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Contribution of Interstitial Valve Cells to Aortic Valve Calcification

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Ai miei genitori,

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Abbreviations

AB: apoptotic bodies
ACE: angiotensin-converting enzyme
ADMA: asymmetric dimethylarginine
ALP: alkaline phosphatase
AngII: angiotensin II
ApoE-/-: apoliproteinE deficient mice
AT1: angiotensin receptor-1
aVIC: activated VIC
BMI: body mass index
BMP: bone morphogenic proteins
BVIC: bovine interstitial valve cells
CAD: coronary artery disease
Cbfa-1: core binding factor alfa-1
Chm-I: chondromodulin-I
CVC: calcifying vascular cells
DDAH: dimethylarginine dimethylaminohydrolase
EC: endothelial cells
ECM: extracellular matrix
EMT: endothelial mesenchymal transition
Ennp1: ectonucleotide pyrophosphatase/phosphodiesterase1/PC-1
Ennp1-/-: Ennp1 deficient mice
ESRD: end-stage renale disease
eVIC: embryonic VIC
FGF-23: fibroblast growth factor-23
FGF-23-/-: FGF-23 deficient mice
HA: hydroxyapatite
Hsp: heat shock protein
ICAM-1: intracellular adhesion molecule-1
IL: interleukin
LDL: low density lipoprotein
LDLr-/-: LDL receptor deficient mice
LPS: lypopolisaccaride/endotoxin
Lrp5: LDL receptor-related protein5
MGP: matrix-gla protein
MGP-/-: MGP deficient mice
MMP: matrix metalloproteinase
Msx2: Msh homeobox 2
MV: matrix vescicles
NAFTc1: Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NMM: non muscle myosin
NO: nitric oxide
NOS: nitric oxide synthase
Notch1: Notch homolog 1, translocation-associated
obVIC: osteoblastic VIC
OC: osteocalcin
ON: osteonectin
OPG: osteoprotegerin
OPG-/-: OPG deficient mice
OPN: osteopontin
OPN-/-: OPN deficient mice
oxLDL: oxidized LDL
Pi: inorganic phosphate
PPi: inorganic pyrophosphate
pVIC: precursor VIC
qVIC: quiescent VIC
RANK: receptor activator of NF-kB
RANKL: receptor activator of NF-kB ligand
RANKL-/-: RANKL deficient mice
RAS: renin-angiotensin system
ROS: reactive oxygen species
Runx2: runt-related transcription factor 2
SMA: smooth muscle α-actin
SMC: smooth muscle cells
SMemb: embryonic smooth muscle heavy chain
SMM: smooth muscle myosin
SOD[Cu-Zn]: superoxide dismutase [Cu-Zn]
Sox-9: SRY-box 9
TGF-β: transforming growth factor-β
TIMP: tissue inhibitor of metalloproteinases
TLR: toll-like receptor
TNFα: tumor necrosis factor-α
VCAM-1: vascular cell adhesion molecole-1
VEC: valve endothelial cells
VEGF: vascular endothelial growth factor
VIC: valve interstitial cell
vWF: von Willebrand Factor

1. Riassunto

Introduzione. Tradizionalmente la calcificazione valvolare aortica viene considerata un processo distrofico, ad evoluzione lenta e non modificabile. Tale visione è stata recentemente messa in discussione da evidenze che sottolineano l'importanza, nel corso della calcificazione vascolare, di un bilanciamento fra fattori promuoventi ed inibenti, nonché del ruolo svolto da processi cellulo-mediati. I lembi valvolari aortici sono popolati da cellule interstiziali valvolari (VIC) fenotipicamente eterogenee, il cui contributo specifico nel corso dei processi degenerativi della valvola è solo parzialmente conosciuto.

Scopo. Scopo del presente studio è quello di ricercare e caratterizzare una sottopopolazione aortica di VIC in grado di acquisire un fenotipo pro-calcifico in seguito ad esposizione a fattori patogeni (quali endotossina [LPS] e fosfato inorganico [Pi]).

Metodi e Risultati. VIC ottenute da espianti di valvole aortiche bovine (BVIC) sono state sottoposte ad un processo di clonazione, mediante tecnica di diluizione limite. I cloni di BVIC sono stati caratterizzati sotto il profilo morfologico ed immunofenotipico mediante l'utilizzo di marcatori tipici per cellule mesenchimali, cellule muscolari lisce (SMC), cellule endoteliali, cellule ematopoietiche e cellule di derivazione ossea. Fra i 40 cloni di BVIC ottenuti sono stati selezionati 4 cloni, morfologicamente rappresentativi delle diverse popolazioni isolate, che mostravano diverse caratteristiche di crescita e di profilo immunofenotipico. Sia la popolazione cellulare di VIC non clonate che i cloni non mostravano la tendenza a fenomeni spontanei di calcificazione *in vitro*. Le cellule sono state quindi trattate con diverse combinazioni di LPS (100 ng/ml) e Pi (2.4 mmol/L concentrazione finale) per 12 giorni. La popolazione non clonale di BVIC ha mostrato un progressivo incremento nei livelli di espressione della fosfatasi alcalina (ALP) dopo trattamento con LPS, mentre la deposizione di calcio è stata osservata solo nelle cellule

trattate con la combinazione di LPS e Pi. Fra i diversi cloni solo il *Clone 1* (fenotipo simil-fibroblasto) ha mostrato un significativo incremento nei livelli di espressione dell'ALP. Tale incremento si accompagnava ad un'aumentata espressione di osteocalcina (OC) e ridotto accumulo di α-actina muscolare liscia (SMA), come documentato da studi in western blotting e citofluorimetria. Il trattamento con LPS non è stato in grado di indurre modifiche simili nel profilo fenotipico del *Clone 4* (fenotipo muscolare liscio differenziato). Nonostante il significativo incremento nell'espressione di ALP ed OC, il *Clone 1* non ha prodotto fenomeni di calcificazione della matrice dopo trattamento con la combinazione di LPS ed Pi. Tuttavia, aspetti di calcificazione sono stati osservati in esperimenti di co-coltura del *Clone 1* e *Clone 4,* quando trattati con la combinazione di LPS e Pi. Inoltre, dopo semina su spugne di collagene di tipo I, il *Clone 1* si è dimostrato in grado di produrre estesi fenomeni di calcificazione della matrice, in seguito a trattamento per 12 giorni con LPS e Pi. Tale combinazione ha indotto solo minimi aspetti di calcificazione nella matrice di collagene popolata dal *Clone 4*. Fenomeni apoptotici sono stati osservati nel *Clone 1* seminato nelle spugne di collagene e trattato con LPS e Pi. Viceversa, nel caso del *Clone 4* non sono stati documentati aspetti apoptotici. L'analisi proteomica della frazione citosolica del *Clone 1* ha permesso di identificare 34 proteine i cui livelli di espressione si modificano con l'acquisizione del profilo pro-calcifico. Fra queste proteine è stata documentata una significativa riduzione nei livelli di molecole antiossidanti, come la superossido dismutasi [Cu-Zn] e la tioredoxina. Un significativo decremento è stato osservato anche per i livelli di dimetilarginina dimetilaminoidrolase (DDAH), un enzima intracellulare che degrada la dimetilarginina asimmetrica (ADMA) (inibitore dell'ossido nitrico sintetasi [NOS]). In linea con questi dati è stato osservato un aumento della produzione di specie reattive dell'ossigeno (ROS) da parte del *Clone 1* trattato con LPS.

Conclusioni. I risultati di questo lavoro dimostrano che le popolazioni clonali di BVIC sono dotate di un diverso potenziale pro-calcifico quando stimolate con uno stesso fattore patogeno. In particolare, è stata identificata una specifica sottopopolazione di BVIC, caratterizzata da un profilo fenotipico simil-fibroblasto, che si è dimostrata in grado di esprimere marcatori osteogenici e di calcificare matrice di collagene, in risposta a trattamento con endotossina ed alti livelli di fosfato inorganico.

2. Summary

Background. The traditional view of aortic valve calcification as a slow, ineluctable event has been recently questioned by evidence showing the importance of a balance between promoting and inhibiting factors, and the relevance of osteogenic cellular-driven processes. The aortic valve leaflets are comprised of a heterogeneous population of interstitial cells (VIC) whose specific contribution to the degenerating valve has not been defined yet.

Aim. The major aim is to identify and describe the phenotypic characteristics of a subpopulation of aortic VIC able to acquire a pro-calcific profile when exposed to pathological stimuli (such as endotoxin [LPS] and inorganic phosphate [Pi]).

