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IDENTIFICATION, CHARACTERIZATION AND PATHOPHYSIOLOGICAL ROLE OF CIRCULATING MYELOID CALCIFYING CELLS IN DIABETIC VASCULOPATHY

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RIASSUNTO

La calcificazione vascolare è una caratteristica tipica della vasculopatia diabetica (Abedin M. et al., 2004; Johnson R.C. et al., 2006). All'interno delle lesioni aterosclerotiche, le microcalcificazioni dell'intima, contribuiscono a destabilizzare la placca aterosclerotica (Virmani R. et al., 2006). I meccanismi che aumentano la calcificazione vascolare nel diabete sono ancora non completamente conosciuti: una eccessiva concentrazione di fattori procalcifici e una riduzione degli inibitori osteogenici potrebbero essere coinvolti in questo processo (Johnson R.C. et al., 2006). Le cellule che iniziano il processo di calcificazione vascolare non sono ancora state identificate definitivamente: cellule vascolari residenti nella parete dei vasi, come cellule muscolari lisce o periciti, potrebbero transdifferenziare e produrre matrice mineralizzata (Iyemere V.P. et al., 2006). Inoltre, Eghbali-Fatourechi et al hanno descritto l'esistenza di una popolazione di cellule osteoblastiche circolanti nel sangue periferico umano capaci di calcificare in vivo e in vitro (Eghbali-Fatourechi G.Z. et al., 1966). Queste cellule, che esprimono osteocalcina (OC) e fosfatasi alcalina ossea (BAP), sono state considerate cellule progenitrici circolanti, ma la loro origine non è ancora stata chiarita. Le cellule osteoprogenitrici classiche, quelle del midollo osseo, originano dal compartimento mesenchimale, ma uno studio recente ha proposto che le cellule OC+ e BAP+ non debbano essere completamente distinte dalle cellule staminali ematopoietiche (Eghbali-Fatourechi G.Z. et al., 2007). E' stato trovato un aumento delle cellule circolanti OC+ BAP+ dopo una frattura (Eghbali-Fatourechi G.Z. et al., 1966). Questo potrebbe essere dovuto ad una stimolazione della nicchia del midollo osseo nel rilasciare cellule con un fenotipo osteogenico (Kumagai K. et al, 2008; Otsuru S. et al., 2008). In relazione alle malattie cardiovascolari (CVD), studi clinici preliminari hanno dimostrato che aterosclerosi coronarica e rigità arteriosa sono associate con l'attivazione di un programma osteogenico nelle cellule derivanti dal midolo osseo (Gossl M. et al., 2008; Pirro M. et al., 2011), ma il ruolo patofisiologico di queste cellule nella calcificazione vascolare non è ancora chiaro.

In questo lavoro abbiamo cercato di determinare la natura, l'origine e l'attività delle cellule procalcifiche circolanti umane. Abbiamo dimostrato per la prima volta che esiste una sottopopolazione di cellule circolanti che esprimono fosfatasi alcalina ossea e osteocalcina (OC+BAP+) e che possiede attività procalcifica in vivo e in

vitro. Le cellule OC+ BAP+ sono di origine mieloide e mantengono i marker tipici dei monociti e dei macrofagi, inoltre una sottopopolazione di queste sono longeve. Queste cellule calcifiche mieloidi (myeloid calcifying cells, MCCs) possono differenziare dalle cellule mononucleate del sangue periferico e formare calcificazione ectopica in vivo. Inoltre la formazione di MCC è stata associata strettamente con l'espressione di Runx2, che è un fattore di trascrizione osteogenico. Nel trapianto di midollo osseo umano gender-mismatched le cellule circolanti MCC hanno un'emivita maggiore rispetto alle cellule OC-BAP-, suggerendo che queste cellule appartengano ad un repertorio cellulare stabile.

Da un punto di vista clinico, abbiamo dimostrato che le MCC sono sovraespresse nel sangue dei pazienti con diabete di tipo 2 e lesioni aterosclerotiche mentre un controllo glicemico è in grado di ridurre queste cellule portandole a livelli normali.

Lo studio di pazienti naïve con leucemia mieloide cronica indica che le cellule OC+BAP+ sono di origine mieloide. Inoltre, alte concentrazioni di glucosio aumentano la calcificazione in vitro, e condizioni di ipossia possono regolare la formazione di MCC in vivo e in vitro.

Presi nel loro insieme questi dati identificano una nuova sorgente di cellule procalcifiche che possono contribuire alla calcificazione vascolare, la quale è coinvolta nell'alto rischio di malattia cardiovascolare associata al diabete.

SUMMARY

Vascular calcification is a hallmark feature of diabetic vasculopathy (Abedin M. et al., 2004; Johnson R.C. et al., 2006). Within atherosclerotic lesions, intimal microcalcifications contribute to destabilize the plaque (Virmani R. et al., 2006). The mechanisms increasing vascular calcification in diabetes are incompletely understood: excess concentrations of procalcific factors and reduction of osteogenic inhibitors may be involved (Johnson R.C. et al., 2006). Cells that initiate vascular calcification are yet to be definitely identified: vascular wall resident cells, such as smooth muscle cells or pericytes, can transdifferentiate and produce a mineralized matrix (Iyemere V.P. et al., 2006). In addition, Eghbali-Fatourechi et al described the existence of circulating osteoblastic cells in human peripheral blood that calcify in vitro and in vivo (Eghbali-Fatourechi G.Z. et al., 1966). These cells, which express the bone protein osteocalcin (OC) and bone alkaline phosphatase (BAP), have been considered circulating osteoprogenitor cells, but their origin is unclear. Classic osteoprogenitor cells in the bone marrow originate from the mesenchymal compartment, but one study proposed that OC+ and BAP+ cells might not be completely distinct from hematopoietic stem cells (Eghbali-Fatourechi G.Z. et al., 2007). Circulating OC+ and BAP+ cells have been found to increase after a fracture (Eghbali-Fatourechi G.Z. et al., 1966), possibly through stimulation of the bone marrow niche to release cells with an osteogenic phenotype (Kumagai K. et al, 2008; Otsuru S. et al., 2008). In relation to cardiovascular disease (CVD), preliminary clinical studies found that coronary atherosclerosis and arterial stiffening are associated with activation of an osteogenic program in bone marrow–derived cells (Gossl M. et al., 2008; Pirro M. et al., 2011), but the pathophysiological role of these cells in vascular calcification is still unknown.

In this work we investigated the nature, origin, and activity of human circulating procalcific cells. We demonstrate for the first time that a distinct subpopulation of circulating cells expressing osteocalcin and bone alkaline phosphatase (OC+BAP+) has procalcific activity in vitro and in vivo. OC+ BAP+ cells originate from the myeloid lineage and mantain monocyte/macrophage markers, and a subpopulation of them is longlived. These "myeloid calcifying cells" (MCCs) can be differentiated from peripheral blood mononuclear cells and form ectopic calcifications in vivo. Moreover, generation of MCCs was closely associated with expression of the osteogenic

transcription factor Runx2. In gender-mismatched bone marrow transplanted humans, circulating MCCs had a much longer half-life compared with OC-BAP- cells, suggesting they belong to a stable cell repertoire.

From a clinical point of view, we provide evidence that MCCs are overrepresented in type 2 diabetes blood and atherosclerotic lesions, whereas glycemic control was able to reduce MCCs toward normal levels.

The study of naïve patients with chronic myeloid leukaemia indicated that OC+BAP+ cells have a myeloid origin. Furthermore, high glucose increased calcification by MCCs in vitro, and hypoxia may regulate MCC generation in vitro and in vivo.

Taken together, these data identify a novel source of procalcific cells that may contribute to vascular calcification, which is involved in the high cardiovascular risk associated with diabetes.

1. INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a common metabolic disease characterized by multiple biochemical disorders whose common denominator is hyperglycemia. Hyperglycemia in diabetes is caused by a defect in secretion and / or insulin activity.

Insulin is a peptide hormone secreted by pancreatic beta cells in response to raising glucose concentrations and other stimuli, and allows glucose to enter the cells and its subsequent use as an energy source. The failure of this mechanism causes glucose to accumulate in the bloodstream.

The endocrine pancreas is composed by pancreatic islets, and each islet is composed by beta cells and other cell types, such as alpha cells (that secrete the counter-regulatory hormone glucagon), delta cells (that contain somatostatin) and cells (that contain a particular peptide that stimulates secretion of gastric and pancreatic enzymes and inhibits the intestinal motility).

The levels of glucose in the blood are normally maintained between 70-110 mg/dl. Diabetes is diagnosed when there is an increase in blood glucose with one of the following criteria: 1) a random plasma glucose ≥200 mg/dl with typical sympthoms of diabetes; 2) a fasting plasma glucose ≥126 mg/dl on at least two occasions; 3) a 2h post-OGTT (Oral glucose Tolerance Test) palsma glucose is ≥200 mg/dl; 4) a glycated hemoglobin ≥6.5%.

All forms of diabetes mellitus have in common hyperglycemia, but the pathogenetic processes involved in the development of hyperglycemia are different. Thus, diabetes can be divided into two main categories:

- **Type 1 diabetes** is characterized by a complete destruction of pancreatic beta cells, whose consequence is the absence of insulin production. In most cases, this type of diabetes is caused by an autoimmune attack to beta cells by CD4+ and CD8+ T lymphocytes. The beta cells that survive express MHC class II molecules as a result of local production of cytokine IFN-γ by T lymphocytes.

- **Type 2 diabetes** is caused by the association between insulin resistance and inadequate secretory response by pancreatic beta cells (Cotran-Robbins, 6th edition).

Diabetes mellitus is associated with several microvascular and macrovascular complication. Retinopathy, neuropathy and nephropathy are microvascular complications, while ischaemic heart disease, cerebrovascular disease and

peripheral vascular diseases are macrovascular complications (Clark C.M. Jr and Lee D.A., 1995; Cooper M.E. et al., 2001; Virsaladze D. and Kipiani V., 2001).

1.2 DIABETIC MACROVASCULOPATHY

Diabetic macroangiopathy is associated with structural and functional changes in large arteries that lead to atherosclerosis, increased arterial stiffnes, irregular pulse wave travel and hypertension. The glycation of wall components, change in endothelial function, increased arterial stiffness or decreased arterial distensibility involve structural changes. The physiological impairment that likely links diabetes with strong increase in atherosclerotic vascular diseases include endothelial dysfunction, smooth muscle cells (SMCs) dysfunction, platelet hyper-reactivity, impaired fibrinolysis associated with thrombosis and coagulation, and enhanced inflammation (Creager M.A. et al., 2003).

Endothelial dysfunction is of paramount importance in the pathogenesis of diabetic vascular disease. The endothelium supervises the tone of the underlying vascular smooth muscle by the production of vasodilators and generally regulating the balance between dilation and constriction. Endothelial dysfunction is characterized by decresed nitric oxide (NO) and by incresed synthesis of vasoconstrictor prostanoids and endothelin (ET-1) (De Vriese A.S. et al., 2000).

ECs synthesize bioactive substrates wich regulate blood vessels structure and function such as NO, reactive oxygen species (ROS), prostaglandins, endothelin and angiotensin II (Verma S. and Anderson T.J., 2001).

The endothelial nitric oxide synthase (eNOS), responsible for NO production in the vessel wall, besides being a major regulator of vascular tone, is also a master gene regulator of endothelial cell development and survival. The defective eNOS activation induced by hyperglycemia involves the impaired phosphorylation via the Akt-PI-3K pathway. Impaired NO production and signaling has several negative consequences on endothelial biology, included impaired endothelial cell survival and excess apoptosis. Endothelial dysfunction/damage is the first step in the pathogenesis of diabetic vasculopathy and atherosclerosis in general (Avogaro A. et al., 2006; Avogaro A. et al., 2008).

Impairment of glycaemic control, hypertension and dyslipidaemia are associated with altered endothelial-dependent vasodilatation, referred to as endothelial dysfunction (Storey A.M. et al., 2001). Hyperglycaemia is associated with increased oxidative

stress, glycation of proteins, lipoproteins, apolipoproteins and clotting factors, and enhanced leucocyte-endothelial interaction (Baynes J.W., 1991; Morigi M. et al., 1998). Hyperglycemia increses the formation of AGEs (Advanced Glycation Ends production), one of the most important links to the development of diabetic complications (Deckert T. et al., 1989; Tesfamariam B. et al., 1991; Zeiher A.M. et al., 1991; Johnstone M.T. et al., 1993; Parving H.H. et al., 1996; Wautier J. et al., 1996). AGEs are produced by the non-enzymatic reaction of glucose and other glycating compounds derived from glucose and increased fatty acid oxidation in arterial endothelial cells and most likely heart (e.g. dicarbonyls such as 3-deoxyglucosone, methylglyoxal and glyoxal) with proteins (Candido R. et al., 2003; Wautier J.L. and Schmidt A.M., 2004). In diabetes, AGEs are found in large quantity in extracellular matrix (Nishino T. et al., 1995; Horie K. et al., 1997; Niwa T. et al., 1997; Stitt A.W. et al., 1997; Stitt A.W. et al., 1998). There are three main mechanism whereby intracellular production of AGE precursors can damage cells: 1) intracellular proteins modified by AGEs have altered function; 2) extracellular matrix components modified by AGE precursors interact abnormally with other matrix components and with integrins that are expressed on the surface of cells; 3) plasma proteins modified by AGE precursors bind to AGE receptors on cells such as macrophages, vascular endothelial cells and vascular smooth muscle cells. One typical consequence of AGE binding to their receptors is the production of ROS (Goldin A. et al., 2006).

Diabetes accelerates the progression of atherosclerotic lesion and alters the vasculature by stimulating the atherogenic activity of vascular SMCs (Beckman J.A. et al., 2002). In patients with diabetes and advanced atherosclerotic lesions the number of vascular SMCs appear to be reduced (Fukumoto H. et al., 1998). In atherosclerotic lesions, the increased migration and cell death of vascular SMCs are regulated by modifications of LDL induced by hyperglycemia (oxidation, glycation, acetylation). In vitro, LDL that are subjected to non-enzymatic glycation induce vascular SMCs migration, whereas oxidized glycated LDL induce SMC death (Taguchi S. et al., 2000). In high glucose concentrations, the formation of hydrogen peroxide (H_2O_2) induces apoptotic and necrotic cell death, that could be as well responsible in the evolution of diabetic vasculopathy (Chaturvedi N. et al., 2001).

In diabetic patients, platelet aggregation and adhesion are enhanced, and the intrinsic platelet activation is increased, together with reduced activity of endogenous platelet inhibitors (Davi G. et al., 1990; Winocour P.D. et al., 1990; Stein B. et al.,

1995; Walsh M.F. et al., 1995). In the initial disease state that precedes the progression of cardiovascular disease, platelet aggregation activity are enchanced in diabetes platelets (Davi G. et al., 1990; Winocour P.D. et al., 1990; Stein B. et al., 1995; Walsh M.F. et al., 1995; Parving H.H. et al., 1996; Vlassara H., 1997; Chaturvedi N. et al., 2001). In vascular diseases, abnormal vascular homeostasis, weakened vasodilatation, excess platelet aggregation, impaired endothelial turnover and inflammation lead to the progression of atherosclerosis and the eventual rupture of the plaque (Vinik A.I. et al. 2001). Platelets can modulate vascular function and they are important in thrombus formation (Winocour P.D. et al., 1990). PDGF (Platelet-derived growth factor), they stimulates migration and proliferation of SMC and extracellular matrix production that concur to the formation of "subendothelial fibrointimal" lesions and the external capsule of fatty lesions inducing the development of atherosclerosis (Myllärnieni M. et al., 1997). Hyperglicaemia stimulates the PDGF-β receptor expression via protein kinase C (PKC) activation in vascular SMCs. This mecchanism implies the increase of superoxide (O_2^-) and the decrease platelet-derived NO, suggesting that PDGF could be involved in the development of diabetic vasculopathy (Inaba T. et al., 1996; Assert R. et al., 2001).

Several factors contribute to the pathogenesis of diabetic vasculopathy. Hyperglycaemia, non-enzymatic protein glycation, lipid modulation, altered expression of cytokines and deranged growth factor activation are all implicated in the development of diabetic vasculopathy.

One of the most important element in the pathogenesis of diabetic vasculopathy is hyperglycaemia (TDCCTRG, 1993; Groundy S.M. et al., 1999; Laakso M., 1999), as it increases oxidative stress due to the production of free radicals, O_2^-, OH^- and H_2O_2 (Baynes J.W., 1991) and with production of AGEs (Brownlee M. et al., 1988; Aronson D. and Rayfield E.J., 2002). The superoxide anion reduces NO bioavailability and inactivates NO in the subendothelial space (Tesfamarian B. and Cohen R.A., 1992). ROS produce alterations in lipids and proteins by cross-linking and fragmentation, they accelerate AGE formation and promote LDL oxidation (Ox-LDL) that are more atherogenic. Ox-LDL increase monocyte recruitment to the subendothelial space which are activated to become macrophages. The macrophages bind and internalize Ox-LDL leading to the formation of foam cells, which stimulate inflammatory mediators and growth factors (Schwartz C.J. et al., 1992; Klein R.L. et al., 1995; Dzau V.J., 2001).

AGEs affect the normal function of protein and lipids by modifing molecular conformation, reducing degradative capacity and recognition of protein and lipids and altering enzymatic activity (Brownlee M. et al., 1988; Aronson D. and Rayfield E.J., 2002). AGEs also have pro-atherosclerotic effects by receptor-mediated mechanisms (Aronson D. and Rayfield E.J., 2002), and they play an important role in the development of vascular dysfunction in general (Brownlee M., 1995).

1.3 THE PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is a fibroproliferative and chronic immunoinflammatory disease, against the large and medium artery wall (Glass C.K. and Witztum J.L., 2001; Libby P. 2002; Hansson G.K., 2005). Endothelial cells, leukocytes, and intimal smooth muscle cells are important players in the development of this disease.

The initial event in the formation of atherosclerosis is endothelial damage and the accumulation and subsequent modification of low density lipoprotein (LDL) in the sub-intimal space of the arteries. The accumulation of LDL is due to increased endothelial permeability and the binding of LDL to the intima extracellular matrix constituents (Fig. 1A). Endothelial dysfunction is followed by the adhesion and migration of monocytes and T cells in the intima in response to the expression of adhesion molecules on the endothelial surface, such as selectin, VCAM-1, ICAM-1, and chemotactic signals released by the endothelium damaged as MCP-1 (Fig.1B). Macrophages ingest oxidized and infiltrated lipoproteins, and they become foam cells that characterize the fatty streaks. Then, atheroma formation involves the recruitment of SMCs from the tunica media to the tunica intima and their proliferation in response to mediators such as PDGF. In the so-called neo-intima, SMCs produce extracellular matrix molecules, such as collagen and elastin, thus forming a fibrous cap covering the plaque. This cap covers a collection of macrophage-derived foam cells, some of which die by apoptosis and release lipids that accumulated extracellulary (Fig. 1C) (Tabas I., 2010). Platelet adhesion to the stripped intima and the formation of thrombi markedly contributes to the rapid growth of the atherosclerotic lesion (Fig. 1D).

Plasma molecules and lipoprotein particles, depending on the size and concentration, extravasate across the damaged endothelium that exposes the underlying tissue and locate within the subendothelial space, where are transformed to become cytotoxic, chemotactic, proatherogenic and proinflammatory. The mechanism of the modification of atherogenic lipoproteins (LDL) is not yet clear but

could be caused by oxidation mediated by myeloperoxidase, 15-lipoxygenase and/or nitric oxide synthase (NOS) (Glass C.K. and Witztum J.L., 2001). Nitric oxide (NO) is a very powerful oxidant which is produced both by endothelial cells and macrophages. NO seems to exert both effects, protective and atherogenic, depending on its sources. NO produced by macrophages by iNOS (inducible NOS) is proatherogenic due to its oxidation by ROS, while NO produced by endothelial cells via eNOS has vasodilator function and it is atheroprotective (Falk E., 2006).

Due to atherogenic and proinflammatory stimuli, the endothelium is activated, the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), are upregulated and they are recruited T lymphocytes and monocytes cause the progression of atherosclerotic disease (Libby P. 2002; Hansson G.K., 2005). Plasma cells and B lymphocytes are rare in the intimal plaque but they may be plentiful in the adventitia (Houtkamp M.A., *et al.*, 2001), while mast cells may be present both in plaque and in adventitia (Kaartinen M., *et al.*, 1998). The adhesion of these cells to the endothelium is not sufficient, while transendothelial migration is needed through sensing of chemokines, such as monocyte chemotactic protein-1 (MCP-1). MCP-1 is the most potent chemokine and its receptor CCR2 is upregulated during the development of atherosclerosis. The trafficking of monocytes and macrophages is also regulated by cytokines/chemokines (Glass C.K. and Witztum J.L., 2001).

