified tissue, which opens the novel possibility to have more information on DNA and proteins composing bone-like tissue. Twenty years ago, in a study on ectopic bone formation, Solari *et al*<sup>[115]</sup> found that osteoclasts undergo amitotic division, and that a budding process is responsible of their division (Figure 4). Recently, these results were fuelled by the finding that some cells with the characteristics of stem cells divide by an amitotic mechanism, using a RNA-DNA intermediate<sup>[116]</sup>. We recently found cells with the same characteristics in adult pathological arteries (Fittipaldi *et al* unpublished data).

Most of the definitions (and incongruity) of the stem cells derive from osteo-chondrogenic differentiation

studies on cultured cells. In these *in vitro* models, cells are induced to differentiate by definite exogenous stimuli, which do not correspond to the vessel wall microenvironment during the *in vivo* calcification process. In our opinion, another way to overcome these incongruities in the future, apart from morphology, is the molecular approach, *i.e.*, the identification of one or more markers to locate *in situ* the progenitor cells and the osteogenic precursors in the vessel wall, as well as the definition of the resident amitotic cells. A promising approach to definitely decipher all the markers characterizing the osteoprogenitor cells could be a combined mRNA profiling and gene set analysis, as already performed on the early and late EPC<sup>[117]</sup>, in order to be able to apply more doable techniques such as immunohistochemistry, immunofluorescence or *in situ* hybridization.

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