Methods and Results. Explants-derived primary bovine VIC (BVIC) were cloned by a limited dilution technique. Characterization of BVIC clones was performed by morphological and functional as well as immunophenotyping using markers specific for mesenchymal cells, smooth muscle (SM) cell lineage, endothelial cells, hematopoietic cells, and bone formation process. Among the 40 BVIC clones obtained we selected four clones, morphologically representative of the isolated populations, which displayed different growth pattern and immunophenotype. Both uncloned and cloned cell populations grown on plastics did not show a spontaneous tendency for calcification in the standard medium and were, hence, treated with different combinations of LPS (100 ng/ml) and Pi (2.4 mmol/L, final concentration) for 12 days. Uncloned BVIC showed a progressive increase of alkaline phosphatase (ALP) activity after treatment with LPS that resulted in calcium deposition after addition of Pi. Among the clones, only *Clone 1* (fibroblast-like phenotype) showed relevant increase in ALP after LPS treatment, which was paralleled by an increased osteocalcin (OC) expression and prevention of smooth muscle (SM) α–actin (SMA) accumulation, as demonstrated by western blotting and cytofluorimetry analysis. The same

treatment had no effect on *Clone 4* cells that showed a more stable SM cell-like phenotype. Despite ALP activity and OC increase *Clone 1* cells did not undergo calcium deposition after treatment with LPS in long-term culture supplemented with Pi. However, mineralization was observed in co-culture of *Clone 1* and *Clone 4* treated with LPS plus Pi. Moreover, when cells of *Clone 1* were grown on type-I collagen sponges and treated with Pi alone or LPS plus Pi for 12 days we observed an extensive mineralization of the collagen-matrix. Instead, only modest calcium deposition was observed in collagen scaffolds seeded with *Clone 4* treated in the same way. A high degree of apoptosis was documented in *Clone 1* cells seeded in the collagen scaffolds and treated with LPS plus Pi. No apoptotic degeneration was observed in *Clone 4* cells. The proteomic analysis of the cytosolic fraction of *Clone 1* cells allowed the identification of 34 proteins which levels of expression were modified with the acquisition the pro-calcific profile. Among these proteins we documented a significant decrease in the expression of antioxidant proteins, such as superoxide dismutase [Cu-Zn] and thioredoxin, together with a downregulation in the level of dimethylarginine dimethylaminohydrolase (DDAH), an intracellular enzyme that degrades asymmetric dimethylarginine (ADMA) (a nitric oxide synthase inhibitor [NOS]). In line with these findings we observed that LPS treatment of *Clone 1* cells was accompanied by increased reactive oxygen species (ROS) production.

Conclusion. The results of this study demonstrate that BVIC clonal subpopulations are endowed with different calcifying potential when stimulated with the same pathogenic factors. In particular, we identified a specific BVIC subset harbouring a fibroblast-like phenotype that express osteogenic markers and promote collagen-matrix calcification in response to endotoxin and elevated phosphate levels.

3. Introduction

Calcific aortic stenosis is the most common valvular heart disease.[1, 2] Once become clinically evident this condition is characterized by a poor prognosis unless patients undergo valve replacement. Unfortunately, most of the mechanisms that drive calcific degeneration of aortic valve leaflets are still incompletely understood, and for this reason no effective therapies are available to slow the disease progression.

The traditional view of valve calcification as a progressive, ineluctable event has been recently questioned by evidence showing the importance of a balance between promoting and inhibiting factors and the relevance of cell-driven processes.[3] Although dystrophic calcification represents the main pathological finding, aspects of mature lamellar bone and endochondral bone formation have been described in the calcified aortic valves.[4] Moreover, analysis of calcified leaflets have shown the expression of bone-related proteins (such as osteopontin [OPN], osteocalcin [OC], alkaline phosphatase [ALP] activity, osteoprotegerin [OPG], RANKL, BMPs), chondro/osteoblast-specific transcription factors (such as Runx2/Cbfa-1 and Sox9) and mediators involved in osteogenic programs (such as Wnt/Lrp5/B-catenin pathway).[4-7] On the whole, these data suggest that processes resembling molecular patterns involved in bone remodelling can take place during aortic valve calcification.

Recent epidemiological and basic science studies suggest that inflammatory molecules, modified lipids, infectious agents, circulating levels of calcium and phosphate could act as potential promoters of valve calcification.[3, 8, 9] Some of these factors have been shown to promote the acquisition of an osteogenic profile by interstitial valve cells (VIC). These are the most prevalent cells in the valve and play a crucial role in maintaining the structural integrity of the leaflets. However, VIC biology, pathobiology and phenotype-

related functions are poorly understood. In particular, we do not know whether specific VIC subsets are endowed with an inherent or inducible pro-calcific potential. If this is the case, the identification of the cell types involved in matrix mineralization, coupled with the elucidation of the mechanisms driving calcium deposition, could represent valuable tools for the development of innovative treatment strategies to prevent calcific aortic valve degeneration.

4. Epidemiology of aortic valve calcification

Calcific aortic stenosis can be observed in 2-4% of the population over 65 years of age, is the third common cause of cardiovascular disease, and represent the most common acquired valvular disease in the industrialized countries.[1, 10, 11] Symptomatic calcific aortic stenosis usually represents the final stage of a slow, progressive disease that in the early phase is characterized by mild valve thickening without significant hemodynamic effects. This initial disease of the valve, called aortic sclerosis, might variably progress towards calcific aortic stenosis, which is characterized by different stages of severity (mild, moderate and severe).[2] As expected, aortic sclerosis is more common than severe aortic stenosis (about 25% of people between 65 and 74 years of age) and is echocardiografically identified as a focal thickening of the valve leaflet that does not impair its mobility.[2] Thus, in patients with aortic sclerosis, the hemodynamic is normal and no clinical symptoms are manifest. However, some important epidemiological studies demonstrated that the presence of aortic sclerosis is accompanied by an increased risk of coronary events.[1, 12, 13] Reasons for this association are still elusive, and the hypothesis of a direct pathogenic role of valve thickening (such embolism or hemodynamic effects) can be considered unlikely. Vice versa, it is plausible that aortic sclerosis might represent a surrogate marker for systemic pro-inflammatory/pro-atherosclerotic process that involves also the coronary vessel wall. In agreement with this theory, several studies have shown an association between the traditional atherosclerotic risk factors and those predicting the onset and progression of aortic stenosis.[11, 14] One of the first study investigating the risk factors associated with aortic valve replacement demonstrated the association of the disease with male gender, hypertension, total triglyceride level, total serum cholesterol level, smoking, diabetes mellitus, and angiographic coronary artery disease.[14] The Cardiovascular Health Study

(including 5621 adults over 65 years) confirmed this findings showing that older age, male gender, smoking, hypertension, LDL and Lp(a) emerged as risk factors associated with the presence of aortic sclerosis.[11] In the following years other population studies confirmed this data and demonstrated the implication of traditional cardiovascular risk factors for the development of aortic stenosis.[15-17]

Only few prospective studies investigated the rate of progression from aortic sclerosis to aortic stenosis. In one of these studies Cosmi et al. observed that the average time after diagnosis for progression from aortic sclerosis to aortic stenosis was 8 years.[18] In this population, of more than 2000 patients, 16% of them developed aortic stenosis (10.5% mild stenosis, 3% moderate stenosis and 2.5% severe aortic stenosis).[18] These findings were confirmed by a smaller study showing a 5% rate of progression towards moderate stenosis, and 2.5% developing severe aortic stenosis.[19] Even if marked individual differences can be observed, once aortic stenosis is identified, the hemodynamic progression of the disease can be estimated in about an increase in aortic jet velocity of 0.3 m/s per year, an increase in mean transaortic pressure gradient of 7 mmHg per year, coupled with a decrease in aortic valve area of 0.1 cm^2 per year.[20-22] The clinical factors predicting the progression of the disease are not so well established, and discrepancies can be documented among the different studies (mainly based on retrospective analysis). As for the prevalence of aortic sclerosis/calcified valve, also the rate of progression has been linked with the presence of traditional atherosclerotic risk factors, such as high LDL, male gender, smoking, hypertension, elevated BMI, diabetes and features of metabolic syndrome.[15-17, 23-26] However, despite several common risk factors a discrepancy exists between the presence of calcific aortic stenosis and coronary artery disease (CAD). In fact, only about half of the patients with aortic stenosis have significant CAD and the majority of patients with CAD do

not have aortic stenosis. These data suggest that additional factors, different from those involved in atherogenesis, might be implicated in the pathogenesis of valve calcification.[27]

The presence of bicuspid aortic valve is considered an important risk factor for the future development of aortic stenosis. Roberts et al. demonstrated that about 50% of the nonreumathic aortic stenotic valves removed at surgery are congenitally bicuspid.[28] This percentage is even higher when looking at the morphology of the calcific valves removed at younger ages (about 70% of patients between 40-60 years of age). The prevalence of the bicuspid valve in the general population is about 1-2%.[29] The majority of patients with bicuspid valve have relatively normal valve function and remain undiagnosed until late in adulthood, when stenosis develops because of superimposed leaflet calcification. Even if the cellular and molecular mechanisms involved in the calcification of the bicuspid aortic valve are thought to be similar to the process within the trileaflet valve,[30] patients with bicuspid valve exhibit an accelerated progression of the calcific disease.[29] Abnormalities in hemodynamic forces and excessive shear/mechanical stresses may account for the increased risk of calcification observed in these patients. However, the recent identification of genetic factors implicated in both the valve development abnormalities and severe aortic stenosis,[31] suggest that perturbation of some biological pathways might be of importance for the high susceptibility towards calcification of the bicuspid valve (see below).