Monocytes differentiate into macrophages within the intima and internalize atherogenic lipoproteins via scavenger receptors. These macrophages contain large amounts of cholesteryl esters. When they die by necrosis or apoptosis, macrophages contribute to the formation of a soft and unstable lipid-rich core within the plaque. Under conditions of low levels of LDL and high levels of HDL, macrophages are able to reduce the flow of cellular cholesterol by taking it out of the cell through membrane transporters and they participate in the reverse cholesterol transport (Glass C.K. and Witztum J.L., 2001; Lewis G.F. and Rader D.J., 2005; Nissen S.E., *et al.*, 2003).

Although lymphocytes are not required for the development of atherosclerosis, the immune system regulates the progression of the disease, indeed T lymphocytes recognize oxLDL that are abundant in atherosclerotic plaques (Hansson G.K., 2005; Nilsson J., *et al.*, 2005).

During the progression of atherosclerosis, the immunoinflammatory response is accompanied by fibroproliferative response mediated by smooth muscle cells, which

are responsible for a temptative repair and healing of damaged arteries. But when atherogenic stimuli continue over the years, the reparative response turns out to be detrimental and leads to narrowing of the lumen, reduced blood flow and finally ischemia. However, the collagen matrix produced by muscle cells within the fibrous cap maintains the plaque stable and protects it from breakage and thrombosis (Kragel A.H., *et al.*, 1989; Schwartz S.M., *et al.*, 2000; Geng Y.J. and Libby P., 2002; Kolodgie F.D., *et al.*, 2003).

The atheromatous core is rich in lipids, avascular, hypoxic, hypocellular, soft and totally devoid of collagen, its size is also a factor often critical for plaque stability (Guyton J.R., 2001). During the progression of atherosclerosis, endothelial cells, macrophages and smooth muscle cells undergo cell death by apoptosis or necrosis. The destruction of foam cells and the loss of smooth muscle cells can cause the destabilization of the atheroma core and the fibrous cap ruptures, owing to the release of metalloproteases (Geng Y.J. and Libby P., 2002).
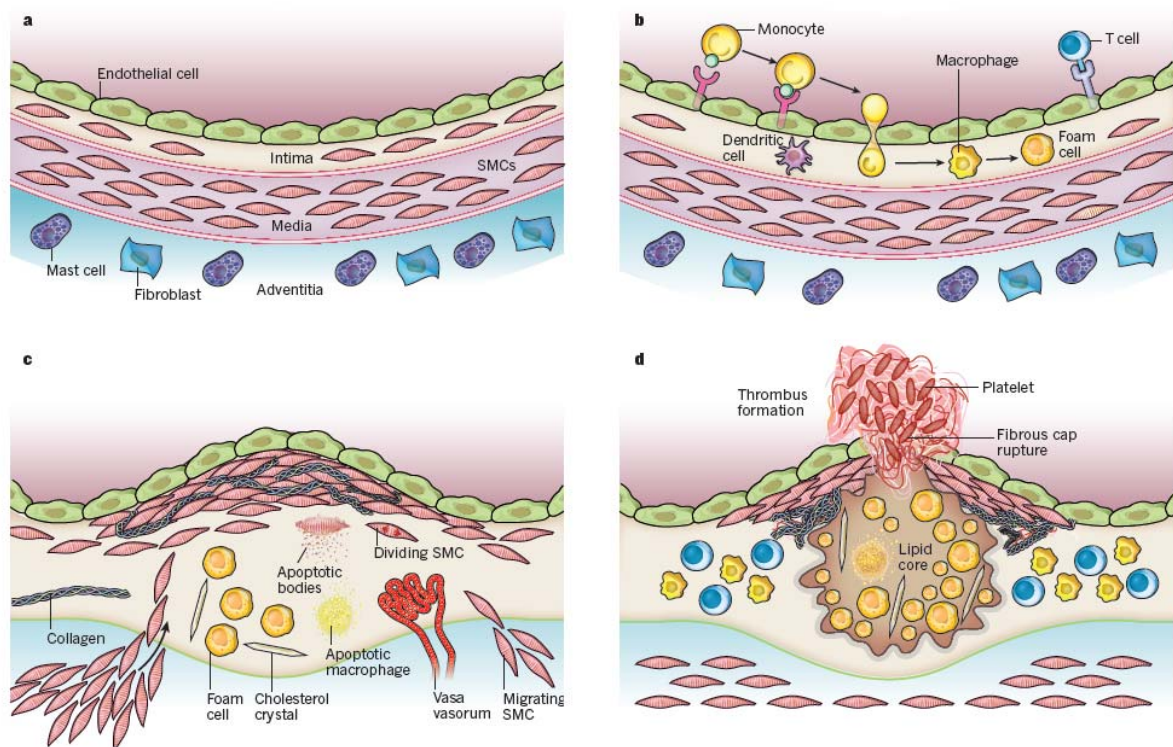
Fig. 1: Stage in the development of atherosclerotic lesions. From Libby P., Nature 2011.
A normal arterial wall and the cell changes that occur during disease progression to thrombosis are shown. **A)** The normal artery contains three layers. The inner layer, the tunica intima, is lined by a monolayer of endothelial cells that is in contact with blood, and overlie a basement membrane. In contrast to many animal species used for atherosclerosis experiments, the human intima contains resident smooth muscle cells (SMCs). The middle layer, or tunica media, contains SMCs embedded in a complex extracellular matrix. Arteries affected by obstructive atherosclerosis generally have the structure of muscular arteries. The arteries often studied in experimental atherosclerosis are elastic arteries, which have clearly demarcated laminae in the tunica media, where layers of elastin lie

between strata of SMCs. The adventitia, the outer layer of arteries, contains mast cells, nerve endings and microvessels. **B)** The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. **C)** Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **D)** Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow.

Plaque may be subject to calcification. Calcification can occur in connective tissue or at the level of lipids that compose it. Excess calcification may increase plaque size and lead to the obstruction of blood flow (Beckman J.A, *et al.*, 2001; Ehara S., *et al.*, 2004).

The process of angiogenesis is frequent in advanced atherosclerosis (Barger A.C., et al., 1984; Kolodgie F.D., et al., 2003; Virmani R., et al., 2005; Casscells W., et al., 2003). Endothelial proliferation and growth originating from adventitial vasa vasorum extend into the plaque, where neovascularization can occur. New microvessels are fragile, leaky, and express cell adhesion molecules (VCAM-1, intercellular adhesion molecule-1). This causes the leakage of plasma proteins, erythrocytes, and inflammatory cells. Angiogenesis and extravasation of plasma components may coexist and they mediate the rapid progression of plaque (Barger A.C., et al., 1984; Kolodgie F.D., et al., 2003; Virmani R., et al., 2005; Casscells W., et al., 2003). When the stability of plaque is compromised, plaques can break and cause thrombosis (Schaar J.A., et al., 2004). In the pathogenesis of arterial thrombosis, platelet aggregation is responsible for the obstruction of blood flow in the early stages, but the formation of fibrin is necessary for the subsequent stabilization of the plaque (Falk E., 2006).

1.4 VASCULAR CALCIFICATION: MECHANISMS

A common complication of several diseases, such as atherosclerosis and diabetes, is the calcification of blood vessels (Wilmer W.A. and Magro C.M., 2002; Chen N.X. and Moe S.M., 2003; Abedin M., *et al.*, 2004; Moe S.M. and Chen N.X., 2004; Speer M.Y. and Giachelli C.M., 2004; Vattikuti R. and Towler D.A., 2004). In several circumstances, vascular calcification is a process reminiscent of endochondral bone

formation and repair, and it involves several regulatory factors, among which the main ones are BMP-2, TGF-beta, TNF-alpha, matrix Gla protein (MGP), osteopontin (OPN), osteoprotegerin (OPG), oxidized lipids, and deregulated calcium and phosphate metabolism (Abedin M., *et al.*, 2004; Collin-Osdoby P., 2004; Speer M.Y. and Giachelli C.M., 2004; Vattikuti R. and Towler D.A., 2004).

The mineralization in vascular calcification may take place by endochondral ossification, which is a calcification mediated by osteoblast-like cells over a cartilaginous metaplasia, or it can be done by intramembranous ossification, which is a calcification of osteoblast-like cells by a collagen extracellular matrix in the absence of a cartilage (Karsenty G., 2003; Abedin M. *et al.*, 2004).

Johnson and colleagues, explaining the main mechanisms in vascular calcification, divided them into two distinct categories: induction of osteogenesis and loss of inhibitors of mineralization (Johnson R.C. *et al.*, 2006). In the opinion of Demer and colleagues (Demer L.L., 1995; Bostrom K, *et al.*, 1993), vascular calcification is due to the active bone formation in situ by osteoblast-like cells. There are many hypotheses on the origin of these cells. Some studies also have shown that vascular smooth muscle cells (VSMCs) are able to change their phenotype giving rise to osteoblastic cells. The reversible differentiation is in response to various stimuli, and allows these cells to enter in the proliferative phase and synthesize collagen extracellular matrix (ECM) (Hruska K.A., *et al.*, 2005).

Demer and other authors (Demer L.L., 1995; Bostrom K, *et al.*, 1993), have shown that the vascular wall contains a subpopulation of smooth muscle cells can form calcified nodules spontaneously and they calcify when cultured for a long time. The nodules of these cells called "calcifying vascular cells" (CVCs) have an increase in alkaline phosphatase (ALP) activity, and increased expression of osteocalcin (OC), OPN and osteonectin, similar to osteoblasts found in the bone (Watson K.E., *et al.*, 1994).

Another hypothesis is that these cells may originate from pericytes, that are a connective tissue cells that surround the endothelial cells of the capillaries and venules, or circulating stem cells.

The pericytes express α- and β-actin and 3G5 epitope of monoclonal antibody-defined ganglioside antigen (Bostrom K., *et al.*, 1993), which are typical markers of CVCs. Pericyte, just as CVCs, are also capable of producing nodules containing OC, MGP, OPN, and type I collagen, and produce ECM when cultured for a long time

(Abedin M. *et al.*, 2004, Bostrom K., *et al.*, 1993, Doherty M.J., *et al.*, 1998; Tintut Y., *et al.*, 1998) .

Some authors suggest that pericytes are mesenchymal progenitors (Tilton R.G., 1991) and they can differentiate into osteoblasts and chondrocytes, but also in adipocyte, fibroblast, and smooth muscle cells. Therefore they may have a role as a source of adult progenitor cells in case of inflammation, repair and disease (Davidoff M.N., *et al.*, 2004; Doherty M.J. and Canfield A.E., 1999; Farrington-Rock C., *et al.*, 2004; Sato K. and Urist M.R., 1985).

1.4.1 INDUCTION OF OSTEOGENESIS

In recent years, it has been clarified that many factors regulate the induction of a osteogenic phenotype. The major are listed here.

BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) are growth factors that belong to the superfamily of TGF-β, and are involved in the formation of ectopic bone (Wang E.A., *et al.*, 1990) and vascular calcification. BMP-2 and BMP-4 are involved in mineralization and in the induction of local inflammation and lead the osteogenic and chondrogenic differentiation of mesenchymal progenitor cells (Shea C.M., *et al.*, 2003), while BMP-7 slows vascular calcification and promotes the phenotype of VSMCs as a result induction of p21 and upregulation of Smad 6 and 7. BMP-7 also upregulates the expression of α-SMA and prevents and reverses the transition to the osteoblastic phenotype (Dorai H., *et al.*, 2000).

BMP-2 is synthesized as a precursor of 60 kDa that becomes a small monomer of 18 kDa through the secretory pathway. The active form is formed by two associated monomers (Israel D.I., et al., 1992). Thus, the active heterodimer binds its receptor. The receptor of BMP is a heterodimer consisting in 2 serine/threonine kinase, type 1 and type 2. When the receptors and BMPR2 and BMPR1 interact, BMPR2 is phosphorylated and activates receptor type 1, which effectively in turn phosphorylates the regulatory Smads (Massague J. and Wotton D., 2000; Chen A.L., et al., 2004; Chen D., et al., 2004) that modulate target gene expression (ten Dijke P., et al., 2003).

BMP-2 is responsible for osteogenic and chondrogenic differentiation in mesenchymal multipotent progenitors (Shea C.M., et al., 2003). When the

atherosclerotic lesions evolve there is a high expression of bone matrix proteins, togther with expression of BMP expressed by endothelial cells, foam cells and SMCs (Bostrom K., et al., 1993; Shin V., et al., 2004; Dhore C.R., et al., 2001; Csiszar A., et al., 2005). Mediators of endothelial dysfunction, such as oxidative stress (Mody N., et al., 2001), turbulent blood flow (Sorescu G.P., et al., 2003; Sorescu G.P., et al., 2004), and hypoxia (Bouletreau P.J., et al., 2002) were associated with increased expression of BMP and increased calcification.

Cbfa1/Runx2

Also known as Cbfa1 or as polyoma enhancer-binding protein 2αA (Pebp2αA), Runx2 (Runt related trascription factor 2) is the most important of all transcription factors involved in bone formation and it is considered the master-gene regulator of osteoblast differentiation (Ducy P., et al., 1997; Komori T., *et al.*, 1997). Runx2 controls the expression of many proteins that allow the osteoblast differentiation such as OC, OPN and collagen type I (Xiao G., *et al.*, 2005). But despite this, there is a weak correlation between Runx2 protein levels and mRNA expression of osteoblast-related genes. Runx2-dependent trascription is regulated by ECM/cell interactions mediated by integrins and by Runx2 protein level. ECM/cell interactions are mediated by the MEK/ERK branch of the mitogen-activated protein kinase (MAPK) pathway. Blocking this pathway inhibits the phosphorylation of ERK and induction of osteocalcin (Franceschi R.T. and Xiao G., 2003). ECM interactions are also required for BMP signal transduction in osteoblasts (Xiao G., et al., 2002). To work properly Runx2 requires activation of osterix, to induce the calcifying phenotype (Suske G., 1999). Osterix is a downstream transcription factor with a C-terminal C2H2 zink-finger, that is a domain containing repeats of cysteine and histidine residues that bind zinc ions and folds in loop structures that bind DNA, characteristic of the family of factors SP1 transcription that recognize the GC-box (Milona M.A., *et al.*, 2003).

Runx2-null mice lack functional osteoblast and are not capable of producing mineralized hypertrophic cartilage or bone (Otto F., et al., 1997). Mutations of Runx2 causes cleidocranial dysplasia in human (Mundlos S., et al., 1997). Runx2 expression in VSMCs is a specific marker of osteoblast differentiation and determines the onset of vascular calcification.

PHOSPHATE METABOLISM

New discoveries have shown that phosphate regulates and coordinates cell signaling and gene expression through a dynamic transport mechanism. For example, in a study conducted by Steitz on VSMCs (Vascular Smooth Muscle Cells) treated with supplemental inorganic phosphate (Pi), the expression of bone regulatory proteins such as Cbfa-1 (Runx2) and OC increases, while markers of muscle line are downregulated (Steitz S.A. et al., 2001). There are two hypotheses for VSMCs in phosphate-induced vascular calcification. The first involves a transition to a bone-forming phenotype. In vitro studies have demonstrate that high levels of phosphate causes loss of smooth muscle markers such as α-SM actin and SM22alpha, and expression of osteochondrogenic markers such as Runx2/Cbfa1, osterix, ALP and OPN (Steitz S., et al., 2001; Jono S., et al., 2000). On the surface of VSMCs was found the presence of matrix vesicles incorporating apatite which act as nucleation sites for calcification, like calcifying collagen fibrils (Wada T., et al., 1999). The second hypothesis involves apoptosis-dependent matrix mineralization. Raynolds and collegues coltured VSMCs in elevated phosphate and calcium conditions, and led to calcification of matrix vesicles and apoptotic bodies (Reynolds J., et al., 2004). Phosphate transport into cells is mediated by 3 types Na/Pi cotransporters. Type 1 and type 2 Na/Pi cotransporters in intestinal epithelium and kidney have a role in whole body phosphate homeostasis (Takeda E., et al., 1999; Werner A., et al., 1998). Type 3 Na/Pi cotransporters, PiT-1 and PiT-2, are ubiquitously expressed (Villa-Bellosta R., et al., 2007). Extracellular phosphate is taken up by VSMC via Pit-1, which is a sodium-dependent phosphate transporter and enhances intracellular phosphate in VSMC, inducing mineralization-related genes (Jono S. et al., 2000). Phosphate uptake in human VSMCs is dependent on a sodium gradient. The treatment with a competitive inhibitor of Na/Pi transport, phosphonoformic acid (PFA), inhibited phosphate uptake and VSMCs osteochondrogenic differentiation (Jono S., et al., 2000). Of particular interest is that PiT-1 is upregulated by inducers of calcification including BMP-2 (Li X., et al., 2008; Suzuki A., et al., 2006), calcium (Yang H., et al., 2004), and platelet-derived growth factor PDGF (Villa-Bellosta R., et al., 2009; Kakita A., et al., 2004).

ALKALINE PHOSPHATASE

Alkaline phosphatase (ALP) is a peculiar marker of osteoblasts and it is widely used as a molecular marker of vascular calcification, it is an early marker of ECM deposition and its activity is essential to bone mineralization. Its activity is pivotal in the formation of hydroxyapatite crystals during endochondral ossification. ALP modulates vascular calcification by reducing the levels of inorganic pyrophosphate. Inorganic pyrophosphate is a ALP substrate and its also a powerful inhibitor of vascular calcification (Johnson R.C., *et al.*, 2006).

1.4.2 LOST OF INHIBITORS OF MINERALIZATION

The other main mechanism that allows vascular calcification is the loss or failure of physiological inhibitors of vascular calcification. The principal mechanisms are described.

MATRIX Gla PROTEIN

Matrix Gla protein (MGP) has been identified as a bone matrix protein (Price P.A., *et al.*, 1983). MGP is a protein expressed in the bone at levels fairly constant in the early stages of development and in adulthood (Otawara Y. and Price P.A., 1986). Its role in calcification is complex and has been shown that it is capable of modulating cell differentiation and calcification (Bostrom K., *et al.*, 2001; Newman B., *et al.*, 2001; Zebboudj A.F., *et al.*, 2002). MGP functions as a noggin-like protein that inhibits the interaction between BMP and its receptor BMPR2 in addition to linking directly to BMP2 (Bostrom K., *et al.*, 2001; Zebboudj A.F., *et al.*, 2002). In this way, the relationship between MGP and BMP2 regulates the mineral deposit and osteogenic differentiation. Low and high levels promote calcification, intermediate levels of MGP inhibit calcification (Zebboudj A.F., *et al.*, 2002).

Recent studies have shown that MPG modulates the mineralization indirectly through effects on cell differentiation. In fact, MGP inhibits the differentiation of mesenchymal cells into an osteogenic phenotype, by sequestering BMP-2 and preventing interaction with its receptor (Bostrom K., et al., 2001; Wallin R., et al., 2000; Zebboudj A.F., et al., 2002). Furthermore, the effects of MGP on BMP-2 are due to the degree of γ-carboxylation (Wallin R., et al., 2000). Insufficient carboxylation could be the key factor that leads to increased calcification (Sweatt A., et al., 2003).

MGP γ-carboxylation is performed by the vitamin K-dependent enzyme, γ-carboxylase, therefore a deficiency of vitamin K may increase vascular calcification because of incomplete γ-carboxylation and reduced function of MGP (Jie K.S., et al., 1995).

OSTEOPONTIN

Osteopontin (OPN) is a matrix protein that contains an RDG motif, that is a tripeptide motif arginine-glycine-aspartate; it is found in the cell matrix protein that binds cells through the integrin α-v-β-3. Its function is to inhibit calcification by blocking the growth of apatite crystals. It has been shown that OPN expression is associated with atherosclerosis (Giachelli C.M., et al., 1993) and it colocalizes with calcified atherosclerotic plaque areas. OPN also appears to be expressed in macrophages, smooth muscle cells and endothelial cells present within the plaque (O'Brien E.R., et al., 1994). In the presence of CAD, plasma levels of OPN are very high (Ohmori R., et al., 2003). The binding of OPN with the integrin α-v-β-3 on osteoblasts results in decreased calcium in the cytosol allowing activation to an osteoclast resorptive phenotype (Miyauchi A., *et al.*, 1991). Other studies show that OPN binding also promotes resorption of ectopic calcification by inducing expression of carbonic anhydrase II, which creates an acidic environment required for resorption (Steitz S.A., et al., 2002). Mice deficient in OPN and MGP develop aortic calcification faster than mice deficient in MGP only, in agreement with the fact that OPN inhibits mineralization (Speer M.Y., et al., 2002).

OSTEOPROTEGERIN

Osteoprotegerin (OPG) is a member of the TNF (Tumor Necrosis Factor) receptor superfamily (Simonet W.S. *et al.*, 1997) that indirectly inhibits osteogenesis. OPG acts as a soluble receptor "decoy" that binds and inhibits RANKL, a TNF superfamily member. Activation of the RANKL receptor, RANK, is required for the maturation of osteoclast progenitors (Boyle W.J., *et al.*, 2003).

OPG is expressed in coltured coronary artery smooth muscle cells (Hofbauer L.C. et al., 2001), and OPG and RANKL are discovered in normal arteries (Dhore C.R. et al., 2001). OPG transgenic mice lack functional osteoclasts, leading to a wrong trabecularized bone (Simonet W.S. et al., 1997). On the contrary OPG-/- mice have serious osteoporosis with multiple fractures, but these mice have vascular

calcification in some arteries (Bucay N., et al., 1998). In the rat model, OPG treatment prevents warfarin-induced vascular calcification (Price P.A., et al., 2001). The physiological role of OPG may be dependent on its levels relative to RANKL. RANKL is responsible for the calcification of cardiac valve myofibroblasts (Kaden J.J., et al., 2004).

INORGANIC PYROPHOSPHATE

The positive effects of inorganic pyrophosphate (PPi) on calcification are highly inhibited by inorganic pyrophosphate. Extracellular inorganic pyrophosphate inhibits the formation of hydroxyapatite crystals and it is generated by nucleotide pyrophosphate/phosphodiesterase (NPP). In fact, mice knock out for NPP1 develop calcification (Johnson K. et al., 1999) and patients with familial idiopathic infantile arterial calcification, a syndrome associated with the recessive mutation of the gene NPP1, show fibrointimal development, premature hyperplasia and calcification of the aortas, and calcified valve disease. Furthermore, there is periarticular calcification similar to that observed in NPP1-deficient mice (Rutsch F. et al., 2003).

Inorganic pyrophosphate is a highly potent inhibitor of vascular calcification, and it is considered increasingly as a vascular paracrine factor that mediates this process. Pi works by inhibiting the formation of hydroxyapatite and prevents the propagation of calcium deposits in tissues (Fleisch H., et al., 1966).

1.5 THE ROLE OF EXTRAPARIETAL CELLS IN VASCULAR

Vascular calcification has been traditionally considered a passive, unavoidable phenomenon, intrinsically connected with aging and atherosclerotic vascular degeneration. However, in the last two decades, this view has been challenged by a series of clinical and basic science findings, which underscore the biological complexity of the processes driving ectopic mineralization (Sage A.P. et al., 2010). Resident vascular cells produce local mediators (such as pyrophosphate and matrix-gla protein, MGP) that in cooperation with circulating factors (i.e. fetuin-A) protect the arteries from deposition and growth of minerals (Luo G. et al., 1997; Harmey D. et al., 2004; Jahnen-Dechent W. et al., 2011). On the other side, cell-death related processes, together with the bone-like activity of vascular cells, stimulate ectopic calcification (Bostrom K.I. et al., 2011; Shroff R.C. et al., 2008). The demonstration that bone markers/transcription factors are expressed within calcified atherosclerotic

plaques suggests that osteogenic programs are activated during vascular mineralization. In addition, cell types showing morphological and biological features close to chondrocyte/osteoblast-like cells have been identified both in mouse models of atherosclerosis and human atherosclerotic samples (Rattazzi M. et al., 2005; Bobryshev Y.V., 2005). These cells might originate from mesenchymal progenitors resident in the vascular wall, trans-differentiation of mature vascular smooth muscle cells (SMC) or arise from circulating cells harbouring a calcifying potential. Several studies have shown that under appropriate stimuli (such as hyperphosphatemia, inflammation, oxidative stress), SMC differentiate into osteoblast/chondrocyte-like cells (Sage A.P. et al., 2010; Steiz S.A. et al., 2001). Other studies have demonstrated the presence of mesenchymal-derived progenitor cells in the vessel wall, including microvascular pericytes, which retain an osteogenic/chondrogenic commitment (Tintut Y. et al., 2003; Farrington-Rock C. et al., 2004). The recent identification of circulating calcifying cells raises the interesting question whether or not these cell types should be added to the list of vascular calcification promoters.

Atherosclerotic lesions in mice are usually characterized by the presence of cartilaginous metaplasia, with chondrocyte-like cells actively depositing calcium within the advanced plaque (Rattazzi M. et al., 2005). Speer et al. investigated the origin of these cells by using a lineage-tracing strategy in the MGP null mouse, a model of medial calcification. They elegantly demonstrated that chondrocyte-like cells in calcified vessels originated from trans-differentiation of resident SMC and not from GFP+ cells used to reconstitute the bone marrow (BM) of MGP deficient mice (Speer M.Y. et al., 2009). More recently, however, Doehring et al. showed a significant contribution of BM-derived myeloid CD34+/CD13+ cells to the population of chondrocyte-like cells observed in the intimal lesions of LDLr null mice (Doehring L.C. et al., 2010). In particular, a significant number of BM-derived cells present in the plaques expressed the chondrogenic marker type II collagen. In addition, the majority of these cells were CD34+ and more than 50% expressed the myeloid marker CD13 (Doehring L.C. et al., 2010). Although the calcifying potential of these BM-derived cells was not clearly established, these two studies suggest a differential origin of chondrogenic cells involved in intimal versus medial calcification, with most of the medial cells originating from trans-differentiation of SMC and BM-derived cells contributing to chondrocyte-like cells recruited into the intimal atherosclerotic plaques.

Similar to arterial cells, aortic interstitial valve cells (VIC) also display phenotypical heterogeneity in term of calcifying potential (Rattazzi M. et al., 2008; Chen J.H. et al., 2009). Of interest, BM transplantation (BMT) studies demonstrated a significant participation of BM-derived cells in normal valve homeostasis. In particular, Visconti et al. demonstrated that BM-derived cells can enter the native valve and differentiate into cells similar to resident VIC (Visconti R.P. et al., 2006). Subsequently, the same group showed that BM-derived cells found in the normal post-natal valve exhibited a myeloid (CD45+/CD11c+/MHCII+/F4/80-) or progenitor-like profile (CD45+/CD133+/Hsp47+), with the first cell type mainly located in the distal portion of the leaflet, and the second type found in proximal areas (Hajdu Z. et al., 2011).

However, the actual contribution of these cells to pathological valve remodelling is currently unknown. Of note, a BMT study in senile ApoE null mice with calcific aortic stenosis, showed that BM-derived cells expressing osteoblast-related proteins (osteopontin and osteocalcin[OCN]) could be identified near site of ectopic calcification (Tanaka K. et al., 2005). This finding is in agreement with the recent observation of CD45+OC+Col1+ cells within calcified human leaflets (Egan K.P. et al., 2011). Although BM-derived CD31+ cells have not been observed in the valvular endocardium of normal leaflets (Hajdu Z. et al., 2011), a significant number of GFP+CD31+ cells were described in the stenotic valve of transplanted ApoE deficient mice (Tanaka K. et al., 2005). Therefore, even if ECs of BM-origin seem not to participate in normal valve homestasis, it cannot be excluded that circulating endothelial progenitors might instead be recruited to the diseased valve and undergo differentiation into chondro/osteoblast-like cells. A recent study conducted in mitral valves showed that a specific subpopulation of endothelial valve cells (VEC) retain a chondrogenic and osteogenic differentiation potential, and might represent a local reservoir of VEC progenitors (Wylie-Sears J. et al., 2011). The relative contribution of local VEC progenitor cells versus circulating EPCs to valve calcification represents an interesting field of research.

Taken together, an increasing amount of data in animal models of vascular/valve disease, confirmed by histopathological studies on human samples, highlights the potentiality of BM-derived cells to engraft into the pathological tissue and participate in the calcification processes. Below we provide a detailed review of circulating cell phenotypes that may contribute to vascular calcification and/or regulation of the bone/vascular axis.

1.6 CIRCULATING MESENCHYMAL OSTEOPROGENITOR CELL

Osteoblast precursors are traditionally considered to reside in the bone marrow mesenchymal stem cell compartment. Increasing evidence indicate the existence of circulating osteoblastic cells, termed circulating osteo-progenitors (COP) (Pignolo R.J. et al., 2011; Eghbali-Fatourechi G.Z. et al., 2005; Eghbali-Fatourechi G.Z. et al., 2007), which appear to be comprised of two populations: one related to hematopoietic stem cells/endothelial progenitor cells and the other to plastic-adherent marrow stromal cells/mesenchymal stem cells (MSCs). MSCs are traditionally isolated in *ex vivo* cultures from the low-density mononuclear fraction of bone marrow by adherence to plastic surfaces (Friedestein A.J. et al., 1968). Using this approach, a number of groups have identified MSC-like cells with osteogenic potential in peripheral blood (Fernandez M. et al., 1997; Zvaifler N.J. et al., 2000; Kuznetsov S.A., et al., 2001; Huss R. et al., 2000). For example, Kuznetsov et al. (Kuznetsov S.A., et al., 2001) identified circulating adherent, clonogenic, fibroblast-like cells with osteogenic and adipogenic potential from the blood of four mammalian species (mouse, rabbit, guinea pig, and human). These cells were osteonectin+, osteopontin+, collagen 1+, α-smooth muscle actin+, and negative for the expression of hematopoietic (CD45) and endothelial markers. Interestingly, however, the human blood-derived adherent cells were negative for Stro-1, as well as for endoglin and Muc-18, all of which are expressed by human bone marrow MSCs, suggesting important differences between bone marrow and peripheral blood MSC-like populations. In addition, the frequency of these cells, especially in humans, was extremely low (<1/10^6 cells). Nonetheless, the MSCs derived from peripheral blood were able to form mineralized nodules *in vitro* as well as bone in an *in vivo* transplantation assay. Consistent with the findings of Kuznetsov, (Kuznetsov S.A., et al., 2001), Zvaifler et al. (Zvaifler N.J. et al., 2000) isolated MSC-like cells from peripheral blood of humans using an elutriation procedure. After 7-14 days, cultures of these cells contained both fibroblast-like cells as well as a small population of large round cells. The cultured cells were negative for the hematopoietic/endothelial markers, CD45, CD14, and CD34, and positive for SH2 (CD105, endoglin), vimentin, collagen 1, BMP-R 1A and BMP-RII. Interestingly, Stro-1 was expressed in the large cells.

Several investigators have examined conditions under which MSCs could be mobilized into peripheral blood, presumably from the bone marrow. Thus, Fernandez

et al. (Fernandez M. et al., 1997) found that administration of G-CSF or GM-CSF to human subjects (traditionally used to mobilize hematopoietic stem cells) also resulted in the mobilization of MSCs. Using a rat model, Rochefort et al. (Rochefort G.Y. et al., 2006) found that the circulating MSC pool increased dramatically (by almost 15-fold) when the animals were exposed to chronic hypoxia. In contrast to G-CSF or GM-CSF administration to humans, however, hypoxia-induced mobilization appeared to be specific for MSCs, since total circulating hematopoietic progenitor cells were not significantly increased.

In subsequent studies, Otsuru and colleagues (Otsuru S. et al., 2007) examined how circulating MSC-like cells contributed to osteogenesis in a BMP-induced model of ectopic bone formation. Following lethal dose-irradiation and subsequent GFP-bone marrow cell-transplantation in mice, a BMP-2-containing collagen pellet was implanted into muscle. Three weeks later, these investigators found a significant number of GFP-positive osteoblastic cells in the newly generated ectopic bone, suggesting that BMP-2 may be another factor leading to the mobilization of MSCs from bone marrow. In a subsequent study, the same group characterized the BMP-2-mobilized circulating MSCs as negative for expression of hematopoietic (CD45, CD11b, Gr-1) and endothelial (CD34, Flk-1, CD31) markers, but positive for expression of the mesenchymal marker, CD44, as well as for CXCR4, a receptor for the chemokine SDF-1, which regulates stem cell trafficking (Otsuru S. et al., 2008). Interestingly, expression of SDF-1 was markedly increased in vascular and osteoblastic cells in and around the BMP-2 implant, suggesting that BMP-2-induced SDF-1 expression was responsible, at least in part, for the mobilization of MSCs into peripheral blood.

In a more recent study, Alm and colleagues (Alm J.J. et al., Res in press) examined the presence of circulating MSCs in fracture patients. They studied three patient groups: elderly females with a femoral neck fracture treated with cemented hemiarthroplasty, an age- and sex-matched group with hip osteoarthritis (OA) treated with cemented total hip arthroplasty (THA), and young adults with surgically-treated lower extremity fractures. Criteria for identifying circulating MSCs included cell surface markers (CD105+, CD73+, CD90+, CD45-, CD14-), proliferation through several passages, and osteogenic, chondrogenic, and adipogenic differentiation. They reported that plastic-adherent MSCs were found in the peripheral blood from 22% of hip fracture patients, 46% of younger fracture patients, and in none of the hip

OA patients, suggesting that mobilization of MSCs into the circulation occurs in response to fracture, even in elderly patients. These findings were also consistent with previous work by Eghbali-Fatourechi et al. (Eghbali-Fatourechi G.Z. et al., 2005) showing that circulating cells expressing osteocalcin increased markedly following fracture, although the COP cells identified in this study likely included cells of both hematopoietic and mesenchymal origin.

1.7 CIRCULATING CALCIFYING CD34+ PROGENITOR CELLS AND EPCs

The hematopoietic system is considered as an organized, hierarchical system spearheaded by multipotent, self-renewing stem cells at the top, followed by lineage-committed progenitor cells in the middle, and lineage-restricted precursor cells, which give rise to terminally differentiated cells, at the bottom (Weissman I.L., 2000). EPCs are bone marrow-derived progenitor cells committed to the endothelial lineage which, once in the bloodstream, participate in endothelial repair and neoangiogenesis (Asahara T. et al., 1999). A huge amount of data has accumulated regarding EPC identity and function, in relation to diagnostic, prognostic and therapeutic uses. The research has been dampened, however, by the elusive phenotype and ambiguous definition of EPCs (Fadini G.P. et al., 2008), while some authors have questioned the actual role played by endogenous EPCs in vascular repair and angiogenesis (Hagensen M.K. et al., 2010; Hagensen M.K. et al., 2011). Moreover, it has been recognized that several putative EPC phenotypes differ in lineage origin and function. True EPCs are supposed to derive from a the hemangioblast recapitulating developmental differentiation steps. Provided that an adult counterpart of the embryonic hemangioblast exists, EPCs descending from this lineage should express hematopoietic stem cell (such as CD34 and CD133) and endothelial (such as VEGFR-2, KDR) markers. When EPCs are isolated from circulating mononuclear cells, two main types of cells can be isolated: so-called "early EPCs" are obtained after a short-term culture and have a monocytic origin, while "late EPCs" or endothelial colony forming cells (ECFC) arise after long-term culture and are almost indistinguishable from proliferating mature endothelial cells (Fadini G.P. et al., 2008). The population of c-Kit-positive, Sca-1-positive, lineage marker-negative (KSL) cells in mouse bone marrow (BM) represents a fraction of hematopoietic stem/progenitor cells (HSCs) (Okada S. et al., 1992). The KSL cell population is believed to be a source of EPCs, which differentiate into endothelial cells and contribute to

vasculogenesis (Sata M. et al., 2002; Bailey A.S. et al., 2004; Sahara M. et al., 2005). An equivalent population of stem cells is composed by adult human peripheral blood (PB) CD34+ cells. These cells reportedly contain EPCs and hematopoietic stem cells HSCs (Asahara T. et al., 1997), and promote vasculogenesis (Asahara T. et al., 1997; Asahara T. et al., 1999). Several reports have shown that CD34+ cells are committed not only to endothelial cells but also to mural perivascular cells (i.e., pericytes and smooth muscle cells) (Yeh E.T. et al., 2003; Iwasaki H. et al., 2006; Howson K.M. et al., 2005). For instance, Zengin et al. reported the existence of EPCs and stem cells in a distinct zone between the smooth muscle and the adventitial layer of human adult vascular wall that are capable of differentiating into mature endothelial cells, as well as hematopoietic and local immune cells (Zengin E. et al., 2006). These findings indicate CD34+ cells and EPCs are heterogeneous populations and potentially committed to multi-lineage cell types. Several lines of evidence indicate that EPCs, either in the bloodstream or in culture, are reduced in patients at risk for or with established CVD (Fadini G.P. et al., 2007), and this is considered to promote vascular disease initiation or progression. Not only are EPCs reduced in CVD patients, but they also display an altered differentiation potential, with impaired generation of endothelial cells and higher yield of inflammatory and smooth muscle-like cells (Loomans C.J. et al., 2009; Fadini G.P. et al., 2010). Along this line of investigation, recent data have demonstrated that circulating CD34+ progenitor cells and CD34+KDR+ EPCs can also express bone-related proteins, especially OC and/or BAP (Gossl M. et al., 2008; Gossl M. et al., 2010). OC is a non-collagenous bone protein implicated in bone mineralization and calcium homeostasis, while BAP is a tetrameric glycoprotein found on the surface of osteoblasts and its function is essential to the mineralization process. Similarly, previous reports showed that CD34+ and CD133+ cells were capable of differentiating into osteoblasts as well as hematopoietic and endothelial cells *in vitro* (Tondreau T. et al., 2005; Chen J.L. et al., 1997). Securing an adequate blood supply to bone fracture site is crucial for bone healing to occur, as evidenced radiographically by the formation of a bridging callus along a former fracture gap. Thus, an emerging focus in regenerative medicine is to foster progenitor cells for simultaneous neoangiogenesis and bone healing. EPCs are appealing for this task because studies on the link between angiogenesis and the bone growth have led to the discovery of a developmental reciprocity between endothelial cells and osteoblasts in vitro. Therefore, a series of studies have explored

the use of PB CD34+ cells or EPCs for bone fracture healing (Matsumoto T. et al., 2006; Matsumoto T. et al., 2008; Mifune Y. et al., 2008; Fukui T. et al., 2011). Using a mouse bone fracture model, these studies first confirmed that fractures trigger EPC mobilization from BM to PB and incorporation into fracture sites (Matsumoto T. et al., 2008). The overlapping origin of endothelial and osteogenic markers was confirmed by Matsumoto et al. (Matsumoto T. et al., 2006) using single cell RT-PCR: specifically, ~20% of human peripheral blood CD34+ cells expressed the mRNA for OC. To investigate clinical feasibility of PB CD34+ cells for bone healing, these investigastors also demonstrated that human PB CD34+ cells recruited to the fracture site following systemic delivery developed a favorable environment for fracture healing by enhancing vasculogenesis/angiogenesis and osteogenesis, and finally led to functional recovery from fractures (Matsumoto T. et al., 2006). In a second series of pre-clinical studies, they seeded the femoral non-union site of immunodeficient rats with human local CD34+ cells that were mobilized with granulocyte colony stimulating factor (G-CSF): this was able to promote successful fracture union per radiographic and histologic assessment. Effective fracture healing occurred as long as there were more than 1×10^4 CD34+ cells per rat implanted at the fracture site (Mifune Y. et al., 2008). To confirm superiority of CD34+ cells over mononuclear cells (MNCs), $1x10^7$ MNCs or $1x10^5$ CD34+ cells were locally transplanted into the fracture site (the ratio of MNCs over CD34+ cells was based on the FACS analysis showing about 1% of CD34+ cells in PB MNCs). In animals transplanted with MNCs fracture healing was achieved less efficiently than when purified CD34+ cells were used, even if PB MNCs contained the same number of CD34+ cells (Fukui T. et al., 2011). Following these series of studies, some researchers have recently focused on the relationship between EPCs and bone fracture healing. In mouse, rat, and human fracture, other groups have reported the mobilization of EPCs for bone healing (Lee D.Y. et al., 2008; Laing A.J. et al., 2007; Laing A.J. et al., 2007). PB EPCs were also reported to contribute to bone healing in rat segmental bone defect model or critical-sized bone defects in sheep (Li G. et al., 2005; Atesok K. et al., 2010; Rozen N. et al., 2009).

Taken together, these findings indicate that EPCs promote healing of bone defects, and can potentially heal nonunions and delayed unions in animals. From a therapeutic perspective, these data pave the way for clinical use of these cells to enhance fracture repairs. From a pathophysiological point of view, the combined

vascular and bone tropism of EPCs highlights the role of circulating cells in the bone/vascular axis. Indeed, several pre-clinical studies and preliminary clinical evidence indicate that EPCs home to sites of vascular damage (Chavakis E. et al., 2005; Aicher A. et al., 2003). Therefore, an osteogenic differentiation of these cells may be involved in the process of vascular calcification. Gossl et al. have found that OC expression on circulating EPCs is significantly associated with coronary artery disease (CAD) in a cohort of 72 patients undergoing invasive coronary assessment. Percentage OC expression on CD34+KDR+ EPCs was inversely correlated with serum MGP, an inhibitor of calcification (Gossl M. et al., 2010). They also showed that culture of CD34+ cells in osteogenic conditions led to formation of mineralized structures and upregulation of bone-related genes, such as osteopontin and Col1a1, especially when CD34+ cells had a high expression of OC prior to culture. Subsequently, by performing blood sampling from the proximal aorta and the coronary sinus in patients undergoing catheterization, the same group demonstrated that OC-expressing EPCs are retained in the coronary circulation of patients with coronary endothelial dysfunction, providing indirect evidence in support of homing of these pro-calcific cells at sites of vascular damage (Gossl M. et al., 2008). The relationship between the osteogenic and the endothelial differentiation programs in EPCs are unknown. Recently, it has been reported that, in type 2 diabetic patients with CAD, circulating CD34+ cells show a drift towards the pro-calcific versus the endothelial commitment, as evidenced by increased OC/KDR expression ratio (G. P. Fadini, manuscript submitted). Increased expression of bone-related antigens, such as OC and BAP, on CD34+ cells, has been associated with both osteoporosis (Pirro M. et al., 2010) and vascular disease (Pirro M. et al., 2011). Indeed, CD34+OC+ cell level correlated with bone mineral density and aortic pulse wave velocity, a measure of vascular stiffness (Pirro M. et al., 2010; Pirro M. et al., 2011). The presence of erectile dysfunction, another marker of vascular disease, is also associated with an higher expression of OC on circulating EPCs (Foresta C. et al., 2010). Moreover, OC-expressing EPCs seem to be regulated by the hormonal status (Pirro M. et al., 2011; Foresta C. et al., 2010), a finding that supports their involvement in the regulation of the bone-vascular axis. The reasons for the phenotypic derangement of EPCs in patients at risk for or with established CVD are unclear, but may be driven by inflammation, as previously suggested in the setting of diabetes (Loomans C.J. et al., 2009). In vitro, calcification of EPCs can be induced by stimulation of the innate

immunity receptors (e.g. CD14 and TRLs) by LPS (Fadini G.P., manuscript submitted), or by treatment with oxidized low-density lipoprotein (Liu L. et al., 2011). However, it should be noted that most cultured EPCs used in such studies retain monocytic features. Therefore, acquisition of an osteogenic phenotype of these culture cells should be viewed in the light of monocyte plasticity, and thus indicates the existence of myeloid calcifying cells.