Patients with symptom-free severe aortic stenosis have a low rate of overall mortality, with less than 1% of the patients that experience sudden death.[32-34] However, after 4 to 5 years about only 30% of the patients remain symptom-free. Predictors of symptom onset included baseline jet velocity, the rate of change in jet velocity over time and the extent of valvular calcification.[33, 34] Angina, heart failure, syncope, decrease exercise tolerance and exertional dyspnea represent cardinal symptoms of severe aortic stenosis. Less

than 20% of the patients are alive 5 years after the onset the symptoms without undergoing valve replacement.[35] After cardiac surgery the long-term survival is about 80% at 3 years, with a postoperatively age-corrected survival that is nearly normalized.[36]

Nowadays surgical therapy remains the only treatment option for patients with severe symptomatic aortic stenosis. It appears that the identification of promoters, cell types and molecular pathways involved in the calcific degeneration of the valves is fundamental for the development of medical therapies able to slow disease progression. However, to meet this scope we need a better definition of the pathological determinants and the mechanisms driving the process of calcium deposition in the valve.

5. Aortic valve developmental biology and functional structure

5.1 The developing aortic valve

The developmental process driving valvulogenesis is characterized by complex spatio-temporal sequences of events that include: i) extracellular matrix (ECM) organization and remodelling, ii) VIC phenotypic maturation, iii) ECM and VIC compartmentalization. After the formation of the heart tube, composed of an outer layer of myocardium and an inner layer of endothelial cells (EC) separated by the cardiac jelly, the initial step driving valve morphogenesis is the formation of the cardiac cushions.[37] The cardiac jelly overlaying the outflow tract expands and protrudes from the myocardium giving rise to the precursor of the future aortic valve leaflets. This initial stage is characterized by the endothelial mesenchymal transition (EMT) of a specific subset of EC. These cells delaminate from the endothelium and penetrate the cardiac jelly, where proliferate and complete the mesenchymal differentiation (Figure 1).

This process is driven by several intracellular pathways, cellular-matrix interactions, and production of soluble factors which create a complex signalling network, including: i) Notch1 and Wnt/β-catenin-driven EMT,[38, 39] ii) BMP/TGF-β regulation of cardiac cushion formation and EMT initiation,[40, 41] iii) NAFTc1-mediated proliferative response of EC,[42] iv) ErbB integration of ECM signals, favouring cells migration into the cardiac jelly,[43] v) neurofibromin/Ras feedback control of EMT, limiting endothelial and/or mesenchymal cell proliferation.[44] All these signals modules act in a coordinate spatio-temporal sequence that has not been completely clarified. However, the initial steps seem to be driven by Notch1 signalling that activate the specific subset of EC undergoing EMT. The cells transdifferention is also promoted by Wnt/β-catenin signalling that controls growth factors production (such as BMPs), and matrix remodelling (i.e versican and hyaluronic acid).

TGF-β and BMP signaling favour the EMT, promoting delamination and migration of the cells inside the cardiac jelly. Through NAFTc1, VEGF signalling prevents excessive EC transdifferation and increases EC proliferation to replace the cells undergoing EMT. The cells migration is also promoted by the hyaluronic acid present in the ECM, through ErbB2/3 heterodimers. The process can be limited by neurofibromin that block NAFTc1 and Ras, controlling both the extent of EMT and endothelial repopulation (Figure 1).[37] Recently, also periostin has been shown to be involved in the post-EMT phase of valvulogenesis, controlling fibroblasts differentiation and collagen synthesis.[45] Even scleraxis has been shown to play a crucial role during post-EMT remodelling for the correct ECM organization and VIC compartmentalization.[46] Final steps of valve remodelling are not known but apoptotic signals can play a role. The analysis of Tie2-cre X ROSA26R mice developing valves confirmed that the majority of aortic VIC are endothelial in origin,[47] while neural crestal-derived cells can populate the outflow tract and contribute to the aortopulmonary septation.[48]

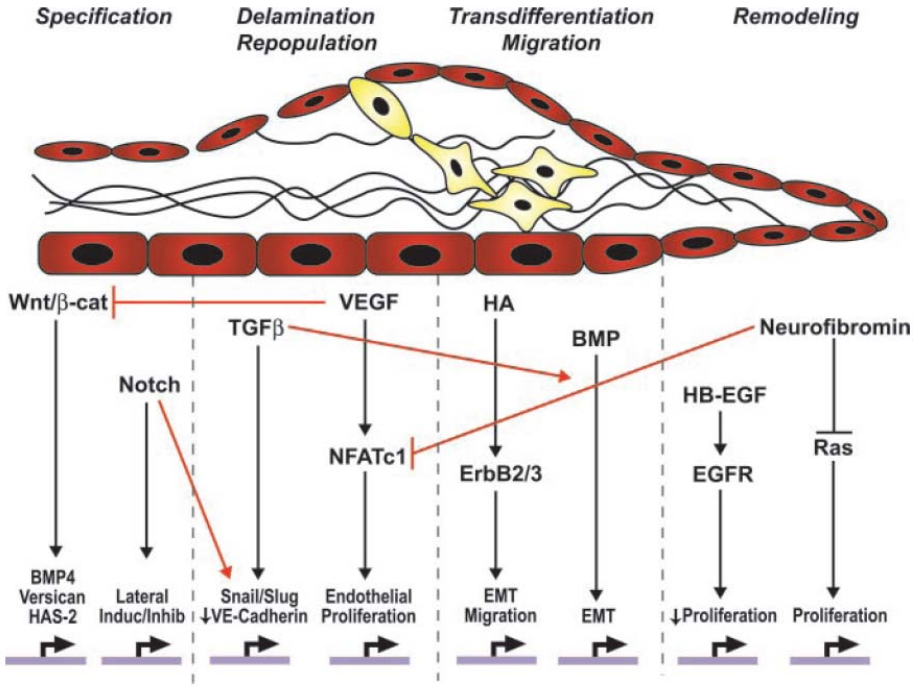

Figure 1. Signaling network model for heart valve development and remodeling (taken from Armstrong et al.).[37]

At the end of the valve maturation the leaflets consist of three well-organized layer called *fibrosa*, *spongiosa* and *ventricularis*, which are different in the cell types content (see below) and ECM composition. The *fibrosa* (aortic side of the leaflet) is a dense layer, containing circumferentially arranged type-I collagen fibers, while the *ventricularis* (ventricular side of the leaflet) is mainly composed of radially orientated elastin fibers. The *spongiosa*, which represents the central layer, is mainly compromised of loose connective tissue containing proteoglycans. Except for some differences in collagen and elastin content, this organization and composition of the valve ECM is maintained across species, including sheep, chicken, rabbit and mouse.[49] ECM stratification is observed late in embryonic remodeling and continues in the postnatal life. During cushion formation and elongation, ECM is mainly composed of proteoglycans. Collagen content can be observed in the *fibrosa* during leaflet remodeling, while elastin appears relatively late in the *ventricularis*. Human valves of fetus between 20 to 39 weeks show a bilaminar structure, containing some elastin in the *ventricularis* and unorganized collagen in the *fibrosa*. The trilaminar structure appears more evident in child, but remains still incomplete compared to the morphology of adult valve leaflets.[50]. After the separation of pulmonary and systemic circulations, happening at birth, the aortic valve is exposed to higher blood pressure, which can play a role in completing valve morphogenesis promoting further collagen and elastin accumulation. Thus, ECM stratification begins at later stages of embryonic development and become more organized during postnatal life

During cardiac cushion formation and elongation, the cells involved in the process show a uniform density and distribution. Differences in cells allocation can be observed at later stages, when ECM stratification appears more evident.[49] With the progression of gestation, human valves show a decline in cell density and proliferation index, a

phenomenon continuing in postnatal life. On the other side, the rate of apoptosis appears to be low throughout life.[50] The majority of VICs populating the fetal valves show an activated/immature myofibroblasts phenotype (expressing α–SM-actin [SMA] and embryonic smooth muscle heavy chain [SMemb]), are distributed homogenously in the leaflet, and express high level of matrix metalloproteinases (MMP-1 and MMP-13). On the contrary, the expression of these markers is almost absent in the adult valve.[50] Thus, during fetal development, VIC are endowed with an activated profile, which could be of great importance for adequate ECM production and remodeling. Phenotypic changes in VIC profile observed in the postnatal life can be partly driven by the changes in the hemodynamic forces. Differently from VIC, valve endothelial cells (VEC) density seems to be constant during the different stages of valve development.[49] However during fetal life VEC show an higher expression of adhesion molecules (such as ICAM-1, VCAM-1), SMemb and MMP compared to VEC of normal adult valve.[50] The meaning and importance of VEC activated profile for normal valvulogenesis is still unclear.

5.2 The adult aortic valve

As mentioned above, only in the postnatal life the three layers compartmentalization of the aortic valve leaflet is completed. This structural organization of the valve is functional to the relevant changes in size and shape that the valve faces during the cardiac cycle. The high content of collagen in the *fibrosa* layer provides strength and stiffness that can withstand high tensile forces and help the valve to maintain coaptation during diastole. On the ventricular side the elastin fibers recoil during the systole and extend in diastole, while the glycosaminoglycans present in the *spongiosa* help to absorb shears and shocks, facilitating the relative arrangement of collagen and elastin fibers during the cardiac cycle. It

appears that the quantitative and qualitative preservation of ECM architecture inside the leaflet is of paramount importance for the adequate functionality and durability of the valve. Every heartbeat provokes shocks and stresses that may induce microscopic forces inside valve leaflets translating in complexes cell-matrix interactions important for the preservation of normal valve function. Nevertheless, abnormalities in the valve stress state can activate cell-mediated molecular processes representing the basis for reparative and pathological remodeling of the valve.