2. AIM OF THE PROJECT

Diabetic vasculopathy is a common complication of diabetes mellitus and is characterized by a general vascular endothelial damage and an arrest of angiogenesis in tissues subjected to ischemia. Another typical feature of diabetic vasculopathy is the development of vascular calcification, resulting in an extensive vascular remodeling process. Since diabetes appears to induce an imbalance in the biology of progenitor cells, reducing the number of endothelial cells and stimulating the development of osteogenic cells and smooth muscle cells, the main purpose is to outline the role of circulating progenitor cells in the development and progression of vascular complications of diabetes.

Here, we investigated the nature, origin, and activity of human circulating procalcific cells. We show that OC+BAP+ cells originate from the myeloid lineage and retain monocyte/macrophage markers, and a subpopulation of them is longlived. These "myeloid calcifying cells" (MCCs) can be differentiated from peripheral blood mononuclear cells and form ectopic calcifications in vivo. From a clinical point of view, we provide evidence that MCCs are overrepresented in type 2 diabetes blood and atherosclerotic lesions, whereas glycemic control was able to reduce MCCs toward normal levels. Taken together, our data identify a novel source of procalcific cells that may contribute to vascular calcification, which is involved in the high cardiovascular risk associated with diabetes.

In particular, we focused on the importance of osteogenic progenitor cells of myeloid origin (MCCs) in reference to their role in vascular calcification.

3. MATERIALS AND METODS

3.1 CELLS ISOLATION FOR OSTEOGENIC DIFFERENTIATION

Peripheral blood derived calcifying cells were isolated from the total peripheral blood mononuclear cells (PBMCs) population using selective culture conditions. PBMCs were separated with Histopaque®1077 (Sigma-Aldrich) density gradient centrifugation. PBMC were plated on 12-well plates at a density of 3 x 10^6/cm^2 in a mesenchymal stem cell medium (MesenCult™ Medium, Stem Cells Technologies Inc., Vancouver, Canada) with Osteogenic Stimulatory Supplements Human (used at a final 15% volume), dexamethasone (used at a final concentration of 10^{-8}M) and ascorbic acid (used at a final concentration of 50μg/mL); only the third week is added the β-glycerophosphate (used at a final concentration of 3.5mM) for a week.

We tried different plate coating strategies: phenol red free Matrigel™ (BD Biosciences), collagen (Sigma-Aldrich), fibronectin (Roche Diagnostics Germany), and plastic. Matrigel enabled the best results as compared to collagen and fibronectin. Matrigel is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparin sulphate proteoglycans, entactin and nidogen. It also contains TGF-beta, fibroblast growth factor, tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumor.

The medium was changed first after 1 week and then every 3 days.

Three weeks after plating, cells coltured in collagen and fibronectin were detached by trypsin/EDTA, and with dispase (BD Bioscience) for cells coltured in matrigel and collected for antigenic characterization by FACS and gene expression analysis.

At the end of the 3-week culture period, von Kossa's and Alizarin red staining were also carried out. Calcification in the culture were quantified after von Kossa's staining using the NIH ImageJ software.

As a positive control we decided to use mesenchymal stem cells (MSCs). Bone marrow mesenchymal stem cells were isolated from human bone marrow aspirates with Histopaque®1077 (Sigma-Aldrich) and expanded by culture on plastic in mesenchymal basal medium (MesenCult, Stem Cells Inc.) with Mesenchymal Stimulatory Supplements Human (used at a final 10% volume) by 3-4 weeks and then subjected to osteogenic differentiation with complete osteogenic stimulatory

supplements plus dexametasone and ascorbic acid. After three weeks is added the β-glycerophosphate. MSCs were characterized by morphology and surface phenotype by FACS. Osteogenic differentiation was confirmed by von Kossa's staining for calcification and expression of OC and BAP.

In another series of experiments, the cells were cultured in presence of high glucose (25 mM) or mannitol 25mM, as osmotic control, or subjected to low oxygen tension using a commercial kit for a week. At the end of the experiment these cells were used for the analysis of calcification.

We also tested other culture conditions: 1) incubation with SDF-1α (50ng/ml final concentration), to observe the effects of CXCR4 stimulation, 2) incubation with levamisole 1mM to test the effects of inhibiting alkaline phosphatase on MCC calcification *in vitro*.

3.2 FACS ANALYSIS

Circulating osteoprogenitor cells were identified and quantified in patients's peripheral blood samples based on the surface expression of selected antigens. Summarizing, after red cell lysis, 150 µL of blood were incubated with 10 µL of phycoerythrin (PE) anti-osteocalcin (OC clone 190125, R&D Systems, Mnneapolis, MN, USA, cat #IC1419P) and 10 µL of allophycocyanin (APC) anti-bone alkaline phosphatase (BAP clone B4-78, R&D Systems, cat #FAB1448A) monoclonal antibodies. OC^+BAP^+ cells were identified and counted in 2D scatter plots after a morphological gate on mononuclear cells. For further characterization, in separate experiments, gated OC^+BAP^+ cells were assessed for binding of FITC-labeled anti-CD34, -CD7, -CD14, -CD45, -CD44, -CD90, -CD29, -CD68, -RankL. Blood samples from healthy blood donors were used to sort OC^+BAP^-, OC^-BAP^+, OC^+BAP^+ and OC^-BAP^- cells to perform gene expression analysis, FISH, or *in vivo* calcification assays.

For phenotypic characterization of culture osteogenic cells, cells were detached with trypsin/EDTA 0.25% and analyzed by flow cytometry for expression of BAP, OC, CD14, CD34, CD45, CD44, CD29, CD90, CD68, RankL. Cultured human mesenchymal stem cells were analysed by flow cytometry for expression of CD34, CD14, CD45, CD90, CD44, CD29, OC and BAP. Identity of mesenchymal stem cells was confirmed by the $CD34^-CD45^-CD14^-CD44^+CD29^+CD90^+$ phenotype.

Intracellular Runx2 expression was analyzed in fresh whole blood samples by staining cells with a primary mouse anti-Runx2 mAb (Clone, 1D8, Sigma Aldrich cat

#WH0000860M1) and a secondary FITC-conjugated anti-mouse IgG2b antibody. Runx2 protein analysis was performed in cultured cells (MCCs) as well as in fresh blood samples and PBMCs. Simultaneous staining with APC-conjugated anti-BAP and PE-conjugated anti-OC allowed the determination of the expression of Runx2 in the four population of cells (OC+BAP+, OC-BAP+, OC+BAP-, OC-BAP-).

3.3 RunX2 PROMOTER REPORTING ASSAY

To study the phenotype of circulating RunX2 expressing cells, weused an EGFP promoter reporting assay. The evolutionary conserved osteoblast specific protection region, -257/-217 on chromosome 6 (45403774-45403814), representing the RunX2 promoter, was inserted into a plasmid upstream of EGFP sequence. Synthetic gene was cloned into AseI/NheI digested pEGFP-C1. Open reading frame orientation is illustrated in Fig. 2.
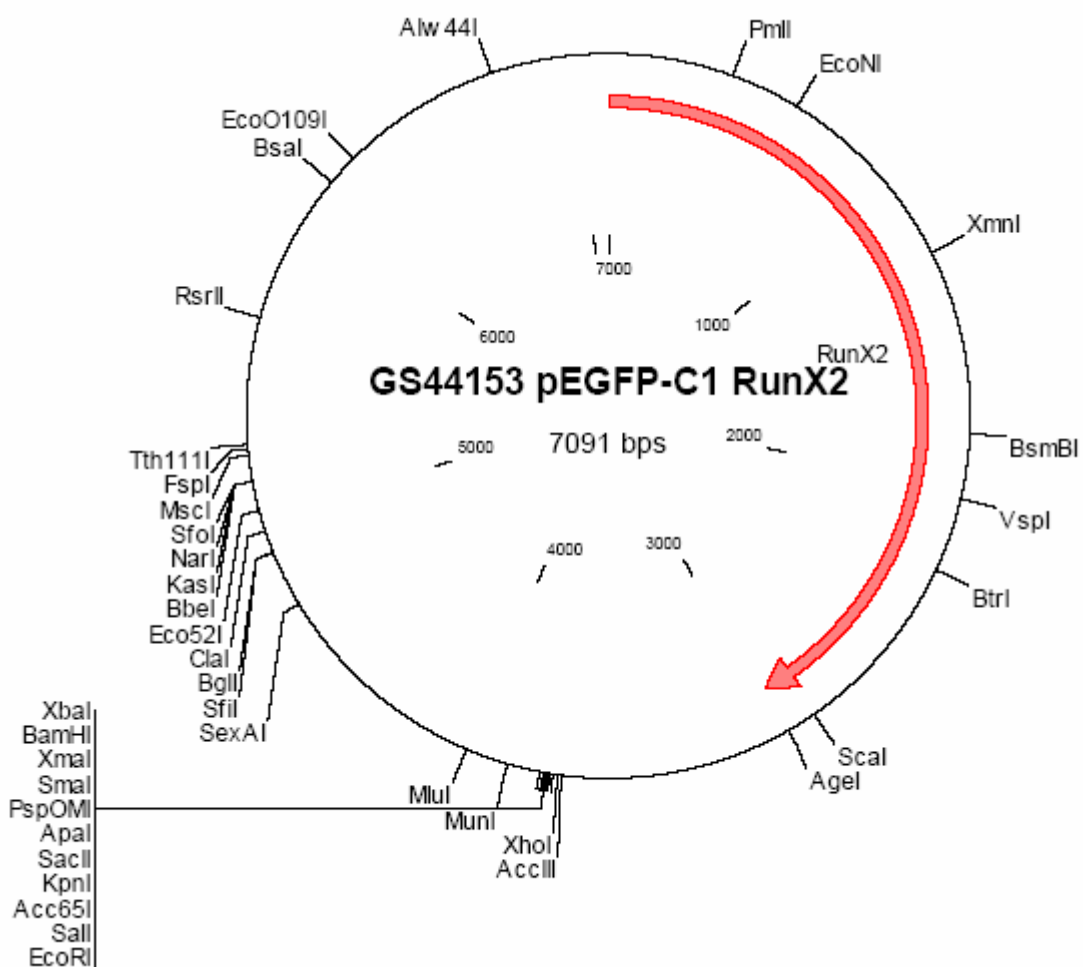
Fig. 2: The synthetic Runx2 gene promoter was cloned into AseI/NheI digested pEGFPC1. Open reading frame orientation is illustrated.

The resulting Runx2 promoter – EGFP reporter plasmid allowed identification of cells supporting high level transcription from osteoblast specific Runx2 promoter region - 257/-217 encoded on chromosome 6.6 PBMC from healthy blood donors were transfected with the pEGFP-C1 RunX2 plasmid or with a control plasmid driving constitutive EGFP expression under a CMV promoter using the Ingenio^{TM} electroporation kit according to manufacturer's instructions. After transfection, cells were seeded for 24 hours on plastic and then analyzed by flow cytometry for EGFP signal and co-expression of OC and BAP, as previously described. Enrichment of cells expressing either OC or BAP or both in the population of RunX2 expressing cells was calculated comparing the percentages of OC BAP-, OC+BAP-, OC-BAP+ and OC+BAP+ cells in the RunX2- EGFP+ gate to those in the control EGFP+ gate.

3.4 FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Peripheral blood samples were obtained form 2 women who received a bone marrow transplantation from a male donor more than 12 months earlier. We chose this criterion to assure complete hematopoietic reconstitution. This approach allowed to determine the donor or recipient origin of cells of interest by analyzing that Y-chromosome signal. For this purpose, OC-BAP- and OC+BAP+ cells were freshly sorted and spotted onto glass slides. A human Y chromosome-painting FITC-conjugate probe (Star*FISH, Cambio, Cambridge, UK) was used. Slides were fixed in formaldehyde in PBS for 5 minutes and pass through three brief rinses in 50, 70 and 100 ethanol grading, after washes in 2X SSC. The DNA probe was applied to the sample and co-denatured at 85°C and then hybridized at 37°C. Washes were performed at 42°C in a solution of 2X SSC/0.3% Nonidet-P40 for 2 minutes. Nuclei were counterstained with TO-PRO 3. Probe was visualized by confocal microscope (TCS-SL Leica, Germany). In the positive and negative controls (represented by male and female cytospin from normal pheripheral blood), at least 200 nuclei were counted to test the efficiency of the probes. The chimerism index was assessed by dividing the number of Y-chromosome-positive cells by the total number of cells of interest. Microphotographs were taken with a TCS-SL laser scanner confocal microscope. A logarithmic curve was used to model the time course of Y-negative cells remaining in the recipient, and half-life of recipient-retained cells was calculated by extrapolating time at 50% Y-negative cells. The curve was fitted to 3 points given

by the two cases plus a time point zero when all recipient cells are assumed to be Y-neg.

3.5 *In vitro* CALCIFICATION ASSAY

ALIZARIN RED STAINING PROTOCOL

Alizarin red S (ARS, Sigma-Aldrich cod. A5533) was used to determine quantitatively by colorimetry the presence of calcific deposition by cells of an osteogenic lineage. Cells or slides were fixed in PFA 4% for 10 minutes at room temperature and then incubated in ARS 2% for 30 seconds to 5 minutes, and observed microscopically. Usually, 2 minutes produced nice red-orange staining of calcium. Samples were finally washed several times in distilled water to remove excess of ARS and mounted in Elvanol.

VON KOSSA'S STAINING PROTOCOL

The Von Kossa staining is used to quantify mineralization in cell culture and tissue sections. The stain principle is a photochemical reaction in which silver ions react with phosphate in the presence of acidic material. Cells or slides were fixed in PFA 4% for 10 minutes at room temperature. The plate with the cells or slides were placed on a sheet of aluminum foil and incubated with 1% silver nitrate (Titolchimica) for 15 minutes under ultraviolet light. After washes in distilled water to remove excess reagent, the samples were incubated with 5% sodium thiosulfate (Bio-optica) for 5 minutes, washed once in tap water and several times in distilled water and finally incubated in Harris hematoxylin for 10 seconds, washed several times in distilled water and mounted in Elvanol.

3.6 *In vivo* CALCIFICATION ASSAY

Cultured osteogenic cells (after the complete 3 week culture protocol), bone marrow mesenchymal stem cells or freshly sorted OC^+BAP^+, OC^+BAP^-, OC^-BAP^+ and OC^-BAP^- cells ($5x10^5$ cells in all cases) were embedded into 500 µL phenol-free Matrigel plugs (BD Biosciences) and implanted subcutaneously in CD1 nude mice (Charles River). After 12 days, plugs were explanted and grossly inspected. Seven µm cryosections were stained with Von Kossa's and positive area quantified using the NIH ImageJ software. In separated experiments, cells embedded in Matrigel were implanted into hind limb skeletal muscles of nude mice with ischemia.

3.7 ATHEROSCLETORIC PLAQUE ANALYSIS

Carotid atherosclerotic plaques were obtained at time of carotid endoarterectomy from 9 non diabetic patients and 12 diabetic patients. Frozen samples were crosssectioned at the equatorial point as well as at 2 upstream and 2 downstream points. Sections were stained with hematoxylin and eosin for plaque type scoring (Stary H.G., 2000) and with Von Kossa's to identify calcified areas. Von Kossa positive areas were quantified in ten randomly selected fields for each section and expressed as percentage of total area. Immunohistochemistry was then performed on serial sections for OC, BAP, CD68 (Dako Cytomation, Denmark) and α-smooth muscle actin (A2547, Sigma Aldrich). Local OC+BAP+ cells were counted in ten randomly selected fields for each section in the plaque areas. Plaque sections were also stained for HIF-1 using a monoclonal mouse anti-human antibody (BD).

3.8 CALCIUM QUANTIFICATION

Matrigel samples with embedded cultured cells in different growth conditions, as well as explanted *in vivo* Matrigel plug were analyzed for calcium content using the HCl extraction method (Calcium CA 0505 CH, Chema Diagnostica, Monsano, Italy).

3.9 SECRETOME PROFILING OF CULTURED MCCs

Secretory activity of cultured MCCs was assayed using a custom human cytokine antibody array. The medium collected from cultured MCCs in control, hypoxia and high glucose conditions, were tested for expression of secreted factors that may regulate their pro-calcific potential. For this purpose, we used a Custom Human Cytokine Antibody Array from RayBiotec, according to manufacturer's istructions. Cells were incubated for twenty-four hours in a serum-free condition before collection of medium. Concentrations of molecules in the MCC medium were then compared with their concentration in the serum-free control medium.

The secreted factors analyzed are:
- BMP-4, Bone Morphogenetic Protein 4, which belongs to the superfamily of TGF-β, implicated in the differentiation of ectodermal tissue;
- BMP-6, Bone Morphogenetic Protein 6, which belongs to the superfamily of TGF-β, induces the development of bone and cartilage, and also induces the expression of osteogenic markers in mesenchymal cells;

- BMP-7, Bone Morphogenetic Protein 7, which belongs to the superfamily of TGF-β, known as osteogenic protein-1, plays an important role in the differentiation of mesenchymal cells into bone and cartilage;
- MMP-1, Matrix Metalloproteinase-1, proteins of the matrix metalloproteinase family also knows as interstitial collagenase, involved in the breakdown of extracellular matrix in normal physiological processes;
- MMP-9, Matrix Metalloproteinase-9, also knows as 92 kDa type IV collagenase is an enzyme that in humans is encoded by the MMP9 gene;
- MMP-13, Matrix Metalloproteinase-13, also called as collagenase 3, is an enzyme that in humans is encoded by the MMP13 gene;
- Osteoprotegerin, belongs to the superfamily of TNF (Tumor necrosis factor),is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL). RANKL is a member of the tumor necrosis factor (TNF) cytokine family which is a ligand for osteoprotegerin and functions as a key factor for osteoclast differentiation and activation;
- Rank, RankL receptor;
- TIMP-1, metallopeptidase inhibitor 1, a tissue inhibitor of metalloproteinases, is a glycoprotein that is expressed from the several tissues of organisms;
- TIMP-4, metalloproteinase inhibitor 4 is an enzyme that in humans is encoded by the TIMP4 gene.

3.10 STATISTICAL ANALYSIS

Experiments were typically performed in triplicate. Data are presented as mean ± standard error. Comparison between 2 means was assessed using unpaired two-tailed Student's t test. When there were more than 2 groups, ANOVA with LSD post-hoc test was used. Differences between categorical data were assessed with Chi square. Linear correlation between two continuous variables was explored with Pearson's r coefficient. A multiple stepwise linear regression analysis was run to verify the independent association of between diabetes and OC+BAP+ cell levels. SPSS ver. 16.0 was used and statistical significance was accepted at $p < 0.05$.
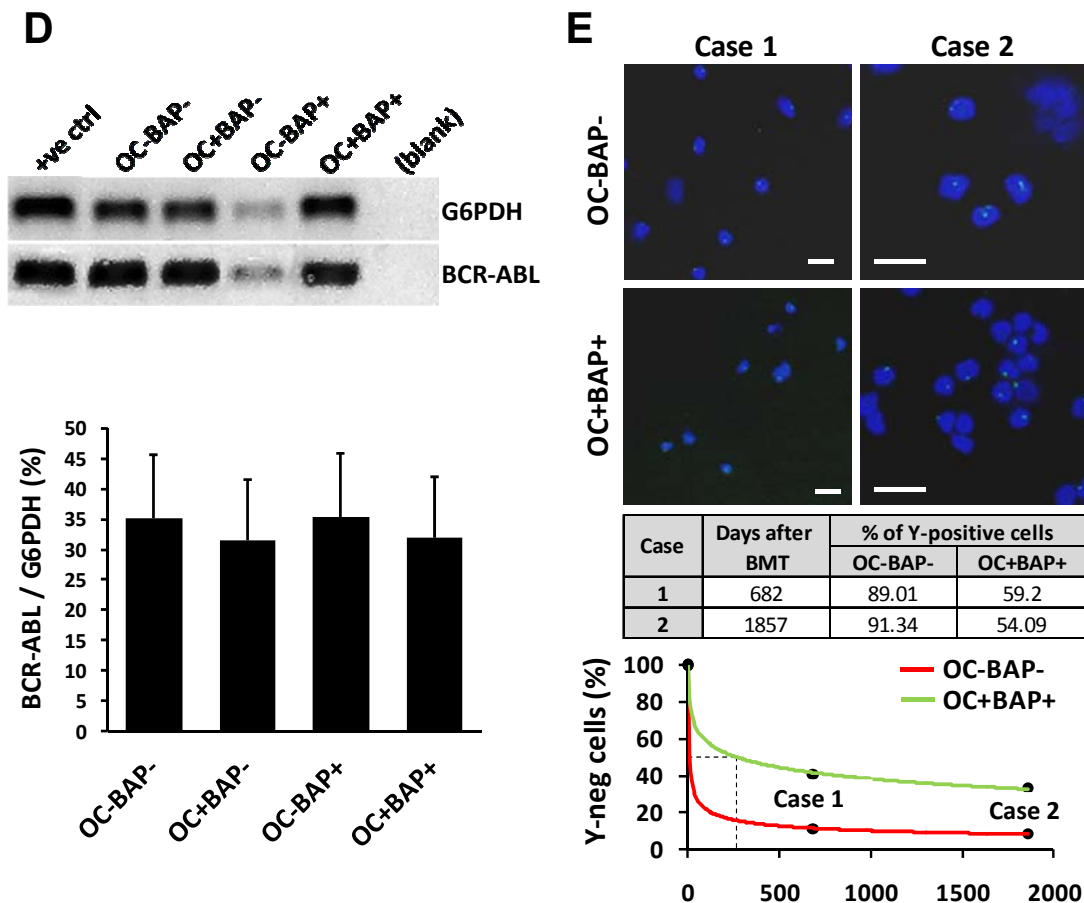
4. RESULTS

4.1 ACTIVITY, IDENTITY, AND ORIGIN OF CIRCULATING CALCIFYNG CELLS

To identify procalcific cells, circulating mononuclear cells were stained with OC and BAP. In 28 healthy subjects, 4.4±0.5% of circulating blood cells were OC^+ and 3.8± 0.5% were BAP^+. In turn, 21.6±2.9% of OC^+ cells coexpressed BAP, and 30.3±4.3% of BAP^+ cells coexpressed OC (Fig. 3A).

D

E
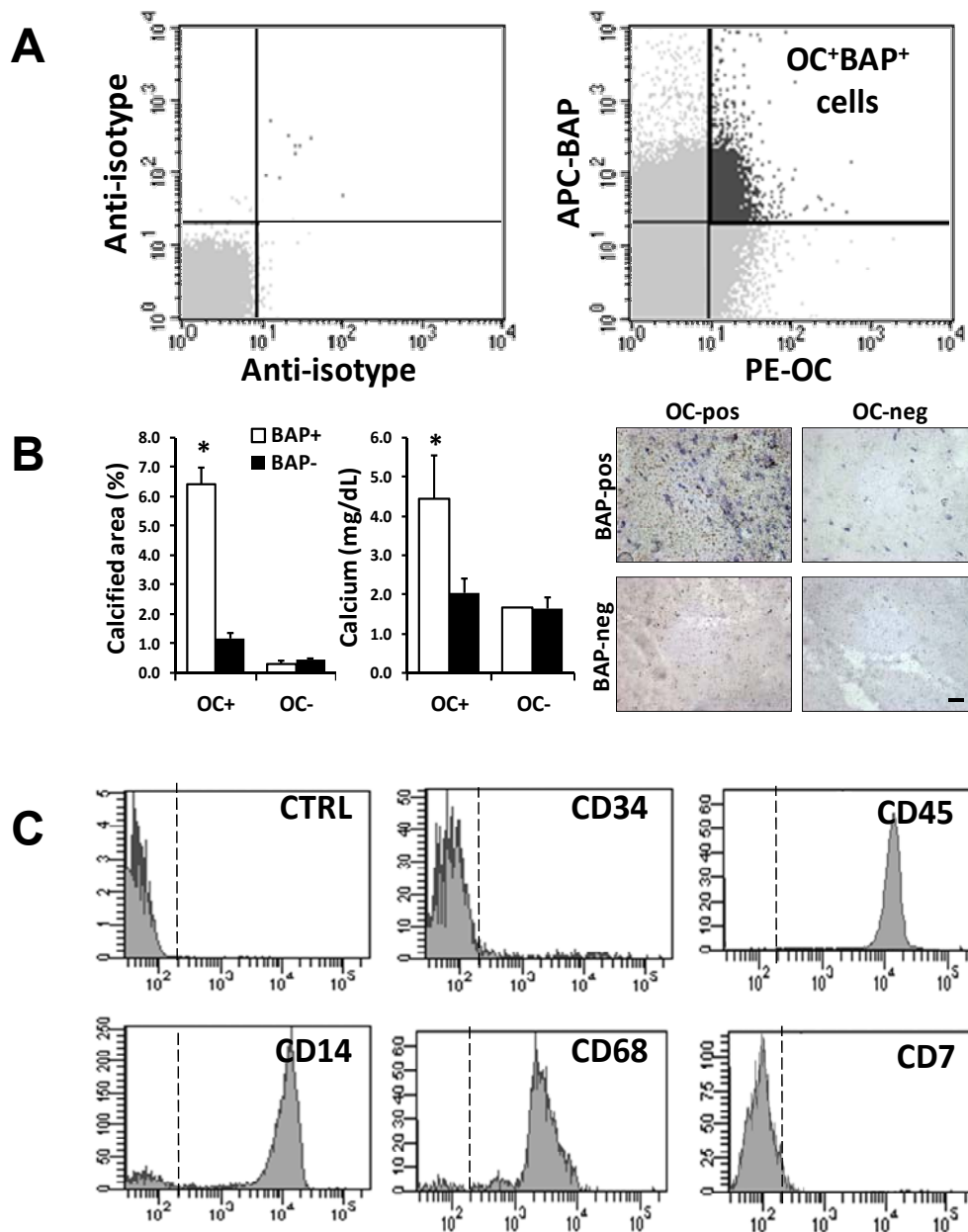
Fig. 3: Activity, identity, and origin of circulating calcifying cells. A) Circulating calcifying cells were identified using flow cytometry relative to the negative isotype control (left) by the expression of OC and BAP (right). APC indicates allophycocyanin; PE, phycoerythrin. **B)** Four populations of circulating cells were freshly sorted according to expression of OC and/or BAP, embedded into Matrigel plugs, and implanted subcutaneously in nude mice. Plugs were explanted 12 days later, and calcification was quantified using von Kossa staining and calcium extraction. OC+BAP- cells showed significantly higher calcification potential (*P<0.05; scale bar, 200 μm). **C)** OC+BAP+ cells were assayed for expression of surface antigens by flow cytometry, with respect to the negative control (CTRL). **D)** OC-BAP-, OC+BAP-, OC-BAP+, and OC+BAP+ cells were freshly sorted from 3 patients with Ph+ CML and assayed for expression of the BCR-ABL mRNA fusion gene. **E)** OC-BAP- and OC+BAP+ cells freshly sorted from 2 cases of male-to-female bone marrow transplantation were analyzed for Y-chromosome signal with FISH (scale bar, 50 μm). Percentages of Y- cells are reported in the table. Rate of disappearance of Y- cells was estimated for OC+BAP+ (green line) and OC-BAP- (red line) cells to calculate their half-life, indicated by the *x*-axis intersection at 50% Y- cells. BMT indicates bone marrow transplantation.

Because OC and BAP did not appear to identify the same cells and were coexpressed by a subset of cells, to understand the procalcific potential of cells expressing either OC or BAP or both, we freshly sorted OC⁻BAP⁻, OC⁺BAP⁻, OC⁻BAP⁺, and OC⁺BAP⁺ cells for calcification assay in vivo. When embedded into Matrigel plugs and injected in nude mice, OC⁺BAP⁺ cells induced higher amounts of calcification compared with cells expressing either OC or BAP and to the OC⁻BAP⁻

negative control (Fig. 3B). Calcified areas were spotty and did not include foci of bone or cartilage formation. The rate of apoptotic cell death, which may trigger pathological cell-mediated calcification (Huitema L.F. and Vaandrager A.B., 2007) was low ($\approx 2\%$ to 3%), but was significantly higher in plugs implanted with OC^+BAP^+ cells than with other phenotypes (Fig. 4A).

C
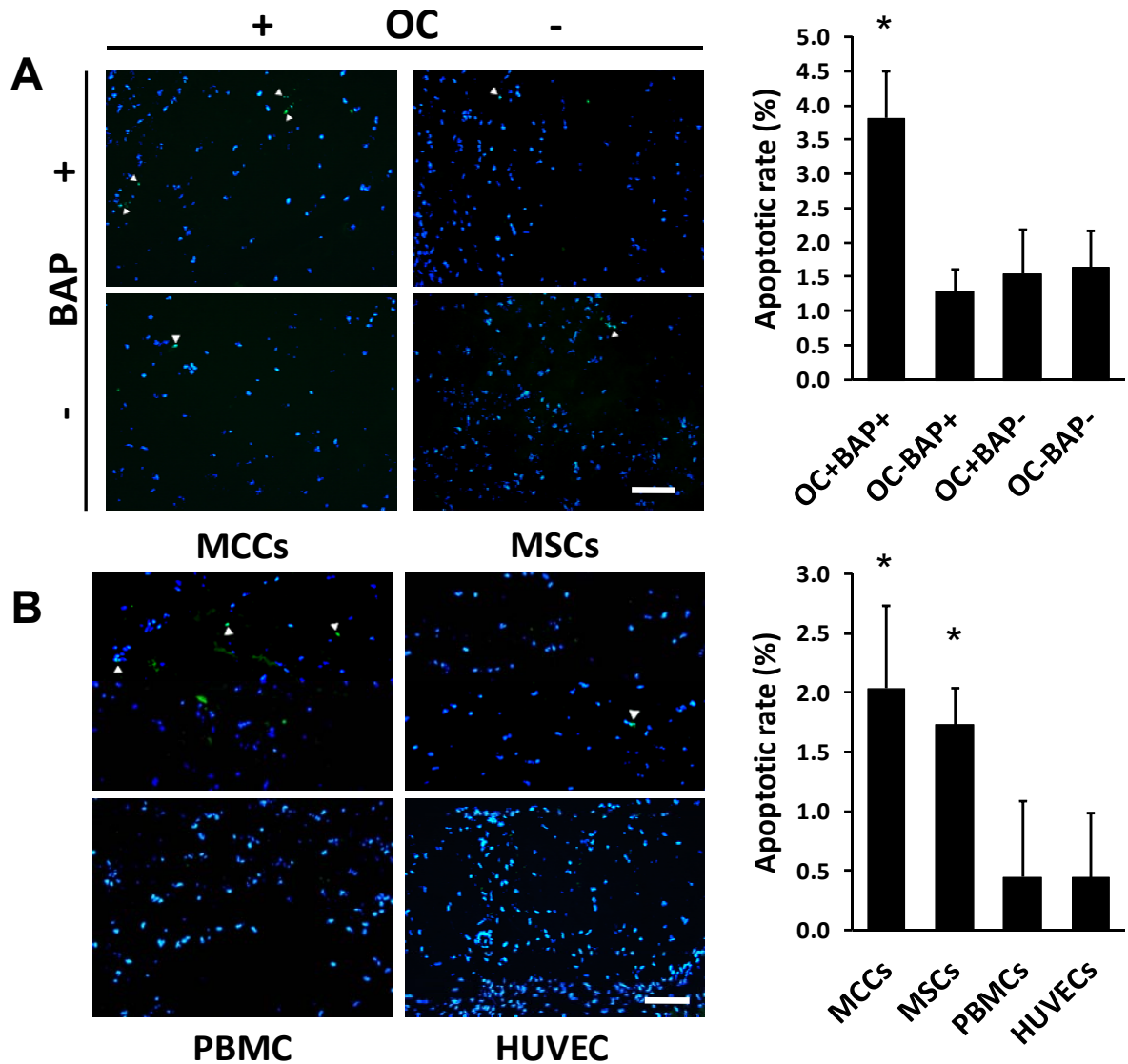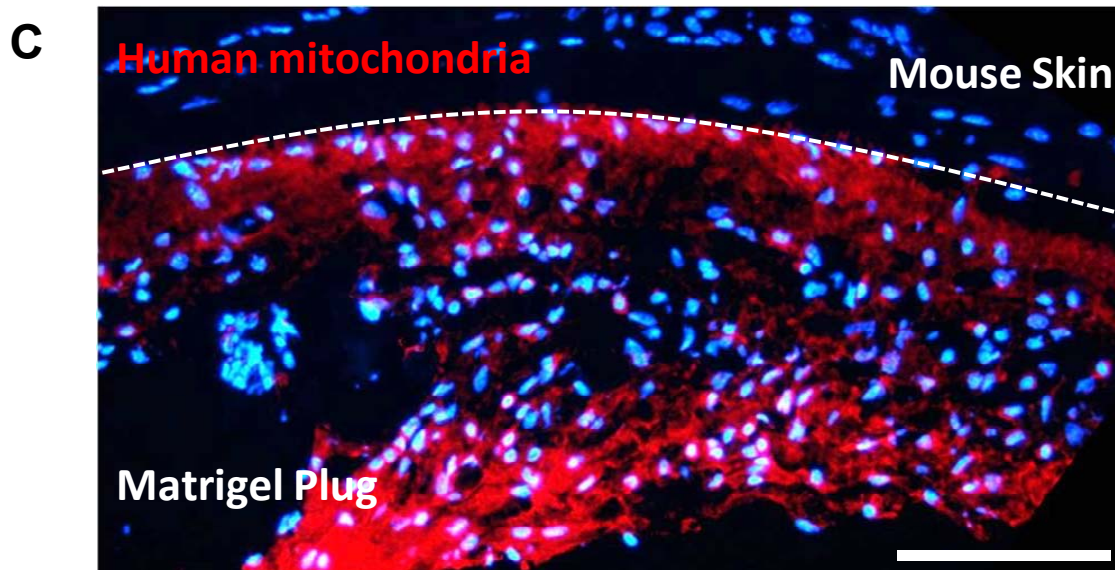
Fig. 4: Apoptosis of calcifying cells in vivo. Different cell types were embedded into Matrigel plugs and implanted subcutaneously in nude mice for calcification assay. After explant, sections of the Matrigel plugs were assessed for apoptosis using an in situ TUNEL assay (green signal, nuclei counterstained in blue with Hoechst). **A)** Fresh cells sorted according to the expression of OC nd/or BAP: OC+BAP+ cells showed a higher percentage of apoptosis when embedded in Matrigel plugs (*p<0.05 vs all other cell types). **B)** Cultured MCCs, MSCs, PBMCs and HUVECs were implanted *in vivo* to compare their calcification potential: MCCs and MSCs showed higher degrees of calcification (*p<0.05 vs PBMCs and HUVECs). White arrowheads indicate apoptotic cells. **C)** A separate staining for human mitochondria (red) at the interphase between mouse skin and the Matrigel plug shows that most cells within the plug are of human origin, while no human mitochondria staining was visible outside the plug.

These preliminary results allowed us to focus on the OC$^+$BAP$^+$ as the most functionally relevant phenotype of circulating calcifying cells. OC$^+$BAP$^+$ cells were then assayed for expression of other markers: lack of CD34 expression indicated they are distinct from hematopoietic stem cells. However, the expression of CD45, CD14, and CD68 suggested that OC$^+$BAP$^+$ cells are derived from cells belonging to the monocyte/macrophage lineage (Fig. 3C). To confirm the myeloid lineage origin of OC$^+$BAP$^+$ cells, we flow-sorted fresh circulating cells according to OC and BAP expression from 3 patients with new-onset Ph$^+$ chronic myeloid leukemia (CML) and looked for the BCR-ABL transcript. We found that OC$^+$BAP$^+$ cells expressed the BCR-ABL transcript to the same extent as cells expressing either OC or BAP and as OC$^-$BAP$^-$ cells, which served as controls (Fig. 3D). This experiment provides reasonably compelling evidence that procalcific OC$^+$BAP$^+$ cells originate from a myeloid progenitor and are not derived from mesenchymal stem cells, which have been previously demonstrated to be BCR-ABL negative in CML patients (Jootar S. et al., 2006). To understand the anatomic location and origin of myeloid OC$^+$BAP$^+$ cells, we analyzed 2 women who received a male bone marrow transplant ≈2 and 5 years

before, respectively. We found that ≈56% of OC$^+$BAP$^+$ cells were Y-chromosome–positive, as compared with ≈90% of OC$^-$BAP$^-$ cells (Fig. 3E).

The existence of Y-negative OC$^+$BAP$^+$ cells years after transplantation indicates that a fraction of these cells is long-lived and rather quiescent, not being affected by myeloablation. Taking into consideration the time passed since bone marrow transplantation, it is possible to describe a logarithmic kinetic of substitution of Y$^-$ recipient cells with Y$^+$ donor cells and estimate a half-life of OC$^+$BAP$^+$ cells in 268 days (8.8 months), whereas the half-life of control OC$^-$BAP$^-$ mononuclear cells was estimated in 7 days (the same order of magnitude as monocyte half-life in humans) (Whitelaw D.M., 1972). Given that >90% of circulating OC$^+$BAP$^+$ cells are CD45$^+$CD68$^+$CD14$^+$CD7$^-$ (Fig. 3C), we hypothesize that these recipient-retained, long-lived, myeloid OC$^+$BAP$^+$ cells belong to the reticuloendothelial system. Collectively, these data indicate that circulating OC$^+$BAP$^+$ cells are procalcific, originate from the myeloid lineage, express monocyte/macrophage markers, and a subpopulation of them is long-lived. Thereafter, we have termed these OC$^+$BAP$^+$ cells MCCs.

4.2 RUNX2 EXPRESSION IN MYELOID CALCIFYNG CELLS

We analyzed expression of a selected range of bone-related genes and found significant upregulation of Col1a1 (3-fold), Osterix (2-fold), and Runx2 (5-fold) in freshly sorted OC$^+$BAP$^+$ cells and, to a lesser extent, in OC$^+$ cells (Fig. 5A). Runx2, a master gene regulator of osteogenic differentiation (Lian J.B. and Stein G.S., 2003), showed the highest differential expression in OC$^+$BAP$^+$ compared with OC$^-$BAP$^-$ cells. Thus, we hypothesized that circulating MCCs could be distinguished from other blood cells by expression of Runx2. Using an EGFP-Runx2 gene promoter reporting assay (Fig. 5B), we demonstrate that the EGFP-Runx2$^+$ population of circulating mononuclear cells express high levels (>90%) of OC and are 70-fold enriched in OC$^+$BAP$^+$ cells as compared with the control mononuclear cell population transfected with a plasmid driving constitutive enhanced green fluorescent protein (EGFP) expression (Fig. 5C).