VIC is the term conventionally used to identify the cells resident in the native normal aortic leaflet. These cells are distributed throughout all the three layers and participate in ECM matrix remodelling, maintaining valve functional and structural integrity. However, till now the biology of the VIC has been poorly clarified. Several differences can exist among the VIC subsets resident in the valve in term of phenotypic profile and participation to reparative/pathological processes inside the leaflet. A first evidence of potential diversity among the VIC arises from studies investigating the immunophenotypical profile of cells resident in the different layers of the leaflet. In fact, studies conducted in humans and other species (such as rabbit and pig) showed different phenotypic profile of VIC populating the three layers.[51, 52] In particular cells resident in the *fibrosa/spongiosa* layers mainly harbour a fibroblast-like profile, being positive for non-smooth muscle myosin (NMM) and vimentin but negative for differentiated smooth muscle markers (such as SMA and smooth muscle myosin [SMM]). Only sparse cells in these two layers of the normal valve are found to be positive for SMA and can be identified as myofibroblasts. On the other side, a high percentage of VIC populating the *ventricularis* layer express mature smooth muscle markers (such as SMA and SMM).[51, 52] The identification in the normal valve of these three immunophenotypical profile (fibroblasts/myofibroblasts/smooth muscle cells [SMC]) has

not been translated yet in biological information about the role played by these cell types during normal/reparative and pathological remodeling of the valve.

In a recent review, Liu et al.[53] proposed a classification of VIC based on the potential cell functions in normal valve physiology and pathological processes. The quiescent VIC (qVIC) might represent the majority of the cells populating the normal valve. These VIC harbour a fibroblast-like phenotype, have a low proliferation rate and participate in maintaining the normal physiology of the valve. The activated VIC (aVIC) are cells expressing higher level of SMA compared to qVIC, suggesting the acquisition of a myofibroblast profile. These cells might proliferate and contribute to ECM remodeling during repair and pathological processes. A phenotypic transition can probably occur from qVIC to aVIC, as a response to pathological stimuli and abnormal mechanical stresses. It is also plausible that the aVIC, in certain condition could acquire again the quiescent profile.

The origin of VICs populating the adult valve is still controversial. Quiescent VIC are probably mainly derived from cells undergoing EMT during valve development (see above). The contribution of precursor cells (pVIC/eVIC) in maintaining the qVIC number after the end of valvulogenesis is not defined. Some recent studies suggest that circulating bone-marrow derived cells and progenitor resident cells can be identified in the valve leaflet.[54] For instance, Visconti et al.[55] demonstrated that bone-marrow derived circulating cells can enter the native valve, express mRNA for procollagenα1 and differentiate into cells similar to those residents in the leaflet. The presence of progenitor cells has been also demonstrated inside the human pulmonary valve.[56] Using a clonal approach, Parachuri et al.[56] showed that a specific subset of VIC, expressing both CD31 (endothelial marker) and SMA, possess the plasticity of differentiating in EC (after treatment with VEFG) or in mesenchymal cells (after stimulation with TGF-β2). However, our knowledge about the contribution of these

pVIC to normal valve physiology or pathological process is still elusive. In particular, we need to clarify whether these cells recruited from the bloodstream or locally "activated" could transdifferentiate in aVIC during physiological and pathological valve remodeling. As described in detail below, recent studies conducted in the calcified valve leaflets hypothesize that cells acquiring an osteoblast-like profile could be actively involved in the calcium deposition inside the pathological leaflets. These data suggest that a subpopulation of VIC, named osteoblastic VIC (obVIC), might be activated under pathological condition and drive ECM mineralization. However, to date, no definitive prove exists demonstrating that obVIC might represent a separate population of VIC.

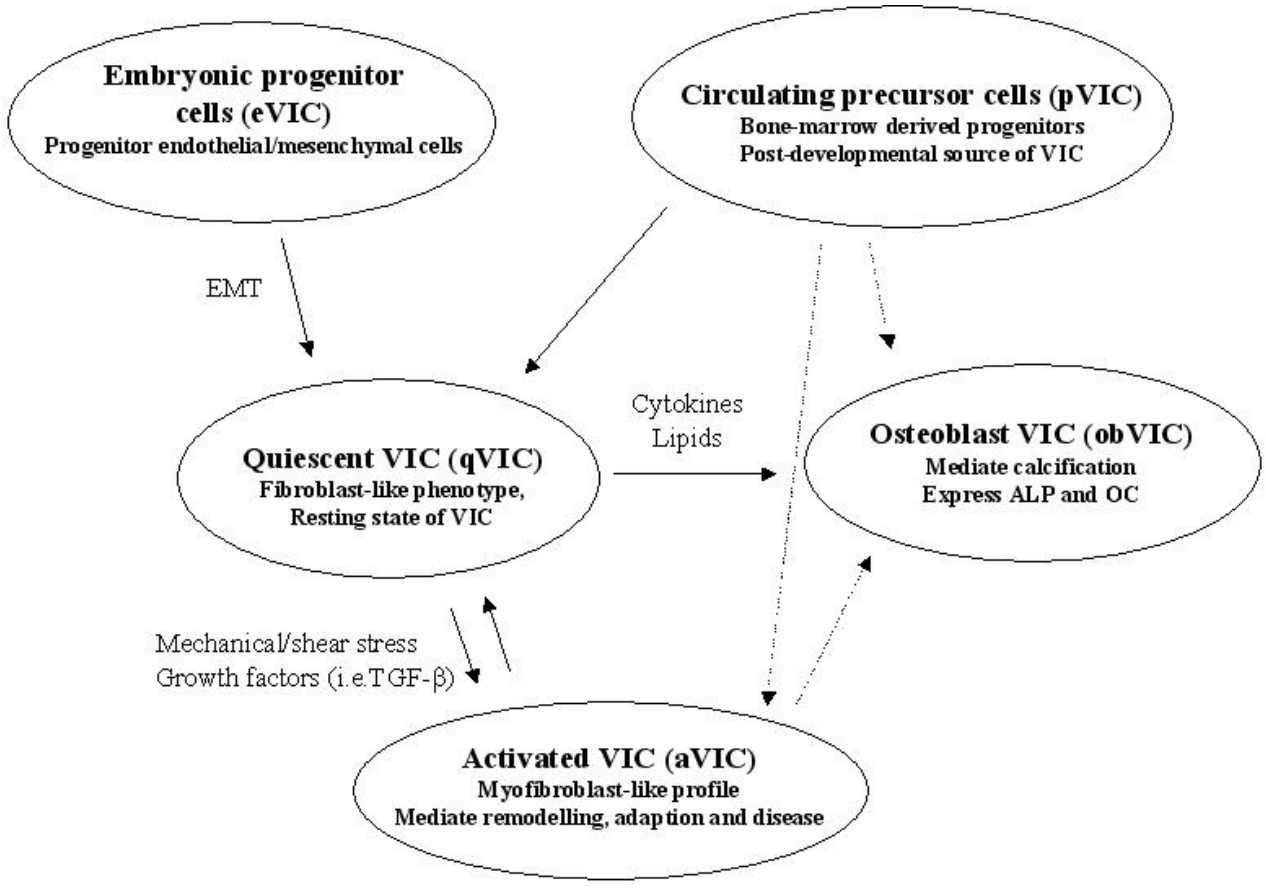

Figure 2. Proposed classification of VIC phenotypes (modified from Liu et al.).[53]

VIC have been shown to produce both MMP (-1,-2 and –9) and TIMP (-1 and -2).[57-59] The expression of MMP during fetal life is suitable for a correct development of the valve structure, while during the postnatal life the expression of inhibitors (TIMPs) might prevent adverse effects of MMP on valve structural integrity. In fact, VIC are capable of expressing not only MMP, but also collagenases and stromelysins, thus showing an intrinsic ability to remodel ECM.[58, 60] This faculty can translate, during pathological processes, in excessive valve damage, eventually leading to valve dysfunction.

Both sides of the leaflets are lined by EC, called VEC. At a first glance, structural and functional properties of VEC appear to be similar to those of EC resident elsewhere in the vascular system. However, recent evidence suggest that VEC might possess peculiar phenotypic characteristics compare to EC populating other vascular sites.[61] If this is the case, the study of VEC functional properties could unveil important information on the interplay between VIC and VEC in maintaining valve integrity. For instance, porcine aortic VEC have been shown to align perpendicularly to the flow, while other vascular EC align in a parallel way.[62] Moreover, VEC are different from the other EC in term of transcriptional gene profile when exposed to the same mechanical stress. A recent study conducted in the porcine valve also showed a different expression of calcification-related genes between the VEC populating the two sides of leaflet.[63] This observation suggests a possible role for VEC in modulating behaviour of underlying VIC during pathological processes.