Runx2 protein content in OC- and/or BAP-expressing cells, analyzed by indirect intracellular flow cytometry, was ≈18- and 10-fold increased in OC$^+$BAP$^+$ and OC$^+$BAP$^-$ cells, respectively, compared with the control OC$^-$BAP$^-$ cells (Fig. 5D), thus confirming results of the gene reporter assay. Despite the low number of

circulating Runx2^{+} cells identified, findings were consistently reproducible among different experiments and with both methods. These data indicate that Runx2 expression is typical of OC[+] and OC[+]BAP^{+} cells, suggest that Runx2 is involved in the differentiation of MCCs from mononuclear cells, and support their procalcific program.
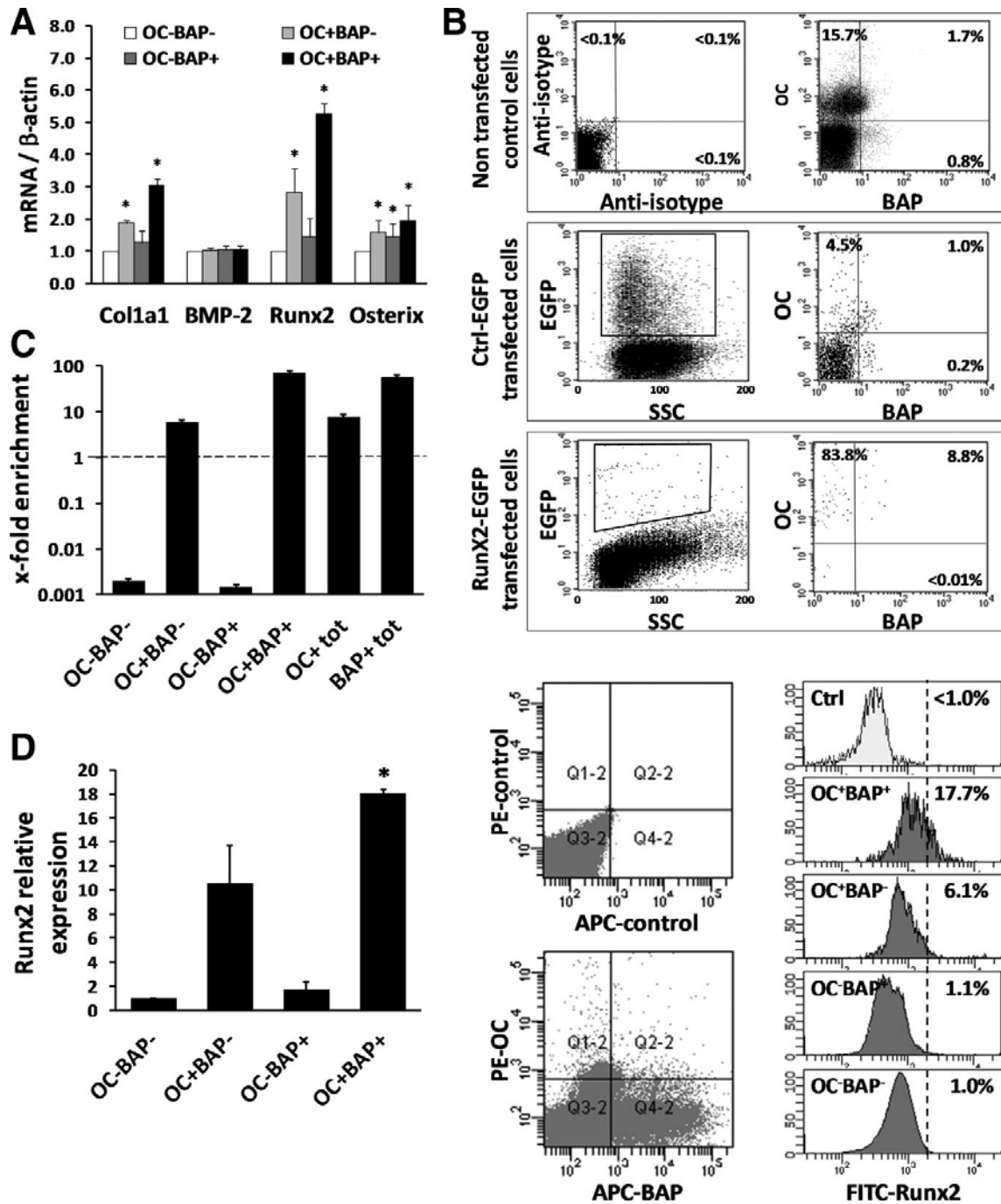

Fig. 5: Runx2 expression in MCCs. A) When freshly sorted from healthy donors, OC+BAP+ cells showed higher expression of osteogenic genes than OC-BAP-, OC+BAP-, and OC-BAP+ cells (*P<0.05). **B)** Expression of OC and BAP was determined by flow cytometry on unselected fresh PBMCs (top), EGFP+ cells transfected with a constitutive EGFP plasmid (middle), or EGFP+ cells

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transfected with a plasmid driving EGFP under the Runx2 promoter (bottom). Bottom, Enrichment of OC+ and BAP+ cells in the small fraction of PBMC expressing Runx2. **C)** Quantification of the enrichment of cells expressing either OC or BAP or both in Runx2-EGFP+ cells as compared with control EGFP+ cells. SSC indicates side scatter. **D)** Runx2 protein content was determined in the 4 populations of cells identified by OC and/or BAP expression (bottom scatter plot) relative to the negative control (top scatter plot) by flow cytometry using an intracellular staining (histograms).

4.3 ISOLATION, CHARACTERIZATION, AND ACTIVITY OF COLTURED MCCs

Human MCCs were obtained after 3 weeks of peripheral blood mononuclear cell (PBMC) culture in 2D Matrigel with osteogenic differentiation medium, where they assumed a rounded or cobblestone morphology. After adding β-glycerophosphate during the last week of culture, MCCs formed in vitro calcifications, as shown by von Kossa and Alizarin red staining, without foci of bone or cartilage formation (Fig. 6A; higher magnification in Fig. 7).
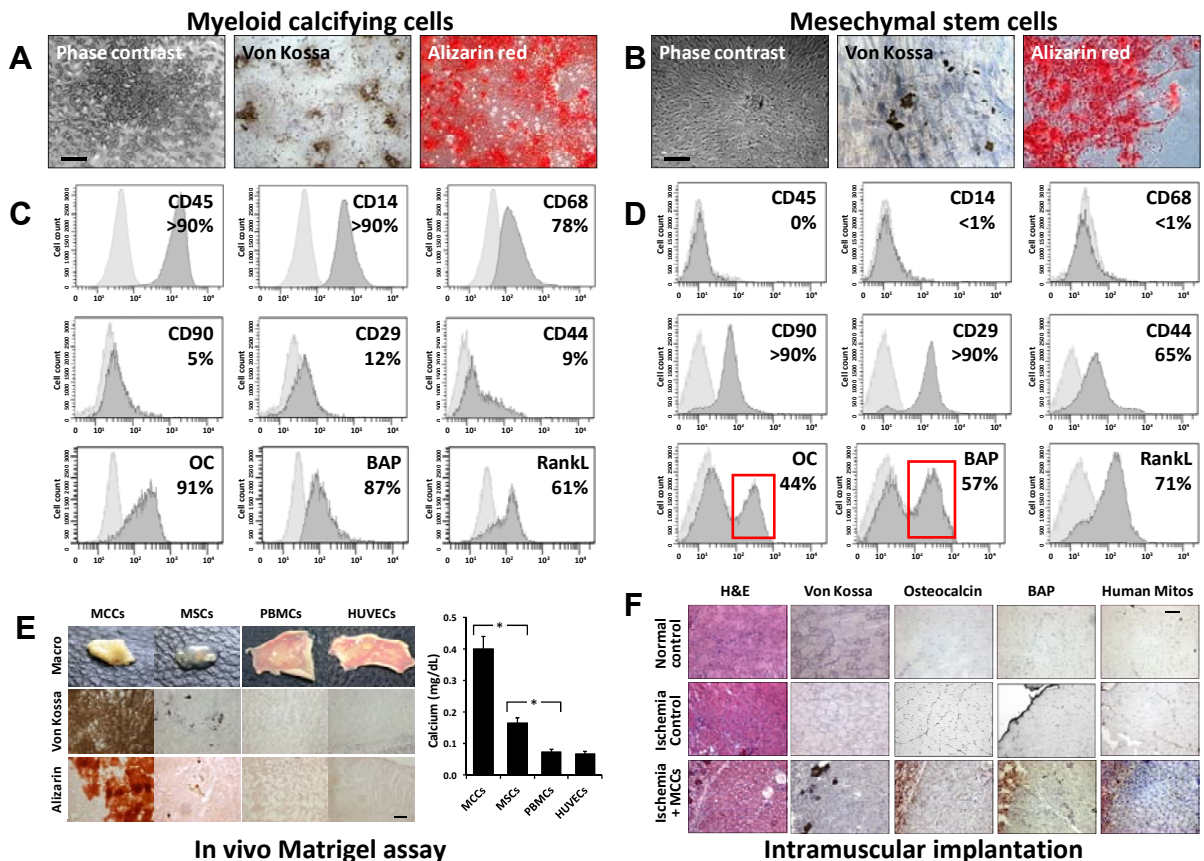
Fig. 6: Isolation, characterization, and activity of cultured MCCs. A) PBMCs cultured for 3 weeks in osteogenic conditions assumed a rounded/cobblestone morphology (left) and calcified in vitro, as shown by von Kossa (middle) and Alizarin red (right) staining (scale bar, 100 µm). **B)** Human bone marrow–derivecells cultured in a mesenchymal medium assume a typical elongated morphology (left), can be induced toward an osteoblast phenotype by osteogenic medium, and calcify in vitro, as shown by von Kossa (middle) and Alizarin red (right) staining. Scale bar, 100 µm. The more intense staining with Alizarin red for calcium than with von Kossa for inorganic phosphate possibly reflects the presence of other calcium–organic complexes in the mineralizing matrix (eg, calcium–phospholipid).

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C) Flow cytometric analysis of peripheral blood osteogenic cells shows positivity for OC, BAP, CD14, CD45, and CD68 and negativity for CD90, CD29, and CD44. D) Flow cytometric analysis of MSCs shows a subpopulation with positivity for OC, BAP (red rectangles), RankL, CD90, CD44, and CD29 and negativity for CD34, CD14, and CD45. E) MCCs embedded into Matrigel plugs developed calcifications 12 days later, which strongly stained with von Kossa and Alizarin red (scale bar, 100 μm). Calcium quantification showed a higher amount of calcium in MCC implanted plugs compared with plugs implanted with MSCs, PBMCs, and HUVECs. Plugs implanted with PBMCs or HUVECs did not calcify and remained adherent to the overlying skin. F) MCCs also formed ectopic calcifications after implantation into ischemic hind limb skeletal muscles, whereas no calcification was seen in control muscle sections with or without ischemia. Calcified areas colocalized with OC, BAP, and human mitochondria (X20; scale bar, 100 μm).
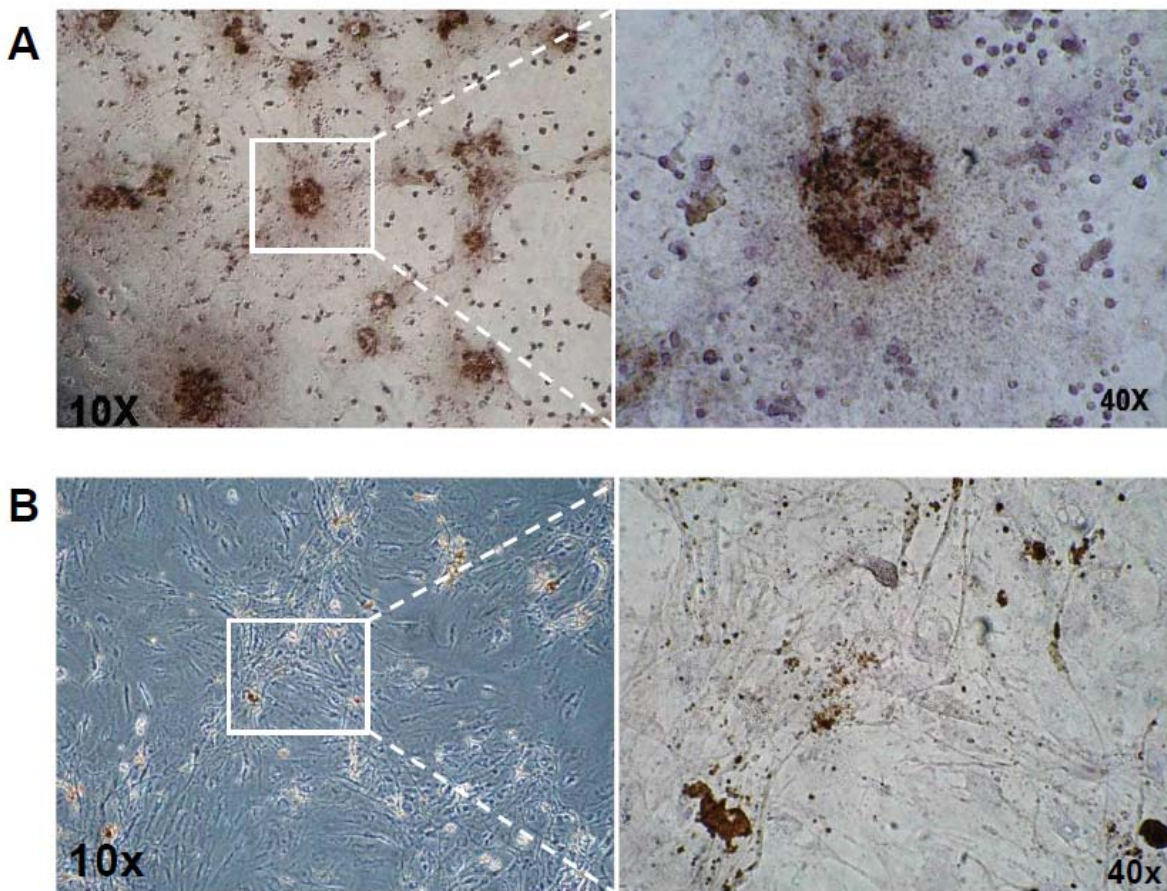
Fig. 7: High magnification von Kossa staining of cultured MCCs and MSCs. A) MCCs were cultured from PBMCs (as described in the method section) for three weeks. At the end of the culture protocol, cells were stained using the Von Kossa method, which identifies inorganic phosphate as brown areas. A calcified nodule at the center of the 10x photo (squared area in the left panel) is magnified to 40x (right panel) to show Von Kossa staining of the extracellular matrix (Matrigel) within which cells are embedded. **B)** MSCs differentiated toward the osteoblast lineage according to the culture protocol described in the method section are shown for comparison with MCCs. At the center of the 10X microphotograph, an area with higher cell density (squared) is magnified to 40X to show spotty microcalcifications and calcified nodules.

The same osteogenic medium protocol allowed differentiation of calcifying cells from human mesenchymal stem cells (MSCs), which served as a positive control (Fig. 6B).

Most cultured MCCs expressed OC and BAP (Fig. 6C), similarly to the subpopulation of MSCs that differentiated toward the osteoblast lineage (Fig. 6D). Cultured MCCs retained features of monocyte/macrophages, such as expression of CD68, CD14, and CD45, and displayed no or very low expression of CD34 and MSC markers CD90, CD29, and CD44 (Fig. 6C). In support of the osteogenic differentiation, we found that cultured MCCs express much higher levels of bone-related genes compared with undifferentiated PBMCs, such as Runx2, Osterix, Col1a1, and BMP-2 (Fig. 8).
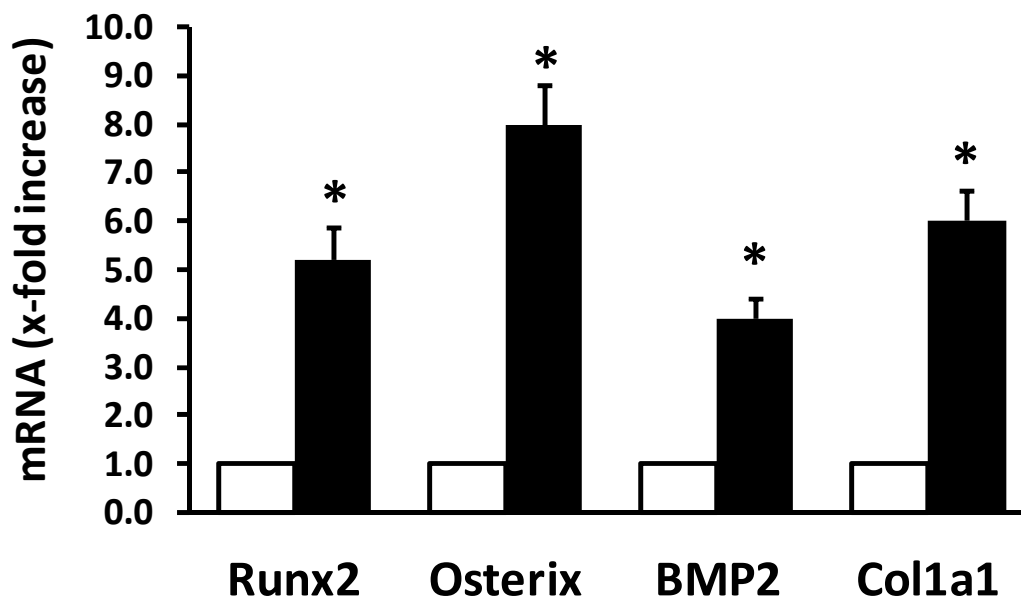
Fig. 8: Expression of bone-related genes in cultured MCCs. mRNA expression level of four bone-related genes was determined by RT-PCR in cultured MCCs (black columns) and fresh undifferentiated PBMCs (white columns). Fresh PBMCs were used as controls because they represent the starting population of cells from which MCCs are differentiated/isolated with the culture protocol. mRNA expression is normalized versus the housekeeping gene -actin and reported relative to control PBMCs, set at 1.0. Means of 3 independent experiments are shown. *$p<0.05$ in MCCs vs PBMCs.

Runx2 upregulation was confirmed at protein level (Fig. 10), and expression of RankL was also in line with activation of an osteogenic program. These data indicate that cultured MCCs represent myeloid cells that have differentiated toward a procalcific phenotype and are different from MSCs. Calcification by MCCs was partly blocked by CXCR4 (C-X chemokine receptor-4) stimulation with stromal-derived factor-1α and was much reduced by the BAP inhibitor levamisole (Fig. 9). Although stromal-derived factor-1α was shown to mediate recruitment of circulating osteogenic

cells (Otsuru S. et al., 2008), it may transiently inhibit their calcification potential. Cultured MCCs embedded in Matrigel plugs and implanted into nude mice formed gross mineralized structures in vivo (without formation of bone), containing higher amounts of calcium compared with MSCs, PBMCs, and HUVECs (Fig. 6E). The apoptotic rate was higher in plugs implanted with MCCs compared with PBMCs or HUVECs (Fig. 3), again suggesting that apoptosis may play a role in this process of calcification.
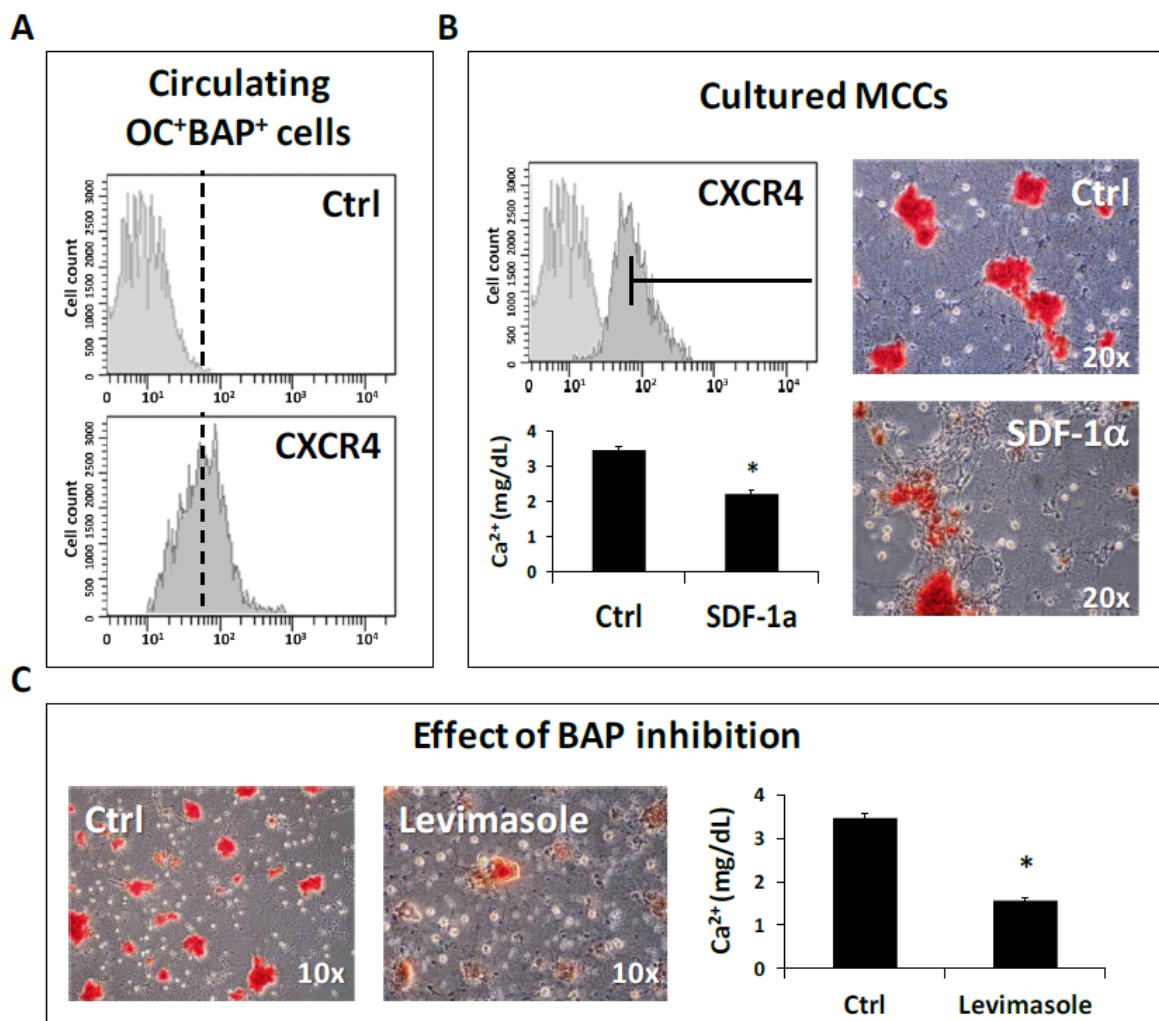

Fig. 9: Effects of the CXCR4/SDF-1α axis and BAP inhibition on MCC calcification. A) The interaction between the chemokine SDF-1α and its receptor CXCR4 has been previously shown to regulate kinetics of circulating osteoprogenitor cells and is also typical of circulating fibrocytes. Herein, we determined CXCR4 expression and effects of CXCR4 activation with SDF-1α on calcification induced by MCCs. Relative to the negative control (upper panel), expression of CXCR4 was analyzed in fresh circulating OC+BAP+ cells using a FITC anti-CXCR4 mAb (lower panel): about 45% of OC+BAP+ cells were CXCR4+. **B)** CXCR4 expression was also analyzed in cultured MCCs, which showed about 50% positivity. Stimulation of the CXCR4 receptor by incubation with its ligand SDF-1α (final concentration 50 ng/mL) reduced *in vitro* calcification of MCCs by about 40% (*p<0.05 in SDF-1α treated cells vs control condition). Representative 20x photos of Alizarin red staining, as well as calcium quantification are shown. **C)** To understand the actual contribution of BAP activity on MCC calcification, we tested the effects of the BAP inhibitor levamisole: 1 mM levimasole significantly

reduced calcium content and Alizarin red staining in MCCs culture by about 60% (*p<0.05 in levamisole-treated cells vs control condition).

MCCs induced ectopic calcifications also when implanted into mouse ischemic skeletal muscles (Fig. 6F). Previous authors have found that a subpopulation of skeletal muscle cells have osteogenic potential (Bosch P. et al., 2000), but we show that calcified areas within muscle tissue colocalized with OC, BAP, and human mitochondria staining in serial sections, indicating that injected cells, not local cells, formed calcifications.

We finally studied the secretome of MCCs regarding release of BMPs, matrix metalloproteinases (MMPs), and TIMPs (tissue inhibitor of matrix metalloproteinases) in the culture medium. In basal conditions, MCCs secreted BMPs and factors that may account for their ability to remodel and calcify the extracellular matrix (Orbe J. et al., 2003), such as MMP-1 and -9 and TIMP-1 and -4 (Fig. 10).
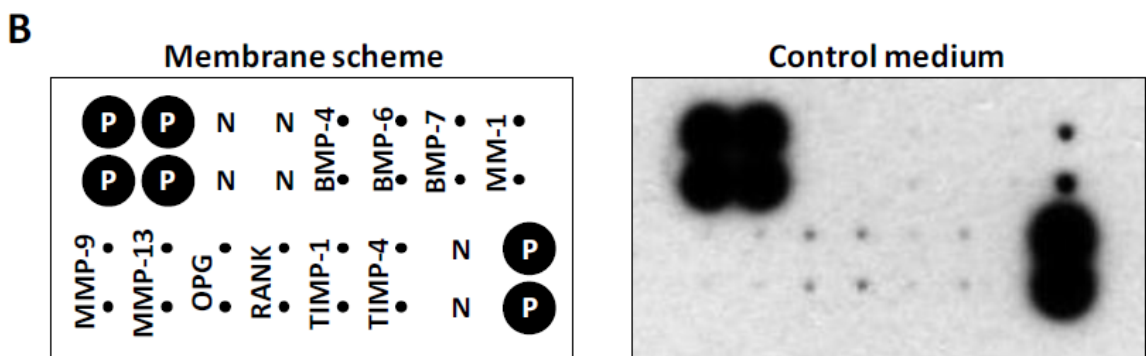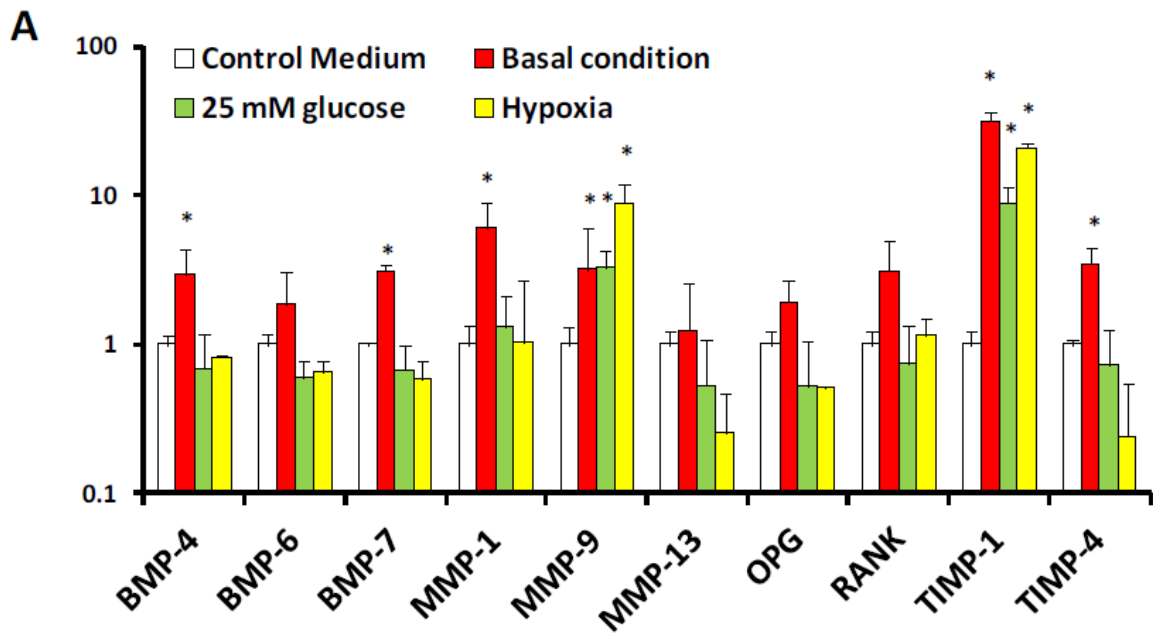
Fig. 10: The secretome of cultured MCCs. Secretory activity of cultured MCCs was assayed using a custom human cytokine antibody array. Cells were incubated for 24 hours in a serumfree conditions to detect molecules released by cultured cells into the medium: the concentration of molecules secreted by cultured cells were compared with the concentrations in the fresh culture medium, which served as a control. **A)** MCCs in basal conditions (i.e. cultured as described in the method section, without additional stimuli) secreted BMP-4, BMP-7, MMP-1, MMP-9, TIMP-1 and TIMP-4, as evidenced by the higher concentration compared with the control medium (arbitrarily set at 1.0, logarithmic scale; *$p<0.05$ vs control). Exposure of MCCs to hypoxia further elevated MMP-9 release, while high glucose and mannitol (not shown) did not significantly modified the MCC secretome. Results are presented as mean of 3 experiments. **B)** The upper left panel shows a schematic representation of the antibody array membrane: positions of the assayed molecules in the membrane are shown (P=positive internal control; N=negative internal control). In the other panels, representative photographs of the MCC secretome in the various culture conditions are shown.

Collectively, these data indicate that cultured MCCs have a phenotype similar to circulating MCCs and form ectopic calcifications in vivo.

4.4 MCCs ARE INCREASED IN CAROTID ATHEROSCLEROTIC PLAQUES OF DIABETIC PATIENT

Carotid atherosclerotic specimen were obtained from diabetic (n=9) and nondiabetic (n=12) patients at time of endoarterectomy and stained for calcium deposits (von Kossa), tissue MCCs (OC and BAP), macrophage infiltration (CD68), and smooth muscle cell localization (αSMA) in serial sections. H&E staining helped in classifying plaques according to American Heart Association guidelines (Stary H.C., 2000). There was no significant difference in the distribution of plaque types from diabetic versus nondiabetic patients (*P*=0.345, Fig. 11) even if more plaques from diabetic patients had predominantly calcified areas (type VII).

U-test p = 0.345
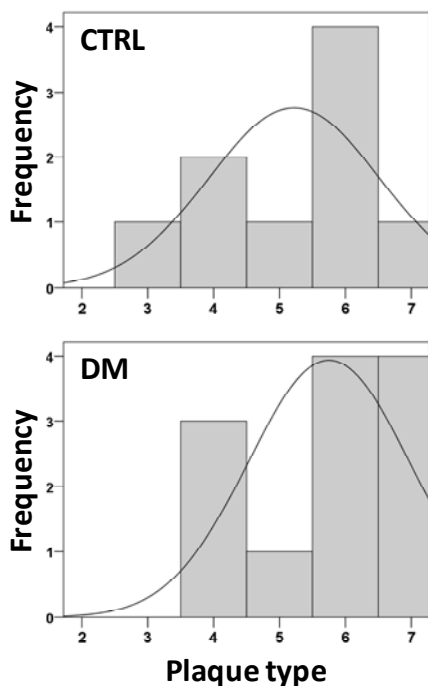
Fig. 11: Distribution of carotid plaque types. Carotid endoarterectomy plaques were histologically analyzed (H&E and von Kossa staining) and scored according to AHA guidelines. Plaque types were plotted against frequency for diabetic (bottom) and non diabetic (top) patients. While there was a higher frequency of prevalently calcific plaques (type VII) in diabetes, the overall distribution was not significantly different between the 2 groups. Mann-Whitney's U test p is shown. These data suggest that, despite more calcium deposition in diabetic plaques, there were no significant differences in the severity of atherosclerotic involvement in analyzed samples from the 2 groups. This was due to the fact that identical criteria were applied to decide surgical intervention in these patients, irrespectively of the presence of diabetes.

Indeed, von Kossa staining showed that specimens from diabetic patients had significantly larger calcified areas (Fig. 12A), especially within the main lesion neointima (Fig. 12B and 12D). Some samples showed intense staining for both OC and BAP, mainly in the main lesion neointima, whereas the far wall was negative for OC and BAP or showed weak positivity for either OC or BAP, but not both (Fig. 12B and 12D). Part of the positive staining appeared to be extracellular, but some cells stained strongly positive for OC and BAP, especially in the vicinity of calcified nodules. The existence of OC+BAP+ cells was also confirmed by 3-color immunofluorescence (Fig. 13).
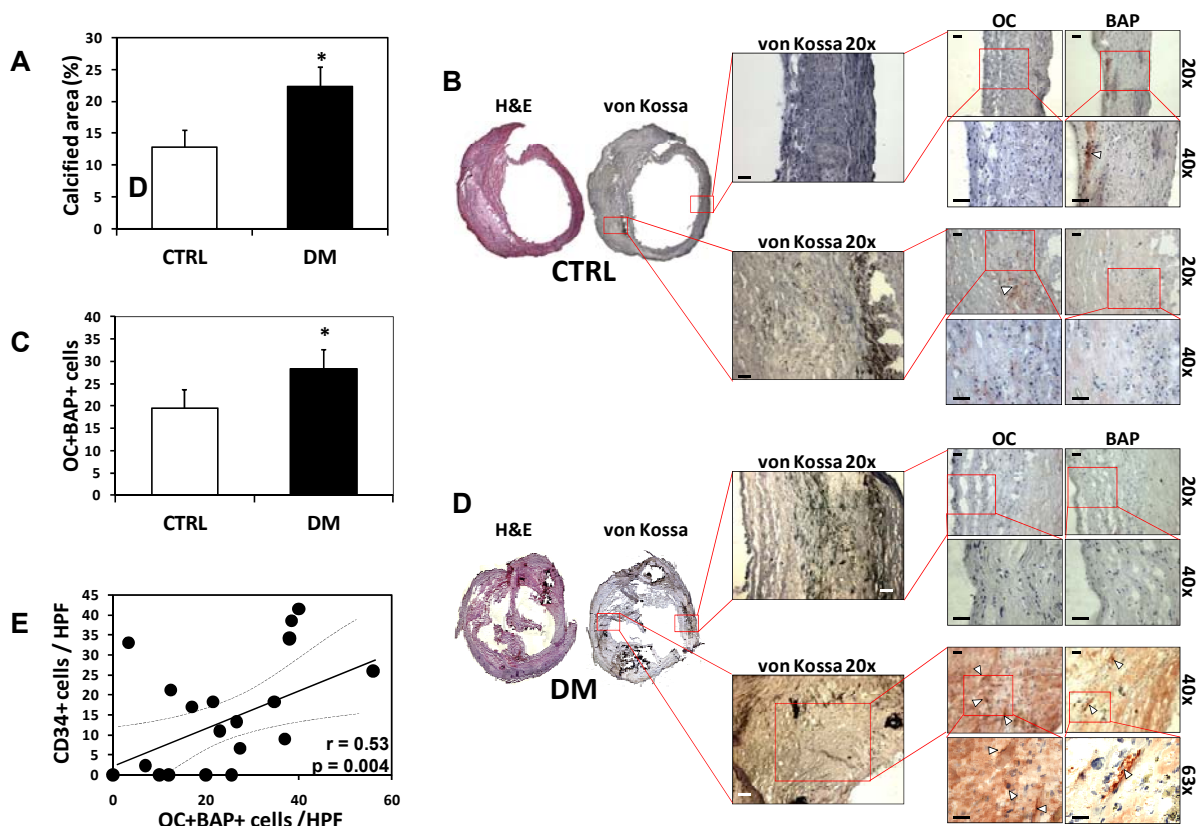
Fig. 12: MCCs in atherosclerotic plaques. A) Calcification was quantified in serial sections of carotid endoarterectomy specimens stained with von Kossa. On average, plaques from diabetic patients showed significantly larger calcified areas (*P<0.05). **B)** Representative histopathology of a calcium-poor specimen from a nondiabetic (CTRL) patient. A few calcified areas are shown in von Kossa X20 in the main lesion shoulder (bottom) and in the far wall (top). Immunohistochemistry shows a few areas staining positive for OC (far wall) or BAP (shoulder) but not both. **C)** The amount of intraplaque OC+BAP+ cells was higher in serial sections of carotid samples from diabetic patients (*P<0.05). **D)** Representative histopathology of a calcium-rich carotid endoarterectomy specimen from a patient with diabetes mellitus (DM). Areas with medial calcification stained with von Kossa in the far wall (top) were negative for OC and BAP, whereas main lesion areas with calcifications were strongly positive for OC and BAP. Some of the staining was extracellular, but distinct OC+ and BAP+ cells could be identified (white arrowheads). **E)** A significant positive correlation was found between calcified area and intraplaque OC+BAP+ cells. Scale bar, 100 μm.
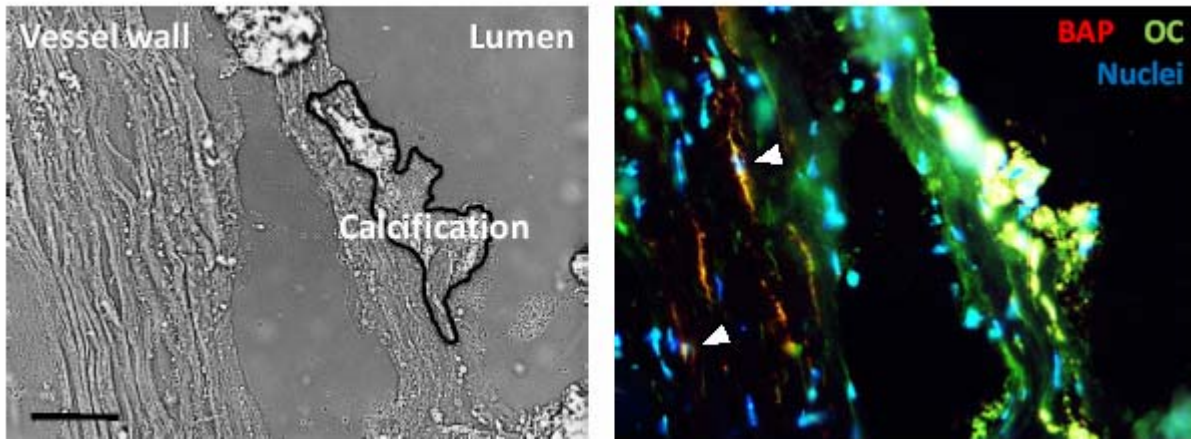
Fig. 13: Fluorescence immunostaining for OC and BAP. Sections of carotid endoarterectomy specimen were examined with phase contrast (left), and fluorescence microscopy after staining for OC (green) and BAP (red). Nuclei are counterstained in blue with Hoechst. The phase contrast image shows anatomy of the section, with identification of the vessel wall and the lumen. A calcified area close to the lumen is bordered. In the immunofluorescence image, this area has strong unspecific fluorescent signal in both green and red channels. In the vicinity of the neointimal calcified area, extracellular OC signal (green areas spatially unrelated cell nuclei) and OC+ cells (green signal in the cytoplasm surrounding nuclei) can be seen. Two OC+BAP+ cells (superimposed red and green signal) are indicated by arrowheads. Scale bar 100 µm.

By quantification in random microscopic fields, OC+BAP+ cells were significantly higher in diabetic versus nondiabetic samples (Fig. 12C). There was also a positive linear correlation between calcified area and the number of local MCCs (Fig. 12D), supporting a possible relationship between MCCs and atherosclerotic calcification.

4.5 EFFECTS OF HIGH GLUCOSE AND HYPOXIA ON MCCs

In separated experiments, MCCs were cultured in the presence of 25 mmol/L glucose or 25 mmol/L mannitol (as osmotic control). Compared with mannitol and control condition (5 mmol/L glucose), high glucose increased the calcified area of MCC culture assessed by von Kossa staining and calcium concentration. When cells were subjected to hypoxia (<1% oxygen tension), calcification by MCCs was increased compared with normoxic control (Fig. 14A and 14C). Interestingly, these stimuli differed in their ability to modulate expression of bone-related genes in MCCs, with high glucose upregulating Runx2 (Fig.10) and hypoxia upregulating Osterix; both induced an increased expression of collagen (Fig. 14B). Stimulation of MCCs with hypoxia induced a further increase in the release of MMP-9 (Fig. 10), which indeed is an hypoxia-sensitive gene and may regulate extracellular matrix remodeling and calcification (Bouvet C. et al., 2008). Although high glucose stimulated BMP-2 gene expression (Fig. 14B), it did not increase secretion of other BMPs (Fig. 10), suggesting that either the effect of high glucose is specific for BMP-2 or the

secretome analysis is not enough sensitive to detect further differences in BMP release. The relationship between hypoxia and MCCs was further explored in atherosclerotic plaques, where hypoxia inducible factor-1α immunostaining was used as a surrogate indicator of local tissue hypoxia. We found that hypoxia inducible factor-1α was more abundant and localized in the necrotic core of plaques with a higher numbers of OC$^+$BAP$^+$ cells (Fig. 14D), suggesting that hypoxia may trigger MCC homing or local differentiation.
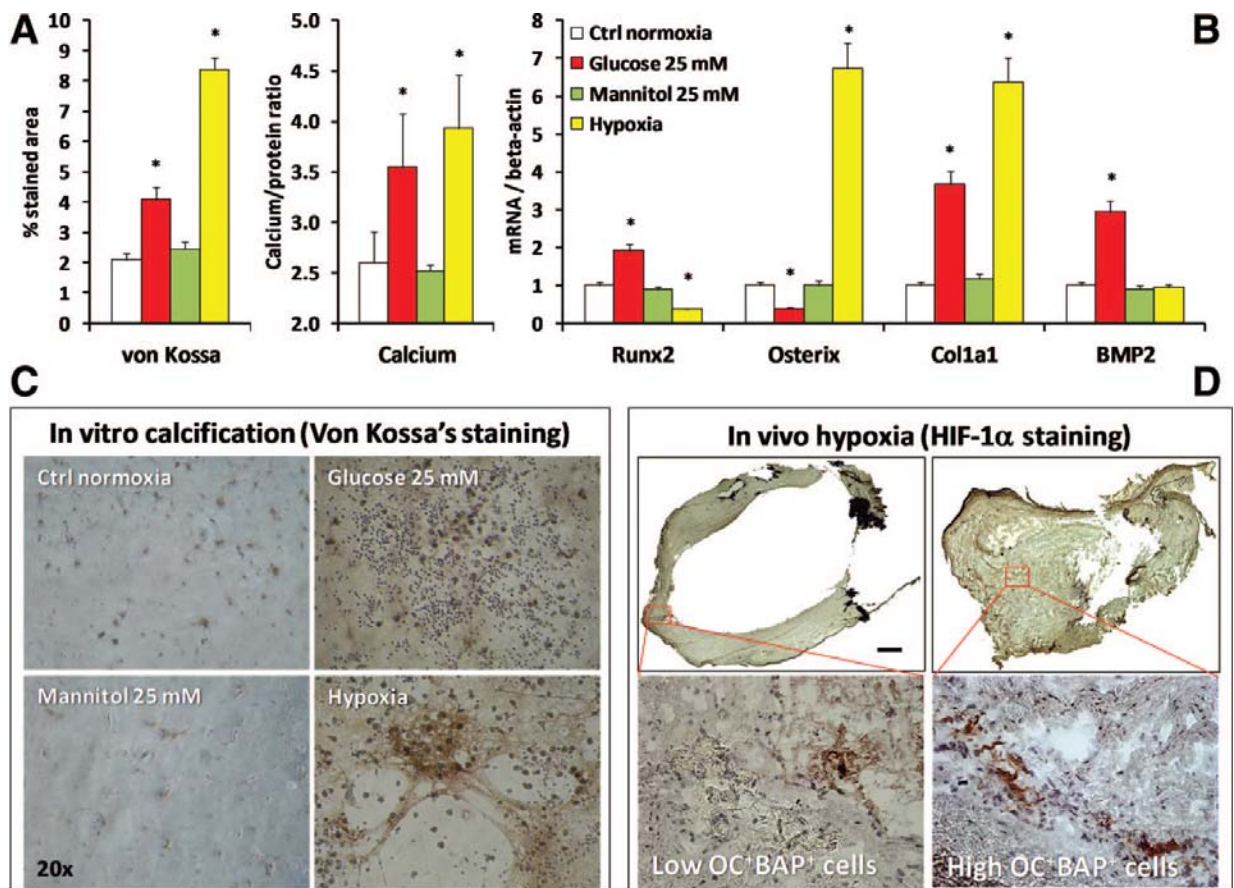
Fig. 14: Effects of high glucose and hypoxia on MCCs. MCCs were cultured in high glucose (25 mmol/L, mannitol 25 mmol/L as osmotic control) or exposed to hypoxia. **A)** Calcification by MCCs exposed to these stimuli was quantified by von Kossa staining and calcium extraction. **B)** Gene expression modulation in MCCs by glucose, mannitol, and hypoxia. **C)** Examples of microphotographs taken showing von Kossa staining from the different conditions. **D)** To understand the relationship between hypoxia and MCCs in vivo, sections of atherosclerotic plaques with low and high OC+BAP+ cells were stained with hypoxia inducible factor-1α (HIF-1α) to show the differential level of tissue hypoxia.