6. Mechanisms of vascular calcification

Vascular calcification can be histopathologically classified into four main types: i) atherosclerotic calcification, ii) medial artery calcification (including the Mönckeberg sclerosis tipical of diabetic patients), iii) cardiac valve calcification, and iv) calciphylaxis (extensive mineralization of arterioles mainly observed in patients with the end-stage renal disease, [ERDS]).

Different hypotheses have been postulated about the mechanisms that start and drive the process of vascular calcification,[64, 65] including both passive and active phenomenon. Recent studies underline a complex regulation of the calcification process based on a potential balance between promoting and inhibiting factors. Other evidences suggest that calcium deposition in the vascular system should be considered a cell-driven process, and that some cellular elements could recapitulate morphological and functional mechanisms typical of bone cells. Promoters of calcium deposition have been identified among cytokines (such as TNFα, IL-6, TGF-β, RANKL),[66-69] adipokines (namely, leptin),[70] morphogenic proteins (such as BMP-2, Wnt/β-catenin),[6, 71, 72] modified lipids,[73] drugs (such as warfarin),[74, 75] and others circulating factors (most importantly, inorganic phosphate [Pi]).[76] At the other side, calcification may be started by a partial or complete loss of inhibitory factors, such as Fetuin-A,[77] OPG,[78] inorganic pyrophosphate (PPi),[79] and Matrix-Gla Protein (MGP),[80] which may occur in the bloodstream or locally in the arterial wall. On these bases four nonmutually exclusive theories can be proposed to summarized the complexity of the mechanisms driving calcium deposition in the vascular wall (Figure 3).[65]

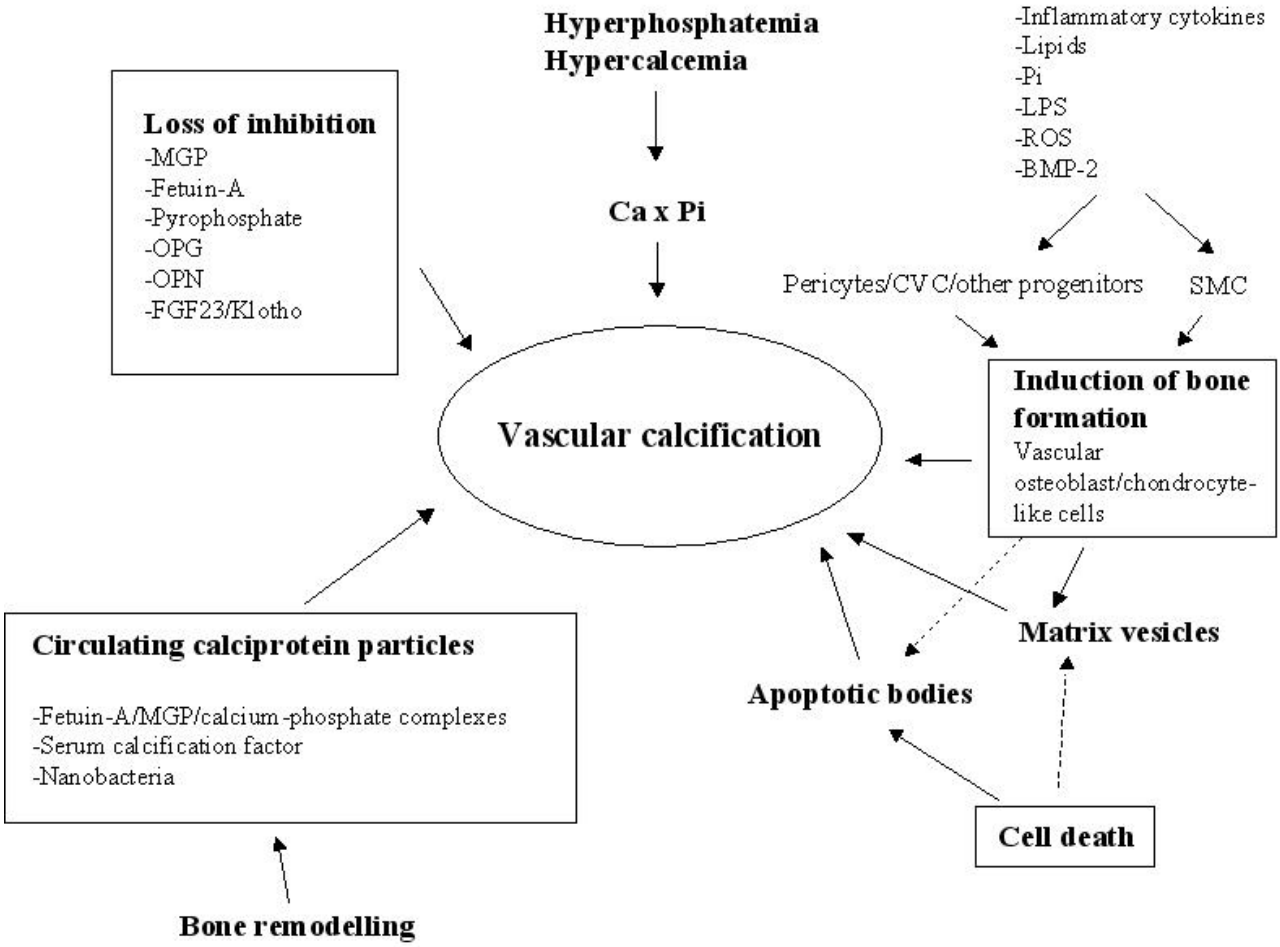

Figure 3. Mechanisms of vascular calcification (modified from Speer and Giachelli).[65]

6.1 Induction of bone formation

The presence of ossified bone within plaques and the expression of osteogenic cell makers have been reported by several studies conducted in both humans and animal models of atherosclerosis. This includes the expression of BMP-2 and -4, osteonectin (ON), OC, OPN, bone sialoprotein, Runx2/Cbfa-1,OPG, RANK, RANKL, MGP, ALP, type II collagen and others.[81-85] All are molecules that are expressed during normal bone development and play a crucial role during the process of bone remodeling. However, it is not completely defined yet which are the cells expressing these markers and which of these molecules can actually play a role in driving or inhibiting calcium deposition in the vessel wall. We recently observed that the advanced atherosclerotic lesions in the innominate arteries of

chow-fed ApoE deficient mice (ApoE-/-) become highly calcified.[86] Inside the atherosclerotic lesions of this animal model of atherosclerosis the wide spread deposition of hydroxyapatite (HA) is preceded by the formation of fibro-fatty nodules populated by so-called chondrocyte-like cells, that are spatially associated with the early stages of calcification.[86] It appears that chondrocyte-like cells are responsible for depositing the HA via a process that may recapitulate the cellular and temporal aspects of endochondral ossification. This conclusion was supported by i) the temporal and spatial association between the chondrocyte-like cells and HA deposits, ii) the concordance in the patterns of expression of osteogenic markers by cells within the developing bone and the advanced atherosclerotic lesions, iii) the presence of active ALP in the chondrocyte-like cells within and adjacent to areas of calcification iv) electron microscopic data showing matrix vesicles (MV) derived from the chondrocyte-like cells containing electron dense crystalline material consistent with HA. Further studies conducted in the same and other animal models of atherosclerosis (such as the LDL receptor deficient mice [LDLr-/-]), confirmed this observation. [87-89] On the whole these data suggest that during atherogenesis some cellular elements of unknown origin can acquire an osteogenic phenotype and drive the process of calcium deposition. At least two main hypotheses delineate the cellular participation in calcium deposit formation: i) recruitment and activation of progenitor cells with osteogenic potential, located in the vessel wall or in the bloodstream; ii) local trans-differentiation of mature SMC toward a chondro/osteogenic phenotype. In fact, several evidence from *in vitro* studies and some *in vivo* observations suggest that a crucial aspect of the vascular calcification may be the conversion of vascular SMC towards an osteoblast/chondrocyte-like phenotype. This phenotypic switch of the SMC results *in vitro* in the deposition of calcium, loss of expression of SMC markers (such as SMA and SM22), and increase

expression of markers typical for osteoblast/chondrocyte cells (such as Runx2/Cbfa-1, OPG and ALP).[66, 76, 90, 91] Jono et al. showed that this effect can be obtained by treating the SMC with Pi,[76] while other studies showed that also inflammatory mediators (such as LPS, TNF-α, IL-6 and other cytokines) can play a role in promoting or accelerating the phenotypic change of the SMC.[66, 92] The importance of cell-driven processes during vascular calcification has been recently underlined also by studies conducted by Towler's group.[72] They elegantly demonstrated the crucial role played by the Msx2/Wnt signalling pathway for the activation of an osteogenic program in the vascular wall of LDLr-/- mice fed high-fat diet.[93] These authors postulated that BMP-2, TNF-α and reactive oxygen species (ROS) can induce the Msx2 (an homeodomain transcription factor) activation in adventitial myofibroblasts, which then favour calcium deposition at the media level through paracrine Wnt3a and Wnt7a signaling.[72] Indeed, the common mesenchymal derivation of vascular and bone cells suggests that SMC/adventitial cells could maintain some degree of the phenotypical plasticity, similar to mesenchymal progenitors. The existence of mesenchymal-derived progenitor cells with osteogenic potential in the vessel wall has been suggested by the seminal works of Demer's group.[68] These authors identified the so-called calcifying vascular cells (CVC), a subpopulation of aortic bovine SMC that spontaneously calcify *in vitro* and retain a mesenchymal multilineage commitment.[94] Remarkably, these cells shared several phenotypic similarities with bovine microvascular pericytes which, as described by Canfield, represent another mesenchymal subpopulation with the ability to differentiate into osteoblasts, chondrocytes and adipocytes.[95] Of note, pericytes, besides being recruited at sites of neoangiogenesis, are thought to participate actively in ectopic calcification.[96] Nevertheless, the relative contribution to calcium deposition inside the

arterial wall of progenitors/pluripotent cells *vs* transdifferation of SMC/adventitial cells is not established.