5. DISCUSSION

We demonstrate the existence of a subpopulation of circulating myeloid cells with the ability to promote calcification in vitro and in vivo. We also found that these procalcific cells are increased in type 2 diabetes and may be involved in atherosclerotic calcification.

5.1 CHARACTERISTICS AND ORIGIN OF CIRCULATING CALCIFYNG CELLS

Our data show that procalcific cells can be distinguished from the total mononuclear cell population by the coexpression of OC and BAP, whereas single positivity for either of these 2 markers identify cells with lower calcifying potential. The ability to calcify in vivo was related to expression of bone-related genes, especially the transcription factor Runx2, a master gene regulator of osteogenesis (Lian J.B. and Stein G.S., 2003). The circulating Runx2$^+$ population was enriched in cells expressing OC with or without BAP, but was deprived of the noncalcific OC$^-$BAP$^+$ subset. Thus, presence of OC driven by Runx2 appears to be necessary for acquisition of a procalcific phenotype by blood cells, which is completed by BAP expression. Detailed characterization of OC$^+$BAP$^+$ cells indicated a monocytic phenotype, and expression of BCR-ABL by OC$^+$BAP$^+$ cells from CML patients confirms their myeloid origin. Therefore, we propose the term "myeloid calcifying cells" (MCCs). Given the hematopoietic nature of myeloid cells, MCCs should originate from the bone marrow and, indeed, they were ≈10-fold enriched in bone marrow aspirates compared with peripheral blood. In 2 cases of male-to-female bone marrow transplantation, we found that a fraction of MCCs was of recipient origin years after transplantation, indicating that these cells survived myeloablation and are long-lived. For this reason, it is possible that MCCs belong to the dispersed reticuloendothelial system. We also demonstrate derivation of MCCs from blood monocytes by culture in osteogenic conditions. Cultured MCCs displayed a phenotype quite similar to fresh circulating MCCs (OC$^+$BAP$^+$ cells), upregulation of bone-related genes, and ability to form calcifications in vitro and in vivo. Fresh and cultured MCCs slightly differed in their gene expression profile: BMP-2 was induced only in cultured MCCs, which are pushed toward the procalcific phenotype by the culture medium containing stimuli for BMP-2 production, whereas fresh MCCs derived from a more physiological environment (the bloodstream) had lower concentration of osteogenic stimuli.

The extent to which the phenotype of MCCs overlaps to other pro-calcific cells remains to be elucidated. For instance, the treatment of cultured monocytes with the cathelicidin-derived peptide LL-37 results in the differentiation of large adherent cells that promote the formation of bone-like structures similar to endochondral bone formation when implanted into immunodeficient mice (Zhang Z. et al., 2010). Upon detailed immunophenotypic analysis, these cells, called monosteophils, express OC and other bone-related proteins (but not BAP) and are distinct from osteoclasts, macrophages, and dendritic cells and MSCs. While the status of the research so far does not provide any evidence for circulating monosteophils, culture MCCs share some degree of homology with these cells from morphologic and antigenic perspectives (Fadini G.P. et al., 2011). Rather, the existence of COP within the common leucokyte antigen CD45+ population has been suggested by Egan et al. (Egan K.P. et al., 2011). Circulating CD45+OC+ cells (about 1% of circulating mononuclear cells) express type 1 collagen at high frequency and the homing receptor CXCR4, just like MCCs (Fadini G.P. et al., 2011), and promote calcification in vitro. Upon immunohistochemistry, these cells were present at sites of heterotopic ossification within specimens of diseased aortic valves. Thus, it is possible that bone marrow-derived COP home to the valve leaflets via CXCR4 signaling and contribute to calcification. On the basis of their frequency in the bloodstream, antigenic profile and local tissue contribution, it is highly probable that these cells overlap with MCCs.

5.2 COMPARISON WITH OTHER CALCIFYING CELLS

In a previous study, blood-derived OC$^+$ cells have been shown to differentiate into osteoblasts after culture on fibronectin using a MSC growth protocol (Eghbali-Fatourechi G.Z. et al., 2005). Although it would be surprising that all circulating OC$^+$ cells (up to 5% of blood cells) are MSCs, our data indicate that MCCs are distinct from MSCs and represent a much larger fraction of blood cells (Kosla S. and Eghbali-Fatourechi G.Z., 2006; Roufosse C.A. et al., 2004). In vitro, MCCs display a different behavior compared with MSCs, because they do not adhere on plastic and do not proliferate steadily. MCCs also appear different from osteogenic cells previously differentiated from blood-derived fibroblasts (Choi Y.H. et al., 2010): despite their mesenchymal behavior, fibrocytes may have a myeloid origin (Shirai K. et al., 2009) and are Col1$^+$CXCR4$^+$CD45$^+$CD14$^-$CD68$^-$.

Thus, MCCs appear as a hitherto unrecognized, distinct and larger population of circulating cells with procalcific potential. Chang et al described a discrete subset of resident macrophages expressing bone-related proteins intercalated throughout endosteal and periosteal cells at sites of bone remodeling, where they regulate osteoblast function (Chang M.K. et al., 2008). It remains to be established whether these so-called "OsteoMacs" correspond to circulating MCCs.

5.3 MCCs IN DIABETES AND ATHEROSCLEROTIC CALCIFICATION

MCCs may be generated in different tissues via differentiation of monocyte/macrophages in the presence of an appropriate microenvironment. Because procalcific stimuli occur within atherosclerotic lesions, we wondered whether MCCs are involved in the development of intimal calcification, focusing on diabetic patients, who experience a high rate of vascular disease and are particularly prone to vascular calcification. Circulating MCCs were ≈2- to 3-fold increased in type 2 diabetic patients, especially in the presence of CVD. Interestingly, diabetic patients without CVD had the same level of MCCs as nondiabetic patients with CVD, resembling the pattern of increased CVD risk associated with diabetes (Almdal T. et al., 2004). MCCs were also ≈3-fold increased in bone marrow aspirates of diabetic versus nondiabetic patients, suggesting that the origin of a higher level of circulating MCCs is in the bone marrow. Mechanisms that favor bone marrow abnormalities in diabetes are still unknown, but accumulating data in animals highlight previously unrecognized alterations of the bone marrow microenvironment, with microangiopathy, neuropathy and defective stem cell niche (Busik J.V. et al., 2009; Oikawa A. et al., 2010). Increased bone marrow MCCs may represent another feature of this niche damage. Interestingly, the increase in circulating MCCs was reversible, because optimization of the antihyperglycemic regimen in type 2 diabetic patients was able to reduce MCCs toward the normal range, depending on the achieved reduction of HbA1c. Because MCCs freshly isolated from the bloodstream promote ectopic calcification in vivo, we reasoned that excess circulating MCCs may cause vascular calcification. We found that carotid atherosclerotic plaques from diabetic patients stained stronger for OC and BAP than plaques from nondiabetic subjects. Some cells surrounding calcified areas were frankly OC$^+$BAP$^+$ and may represent local MCCs. Whether these cells truly correspond to circulating MCCs homed to the plaque has not been definitely demonstrated in this study, but the

colocalization of αSMA⁻CD68⁺ areas with OC⁺ and BAP⁺ cells in the neointima supports this hypothesis. In addition, Aikawa et al, using in vivo molecular imaging of apoE$^{-/-}$ murine aortas, demonstrated a spatial association between osteogenic activity and macrophages (Aikawa E. et al., 2007), which may regulate calcification (Tintut Y. et al., 2002). Given that medial calcification is highly prevalent in diabetes, we also evaluated calcified tibial arteries from amputation specimens and found little or no staining for MCC markers (not shown), suggesting that this cell-mediated process occurs preferentially within the atherosclerotic neointima. In future studies, it will be of interest to determine the possible contribution of MCCs to other sites of ectopic calcification in humans, such as heart valves and damaged skeletal muscle.

A possible scenario resulting from our set of experiments is that diabetes increases bone marrow generation and release of MCCs, which home to sites of vascular disease and promote ectopic calcification. There are alternatives to this hypothesis. For instance, OC⁺ and BAP⁺ cells have been found to be higher in humans with osteoporosis or after bone fractures (Eghbali-Fatourechi G.Z. et al., 2005; Pirro M. et al., 2010) which stimulate bone marrow to release cells contributing to bone healing (Kumagai K. et al., 2008; Otsuru S. et al., 2008). We found no correlation between MCCs and markers of bone metabolism; thus, our data do not support that an altered bone microarchitecture triggers an increased demand of MCCs as a physiological skeletal response. A final alternative is that osteogenic stimuli act independently in the bloodstream and within several tissues, leading to an excess differentiation of MCCs from monocyte/macrophages. We have shown that high glucose and hypoxia increase calcification of MCCs in vitro, likely through different mechanisms involving Runx2 and Osterix. Hypoxia, occurring within the core of atherosclerotic plaques, may be one important driver of MCC differentiation acting in association with high glucose to increase MCCs in diabetic patients.

5.4 PATHOPHYSIOLOGICAL AND CLINICAL IMPLICATIONS

The identification of circulating cells harboring an osteogenic potential raises a series of important questions about their pathophysiological role in the bone-vascular axis, their clinical significance and therapeutic potential (Fig. 15).
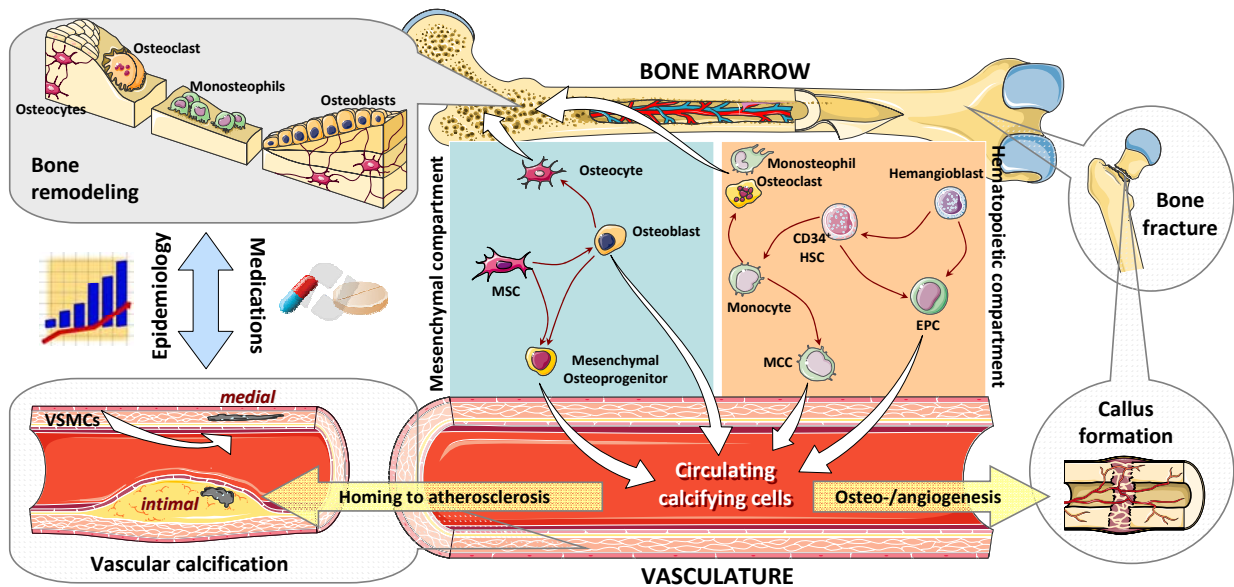
Fig. 15: The role of circulating calcifying cells in the bone vascular axis. The bone marrow mesenchymal and hematopoietic compartments can give rise to osteogenic cells that contribute to the pool of circulating calcifying cells. Bone fracture triggers mobilization of calcifying cells from the bone marrow into the bloodstream. In turn, combined osteogenesis and angiogenesis by calcifying cells and/or endothelial progenitor cells (EPCs) contribute to formation of the bone callus. On the other site, circulating calcifying cells can home to atherosclerotic lesions and contribute to intimal calcification, while medial calcification may arise from transdifferentiation of vascular smooth muscle cells (VSMC). Cells of mesenchymal (osteoblasts/osteocytes) or hematopoietic (osteoclasts and monosteophils) origin contribute to bone remodeling. Epidemiological data link bone remodeling to vascular calcification and animal models show that the two conditions share common drugs targets and effects of medications.

Pathology of the bone-vascular axis (osteoporosis and vascular calcification) is typically associated with aging and could be accelerated in some clinical conditions, such as chronic kidney disease, diabetes and chronic obstructive pulmonary disease (Sabit R. et al., 2007; Demer L. and Tintut Y., 2010; Khosla S., 2011). A common substrate of these clinical entities is systemic inflammation, which drives bone resorption and promotes vascular disease (Khosla S., 2011). BM-derived cells cooperate with parietal progenitors cells in the normal homeostasis of the vascular/valve tissue. However, perturbation of vascular integrity might induce recruitment and engrafting of BM derived-cells as part of a response to injury. Of note, cell tracking studies were not able to identify a contribution of circulating

calcifying cells in medial calcification (Speer M.Y. et al., 2009). On the other hand, a significant participation of circulating calcifying cells in calcium deposition has been described within intimal and valve lesions (Doehring L.C. et al., 2010; Tanaka K. et al., 2005). In contrast to medial calcification, atherosclerosis and aortic valve calcification share inflammatory mechanisms of disease progression. Together with resident cells, circulating calcifying cells might be recruited and deposit calcium in the tissue in an attempt to resolve the inflammation in the vascular/valve wall. From this perspective, it is not surprising that mesenchymal and hematopoietic phenotypes have been described for these circulating cells (Table 1, Fig. 16), as both immune and mesenchymal-derived cells (e.g. fibroblasts) are actively involved in the inflammatory response.

Cell type	Origin	Antigenic phenotype	Calcification in vitro	Calcification in vivo	Vascular Calcification
Mesenchymal osteoprogenitor cells	Mesenchymal stem cell compartment	CD44+ CD105+ CD73+ CD90+ CD34- CD45- CD14-	Yes	Yes	?
CD34+ hematopoietic progenitor cells	Hematopoietic stem cell compartment	CD34+ CD45+ OC+ BAP+	Yes	Yes	Yes
Endothelial progenitor cells	Hematopoietic stem cell compartment	CD34+ KDR+ OC+	Yes	?	?
Myeloid calcifying cells	Hematopoietic compartment	CD14+ CD68+ CD45+ CD34- CD44- CD90- CD29- OC+ BAP+	Yes	Yes	Yes

Table 1: Characteristics of the different circulating calcifying cells populations.
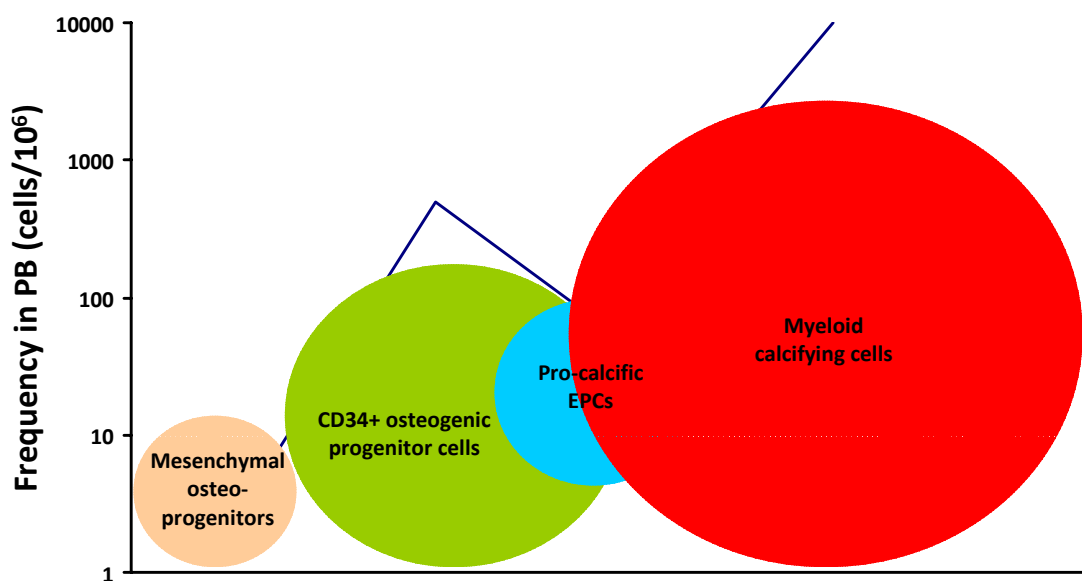
Fig. 16: Frequency and inter-relations of circulating calcifying cells phenotypes. Diameters of the circle are roughly proportional to frequency of each cell type in the bloodstream. Moreover, exact cell frequency is depicted by the line and the logarithmic y-axis scale. A clear overlap exists between CD34+, ECPs and myeloid calcifying cells. For phenotypic characterization see table 1.

The significance of calcifying EPCs may be viewed as a sort of endothelial-to-mesenchymal transition-like phenomenon, typically driven by inflammatory/pro-fibrotic mechanisms. (Gossl M. et al., 2008; Diez M. et al., 2010).

Thus, it is tempting to speculate that systemic inflammation represents a key link between bone disease, circulating calcifying cells and vascular pathology.

In contrast to from aortic stenosis, where calcification is invariably associated with negative clinical outcomes, the significance of intimal calcification is still controversial. While microcalcifications can amplify inflammation and destabilize the plaque, extensive calcium deposits have been associated with a stable plaque phenotype (Ehara S. et al., 2004). Clarification of the role of circulating calcifying cells in the different phases of atherogenesis is mandatory in order to establish whether or not they represent a target for therapeutic interventions. Additional studies are also needed to determine their clinical utility for prediction of future cardiovascular disease, as prospective data are currently lacking. It appears that all these aspects are of great relevance to establishing whether or not circulating calcifying cells could be also safely used for bone-healing interventions. As mentioned above, Matsumoto and colleagues have reported successful outcomes when utilizing CD34+ cells/EPCs for fracture healing (Hajdu Z. et al., 2011; Tanaka K. et al., 2005; Egan K.P. et al., 2011; Wylie-Sears J. et al., 2011). Based on the pre-clinical study, they have initiated

a phase I/IIa clinical trial of autologous local transplantation of G-CSF mobilized CD34-positive cells for patients with tibial or femoral nonunion. The first case exhibited a promising result (Kuroda R. et al., 2010), but efficacy of this cell therapy should be further elucidated in a randomized controlled clinical trial.

6. CONCLUSIONS

With ineluctable aging of the general population, it is of paramount importance to identify mechanisms that link together distant end-organ damage due to the senescence process. Mounting evidence support the existence of circulating calcifying cells of mesenchymal and hematopoietic origin. By trafficking through the bloodstream, cells with an osteogenic potential are prime candidate regulators of the bone-vascular axis, with potential implications in bone remodeling and vascular calcification. While the ability of these cells to promote calcification in vitro is established, their activity in vivo is not fully elucidated, especially in relation to vascular biology. Data so far available trace the way for future development of this area of research, which has the potential to identify a novel pathophysiological link between bone and vascular disease, as well as a possible new therapeutic target.

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