6.2 Loss of inhibition

Recent evidence suggest that the arterial wall normally express a series of molecules that, together with some circulating factors, can act as inhibitors of calcium deposition. The lack or the decrease production of these molecules can lead to a "loss of inhibition", with subsequent spontaneous calcium deposition in the vascular wall. Several studies conducted in animal models allow the identification of proteins whose absence is accompanied by calcium deposition in the arterial wall and other sites.

MGP is a γ-carboxylated protein express in cartilage, lung, heart, kidney and arteries that act as inhibitor of soft-tissue calcification.[97] MGP deficient mice (MGP-/-) die within six to eight weeks for rupture of large arteries due to extensive mineralization of elastic lamellae.[80] The histological analysis of the arteries of the MGP-/- mice also shows the presence of chondroplastic metaplasia inside the medial layer, suggesting that MGP can modulate the phenotypical behaviour of SMC. MGP could be of importance for the maintenance of SMC contractile profile, blocking their trans-differentiation into chondrocyte-like cells. In agreement with this hypothesis, it has been shown that MGP could interact with BMP-2 preventing its osteo-inductive effects on vascular SMC.[71, 98] Besides acting modulating SMC profile, MGP might exert the anti-mineralization effect also by binding to elastin, masking mineral nucleation sites[99] and blocking further crystal growth.[74] In any case, protective effects of MGP seem to be attributable to local vascular actions, as increased systemic level of the protein do not inhibit arterial calcification.[100]

MGP circulate in the blood in a complex with Fetuin-A, calcium and phosphate.[101] Fetuin-A, also known as α2-HS-glycoprotein, is another inhibitor of calcium precipitation. In fact, Fetuin-A deficient mice develop soft tissue calcification in several districts including the arterial wall.[77] Several clinical studies, mainly conducted in patients with ESRD, showed that low serum levels of Fetuin-A are linked with higher vascular calcification and correlate with increased risk of cardiovascular death.[77, 102] The physiological role of the circulating MGP/Fetuin-A/calcium-phosphate complex is still unclear. It could be part of the circulating calciprotein particle system linking bone metabolism and the vascular system (see below).[103]

Indeed, some epidemiological studies have shown that atherosclerosis and osteoporosis are linked, and that bone loss is accompanied by an increase in cardiovascular events.[104, 105] For this reason, in the last few years, the study of bone biology has been of increasing importance in vascular biology and vice-versa. In this context, an emerging field of research is represented by the study of the OPG/RANK/RANKL axis. The RANKL/RANK interaction that occurs in the bone promotes the complete development of multinucleated bone-resorptive osteoclasts from monocytic precursors. OPG acting as a decoy receptor and interacting with RANKL inhibits osteoclast differentiation and activation. Imbalances in the RANKL/OPG ratio and its effect on RANK signaling appear to underlie the pathology of bone diseases characterized by bone loss, such as osteoporosis.[106] For example, the OPG deficient mouse (OPG-/-) is characterized by an osteoporotic phenotype due to excess bone resorption by osteoclasts,[78] whereas the RANKL-/- mouse exhibits osteopetrosis.[107] Interestingly, OPG-/- mice also develop extensive medial calcification of blood vessels with no evidence of atherosclerosis.[78] Even if the exact biological roles of OPG, RANK and RANKL in regulating vascular calcification

are still unclear, this and other evidences suggest an emerging role for these molecules in the vascular system.[108] OPG is constitutively expressed by EC, SMC in a wide range of tissues (heart, veins, arteries), whereas RANKL and RANK are frequently undetectable in normal vessels and noncalcified arteries or valves.[109] RANKL and OPG have been detected in early and advanced atherosclerosis lesions both in human and animals models of atherosclerosis.[81, 86] The original observation that OPG inactivation promotes vascular calcification has been confirmed in the setting of atherosclerosis development by Bennett et. al., showing that the OPG – ApoE double deficient mice experience a higher degree of calcium deposition in the vasculature compared to the ApoE-/- control mice.[110] In addition, more recently, also OPG injection has been shown to reduce calcification of the atherosclerotic lesions in LDLr-/- mice, with no significant modification in the lesion size.[88] How OPG exerts protective effect on calcium deposition inside the arterial wall is not defined yet. In particular, we do not known whether OPG is able to modulate phenotypical behaviour of cells acquiring a pro-calcific profile or if its protective role should be ascribed to the block of detrimental RANKL activities. In fact, the latter has been shown to promote the acquisition of an osteogenic phenotype in medial adventitial cells[109] and valve cells.[69] In agreement, we also documented that the expression of RANKL is increased within atherosclerotic plaque showing high degree of calcification.[89]

Among the possible contributors to the bone-vascular axis an increasing interest is now focusing on the role of the Fibroblast Growth Factor-23 (FGF-23)/Klotho pathway. FGF-23 is a bone-derived circulating peptide protein controlling renal phosphate excretion and Vitamin D metabolism. Increased levels of FGF-23 determine hypophosphatemia and reduction in serum concentrations of $1,25(OH)_2D$, vice-versa FGF-23 gene deletion in mice (FGF-23-/-) determines hyperphosphatemia and abnormal bone development.[111, 112] In

addition, FGF-23-/- mice also show an extensive soft-tissue and vascular calcification. Interestingly the phenotype seen in these mice is similar to that observed in the Klotho deficient mice (Klotho-/-).[113] Klotho has been recently identified to be a co-receptor essential for FGF-23 biological function. In fact, despite high serum levels, FGF-23 cannot exert its phosphaturic effect in the Klotho-/- mice.[114] On the whole, these initial observations suggest that FGF-23/Klotho activity can slow vascular calcification by negatively impacting the serum phosphate balance.

Osteopontin is a glycoprotein normally expressed in bone that has been shown to play a role in regulating the process of mineralization. Besides acting as modulator of osteoclast function, both *in vivo* and *in vitro*, OPN inhibits HA crystal growth. OPN is expressed by SMC, EC and macrophages and is abundant at sites of calcification in human atherosclerotic plaques and in calcified aortic valves.[85, 115] In the atherosclerotic lesions of the ApoE-/- mice the chondrocyte-like cells show the same pattern of OPN expression seen in the bone, staining positive for OPN only when associated with areas of calcification.[86] In this regard, the combined deletion of ApoE and OPN has been reported to increase calcification of atherosclerotic plaques in 60 week old mice.[116] These results suggest that OPN may act locally to inhibit calcification through its ability to bind HA and reduce crystal growth. The protective effect of OPN on ectopic mineralization has been confirmed by Steitz et al., in a subcutaneous implantation model, showing that glutaraldehyde-fixed porcine aortic valve leaflets explanted from OPN deficient mice (OPN-/-) exhibited greater calcification compared to leaflets implanted in OPN wild types.[117] In addition, MGP-/-OPN-/- mice showed a higher degree of vascular calcification compared to the MGP-/-OPN+/+ mice.[118]

The activity of ALP is generally used to demonstrate cell osteogenic differentiation in studies of vascular calcification.[66] ALP is needed to prevent the inhibitory effect on calcium deposition by PPi, a small molecule constitutively produces in the vascular cells by the ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1) also known as PC-1.[119] The latter is a plasmamembrane glycoprotein that plays a key role in modulating orthotopic and heterotopic calcification. Deficiency of PC-1 causes a rare disorder called "idiopathic infantile arterial calcification", which is characterized by the early development of extensive medial calcification in large arteries with massive hyperplasia due to proliferation of SMC.[120] This human model suggests that PPi could also act as a phenotypic modulator of SMC. Consistent with this hypothesis, mice lacking PC-1 (Ennp1-/-) develop cartilaginous metaplasia in the medial layer, mediated by the lost inhibitory effect of PPi.[121] The optimal concentration of PPi is ensured by a balance between PC-1 and ALP activity, thus we could postulate that a concomitant increase in ALP activity may antagonizes the production of PPi, determining a parallel increase in Pi availability for modulation of SMC phenotype and calcium deposition.

Other molecules now under investigation for their potential inhibitor effects on vascular calcification include: carbonic anhydrase II,[122] desmin,[123] Smad6,[124] and Abcc6.[125]

6.3 Cell death

Cell death has been long considered as the major contributor to dystrophic calcification observed in atherosclerotic lesions and calcified valves. The dying cells can become permeable to calcium and phosphate which concentrate inside the cells overcoming their solubility product and favouring the nucleation of crystals. Moreover, phospholipids from membranes of the dying cells may represent sites for heterogeneous nucleation and/or

epitactic growth of calcium phosphate crystals.[126] Other events associated with calcification are secretion of MV from living SMC[91] as well as release of apoptotic bodies (AB) by dying cells.[127] Morphological and structural differences between AB and MV are not completely defined, even if both of them have been shown to be elective sites for initial nucleation of crystals. The presence of MV has been related to calcium deposition in both human plaques and animal models of atherosclerosis.[86, 128, 129] Moreover, *in vitro* evidence suggests that SMC can release MV which then play a role in driving matrix mineralization.[91] Elegant studies by Anderson and others showed that MV should be considered highly specialized organelles containing a variety of proteins associated with the mineralization process, including: ALP, the calcium binding proteins annexins II, V and VI, ATPase, the type III sodium-dependent Pi transporter and PC-1.[79, 130] The refined machinery characterizing the MV underlines the complexity of biochemical/biological processes driven by these organelles at the early stages of calcium deposition. Which are the factors that promote and regulate the release of MV from SMC acquiring a pro-calcific profile is not defined. In vitro studies have also confirmed the important role of AB bodies released by dying SMC in initiating mineralization. In fact, when SMC are grown in culture they could form multicellular, calcific nodules enriched in AB.[127] It is unclear whether AB carry the same biological complexity of MV in regulating mineral deposition. However, several *in vitro* studies demonstrated that blocking apoptotic pathway can prevent calcium deposition.[127, 131] In addition, an elegant study by Clarke et al. showed that *in vivo* induction of apoptosis can accelerate the calcium deposition inside the intimal lesions of ApoE-/- mice.[132] Interestingly, the induction of apoptosis was not accompanied by calcification of the normal arterial wall, suggesting that cellular or microenviromental phenomenon typical of atherogenesis are needed to allow calcium deposition. In a recent paper, Shroff et al.

showed that SMC apoptosis can be observed in arteries obtained from dialysis patients, and that areas of apoptosis co-localized with region of calcium deposition.[133] Moreover, electron microscopy analysis demonstrated that SMC in dialysis vessels showed apoptosis and damage characterized by increased electron density of nuclear heterochromatin, cell shrinkage, and/or vesicle release. Vesicles containing microcrystalline structures consistent with HA were deposited in the ECM and their size range (0.1 to 1.0 μm) was consistent with their derivation from both AB and plasma membrane budding of MV.[133] Further studies are needed to clarify the relative contribution of AB and MV to vascular calcification and their relationship with the acquisition of an osteoblast-like profile by the vascular cells.

6.4 Circulating calciprotein particles

In the last few years some authors postulated the presence of circulating nucleational complexes, release during bone turnover, whose serum levels correlate with calcification of the vascular wall.[134] This theory is supported by the observation that, under some circumstances, a complex of calcium-phosphate mineral phase and proteins (Fetuin-A and MGP) is released from bone and can be detected in blood.[103] The bone-origin of this complex is confirmed by the observation that the treatment with inhibitors of bone resorption (such as bisphosphonates and OPG) can reduce its circulating levels.[103, 135] The physiological functions of this high molecular weight circulating calciprotein (CCP) complex is currently unknown. In particular, it is not defined where the circulating complex is retained (in the bone itself or in other tissues), and how it might be metabolized and excreted by the kidney. It would be tempting to speculate that Fetuin-A may serve for the transport of small mineral complexes in the same way as apolipoproteins are fundamental for the transportation of water-insoluble lipids.[136] In theory, CCP may form spontaneously

and be present in all tissues, but their circulating levels can be particularly increased during bone resorption by osteoclasts. In particular, during certain pathological conditions, their increase in circulating levels could overwhelm the clearance capacity of the reticuloendothelial system and the kidney. Thus, the combination of increased serum levels and low phagocytosis can result in ectopic calcification.[136] Several studies are needed to confirm this hypothesis and clarified the mechanisms. Nevertheless, Price's group demonstrated the existence of a serum calcification activity, highly preserved among species, that consist of one or more proteins (50 to 150 kDa in size).[137, 138] The nature of this serum nucleator is currently undefined and further researches should clarify how the activated nucleator is able to produce apatite-like crystals at the concentrations of calcium and phosphate in serum.

An integrated overview about the mechanisms driving calcium deposition in the vascular system should take into consideration the relative contribution of all the factors listed above. It is plausible that the acquisition of an osteoblast/chondrocyte-like profile by cells recruited or resident in the arterial wall is accompanied by a complete or partial loss in the expression of physiological inhibitors by the calcifying cells itself or the "adjacent" vascular elements. In addition, perturbance in circulating factors homeostasis (increased of pro-calfic factors and decreased of inhibitors) may overwhelm the defensive capacities of the vascular system, eventually leading to calcium-phosphate deposition. Cell death pathways may be part of the differentiation processes driving the acquisition of the pro-calcific profile by vascular cells. Alternatively, cell debris, AB and MV may simply furnish the initial nucleation site for HA crystal. In a situation of anti-calcific defences impairment

these crystal may growth without hindrance along the collagen and elastin fibers of the arterial wall (these concepts and the mechanisms potentially involved are summarized in figure 4).[64] Additional studies are needed to complete this picture and identify further "bad" and "good" players in the process of vascular calcification.

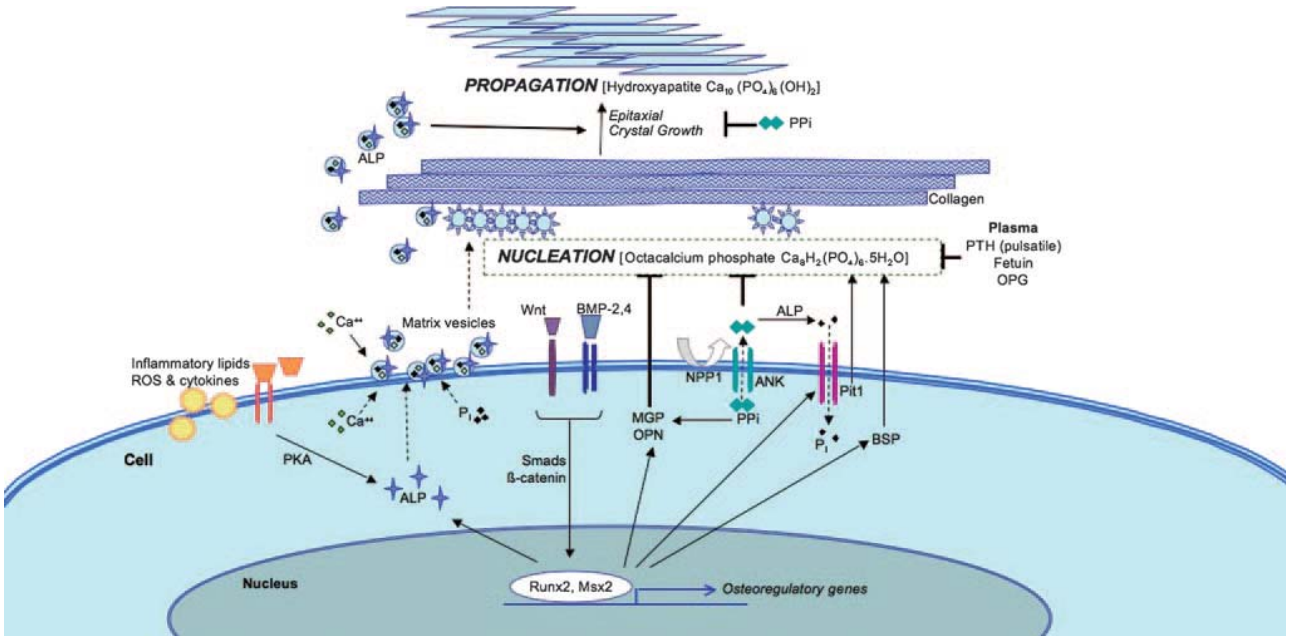

Figure 4. Overview of the mechanisms potentially involved in controlling calcium deposition inside the arterial wall (taken from Demer and Tintut).[64]

7. Pathogenesis of aortic valve calcification

Traditionally, calcium deposition in the aortic valve has been considered an ineluctable event, mainly driven by passive, dystrophic phenomenon. However, as mentioned above, recent evidences underline the complexity of the mechanisms driving vascular calcification, including calcium deposition in the aortic valve. Histopathological analysis of valve tissues, experiments on animal models, and *in vitro* data obtained from VIC cultures, suggest that "active", cell-mediated processes can be of pathophysiological relevance for calcium deposition in the valve leaflets.

Histopathological analyses of early lesions of the valve (aortic sclerosis) have shown that the disease usually start on the aortic side of the leaflet and extend to the *fibrosa/spongiosa* layers.[139] This initial stage is described as a subendothelial thickening and is characterized by accumulation of modified/oxidized lipoproteins (including Lp(a)), inflammatory cells (macrophages, lymphocytes, mast cells) and formation of calcified microscopic foci.[54, 139-142] Inflammatory cells are usually located near the surface of the lesion, while initial foci of calcification are deeper.[139] As it happens during atherogenesis, VEC damage/dysfunction represent an initiating factor for lipid infiltration beneath the lamina elastica, followed by recruitment of inflammatory cells.[143] The expression of adhesion molecules (such as VCAM-1) on the VEC's surface might favour monocytes/lymphocytes recruitment. LDL and Lp(a) are retained in the valve leaflets probably interacting with proteoglycans, and their trapping might favour oxidative modification.[3] The importance of high lipid levels in promoting valve disease has been recently confirmed by studies conducted using animal models of atherosclerosis, such as ApoE-/- mice,[143, 144] LDLr-/- mice,[145] and New Zealand White rabbits fed high-cholesterol diet.[146] In these animals, the hyperlipidemia promotes structural changes of the leaflets

(such as thickening and calcification), which are accompanied by VEC activation and inflammatory cells accumulation. However, the effects of lipids accumulation and modification on VIC phenotypic behaviour are not defined. Only one *in vitro* study showed that treatment of VIC with oxLDL promotes the formation of cellular calcified nodules.[147] Lipoproteins retained in the ECM of the pathologic valve has been shown to co-localize with enzymatically active Angiotensin-Converting Enzyme (ACE),[148] suggesting the local production of Angiotensin II (AngII). Other sources of AngII can be probably identified in macrophages expressing ACE and mast cell chymase.[148, 149] Angiotensin receptor-1 (AT1) is not express by VIC in the normal valve, but its presence can be detected in the pathological leaflets.[149] However, the impact of cellular mechanisms driven by renin-angiotensin system (RAS) activation during valve calcific degeneration is not known.

In the advance stages of aortic valve calcification, calcium deposits can be identified in the form of different combination of calcium-phosphate, including HA, the form of mineral presents in the bone. In addition, up to 13% of advanced calcified valve contain lamellar or endochondral bone tissue including in some cases haematopoietic marrow.[4] In the last few years, histopathological and gene expression analysis of calcified valve tissues demonstrated the expression in the pathological valve of bone-related proteins, such as ALP, OPN, OC, BMPs, RANKL, Sox-9, tenascin-C, and Runx2/Cbfa1.[4-7, 143] Caira et al.[6] demonstrated that also the Wnt/Lrp5/β-catenin pathway, usually activated during bone remodelling, is upregulated in the human calcified valve. On the whole, these data suggest that active biological processes, resembling those happening during bone formation, can be involved in calcium accumulation inside the aortic valve. The presence in the valve of a subset of VIC able to acquire an osteoblast-like profile has been first proposed by Mohler et al.[147] Using VIC obtained from human bicuspid valve and canine aortic valve, these authors

demonstrated the formation *in vitro* of calcified nodules expressing BMP-2 and ALP. In the following years other studies confirmed that VIC, stimulated with oxLDL, TGF-β, LPS, BMP-2, IL-1 can express bone-related proteins and induce calcium deposition.[131, 147, 150, 151] As it has been shown for SMC, also VIC-induced calcification depends on the increase in ALP activity.[152] However, we are still lacking in a comprehensive phenotypical characterization of the VIC acquiring the pro-calcific profile. In particular, we do not actually know whether a specific subset of VIC exists in the valve that, under pathological stimuli, can undergo osteoblastic differentiation and drive calcium deposition. This specific subpopulation, if present, could be identified as osteoblastic VIC (obVIC)[53] and become a preferential target for development of future therapeutic strategies. Additional information are needed to define the relationship between obVIC and other VIC, in particular we do not known whether obVIC represent a separate subgroup of qVIC, and how these cells are related to the aVIC or the pVIC (see Figure 2). Inflammatory mediators, modified lipids and other factors (such as infectious agents or Pi) might preferentially drive the acquisition of the osteoblast-like profile by specifically targeting this subset of VIC.

As mentioned above, macrophages are recruited within the pathological valve, and could represent the source of inflammatory mediators (such as cytokines), MMP, and ROS, which have been all implicated in calcium deposition.[64] The major determinants of macrophages activation are not clarified. In fact, inside the pathological valve, these cells could interact with modified lipoproteins (such as oxLDL), engulf them and become foam cells.[3] Alternatively macrophages could be activated by infectious agents-derived products, which have been advocated playing a role in the aortic valve calcification by some epidemiological and experimental evidence.[9, 153] All the factors released by activated macrophages can be potentially implicated in modulating the calcium deposition inside the

valve leaflets. Evidence obtained with both SMC and VIC showed that some cytokines (such as IL-6, IL-1 or TNF-α) could play a role in promoting or accelerating the acquisition of an osteoblast/chondrogenic phenotype by SMC and VIC.[66, 90, 151] Increased MMP activity have been documented within the calcific valve, including MMP-9, usually released by macrophages[154] and MMP-2, which can be mainly produced by VIC stimulated with tenascin-C.[58, 151, 155] However, the actual participation of MMP to the pathological remodelling of the valve is still elusive.

As mentioned above apoptotic cell death can be of importance for calcium deposition. TGF-β has been identified *in vitro* as a crucial mediator of calcification through induction of VIC apoptosis. In fact, the block of the apoptotic pathway by using ZVAD-FKM, prevent TGF-β induced *in vitro* mineralization by VIC.[131] However, further studies are needed to clarify the importance of apoptotic pathways activation during valve calcification.

More recently, also ROS have been proposed as critical mediators of calcium deposition inside the valve leaflets. A study by Liberman et al. demonstrated that superoxide and hydrogen peroxide generation is increased in human stenotic valve around calcifying foci.[156] The increased ROS production was accompanied by higher expression of p22phox and Nox2 NAD(P)H oxidase. This observation was confirmed by Miller et al. which demonstrated in the calcified valve the presence of an unbalance between ROS production (increase superoxide and hydrogen peroxide) and expression of antioxidant mechanisms (decrease of superoxide dismutase and catalase).[157] Interestingly, these authors also demonstrated that, inside the calcified valve, nitric oxide synthase (NOS) uncoupling could contribute to ROS generation.[157] Thus, it is plausible that the calcifying aortic valve generates an excess of superoxide and peroxide in the setting of impaired antioxidant

defences. Nevertheless, mechanisms whereby ROS enhance valve calcification are yet poorly understood. In particular, we do not know which cell types (VIC or inflammatory cells) are producing ROS, and how the excess of ROS production impact on cell-mediated process of calcium deposition. Recent evidence suggest that ROS underlie signaling and expression of BMP-2, ALP as well as Runx2/Cbfa-1.[158-160] Thus, ROS might act as secondary messages able to promote the acquisition of an osteoblast-like profile in cells resident/recruited inside the pathological leaflets. Further mechanistic studies are needed to clarify whether oxidative stress plays a causative role in valve calcification or should be considered as an epiphenomenon.

While normal aortic valve is an avascular tissue, neoangiogenetic processes are commonly observed within the calcified leaflets. VEGF levels and its receptor expression are increased in the pathological valves[161] and an enriched presence of endothelial progenitor cells is observed inside the *fibrosa* layer of calcific valves.[162] A recent paper by Yoshioka et al. demonstrated that chondromodulin-I (chm-I) is highly expressed in the normal valve and downregulated in areas of calcification.[163] The antiangiogenetic effects of chm-I were confirmed by *in vitro* studies showing that chm-I, produced by VIC, can inhibit endothelial tubes formation. In addition, analysis of valves obtained from chm-I deficient mice showed the presence of angiogenesis, high VEGF levels and calcification inside the leaflets.[163] The link between neoangiogenesis and calcification is still unknown. As mentioned above, pericytes are cells that follow the formation of new vessels and possess the ability to differentiate into osteoblast-like cells.[95] However, their specific contribution to valve calcification remain undefined. Alternatively, the formation of new vessels can favour the recruitment inside the leaflets of inflammatory cells and other bone-marrow derived progenitor cells which could be involved in the pathological processes.

More recently, a few studies investigated the potential contribution of genetic factors to valve disease pathogenesis. Some polymorphisms related to the VitaminD receptor,[164] the apolipoproteinE4 allele,[165] the estrogen receptor and TGF-β1[166] have been associated with increased risk of aortic stenosis. Other studies suggest that genetic mutation might contribute to both valvular morphological abnormalities and valvular calcification. In this context mutation of Notch1 transcriptional factor have been recently linked, in two families, to developmental aortic valve anomalies (including bicuspid valve) and severe valve calcification.[31] Interestingly, Notch1 is known to represses Runx2/Cbfa1 (osteoblast transcription factor), allowing normal fibroblasts differentiation and ECM organization. Thus, mutations in this gene might lead to impaired fibroblast phenotypical maturation and favour the acquisition of an osteoblast-like profile by VIC. Even if this observation does not furnish a definitive explanation for the pathophysiological connection between bicuspid valve and calcification, it opens new scenario on the relevance of genetic factors for valve disease progression.

Calcification represents one of the major culprits for functional failure of bioprostheses.[167, 168] The fixation procedure with glutaraldehyde, used for the preparation of porcine bioprostheses and bovine pericardial valves, induces death of the cells resident in the tissue. Differently from calcification happening in the native valve, immunological phenomenon are not considered of relevance for calcification of the bioprostheses.[168] Initial site of mineralization within the cusps of bioprosthetic valves are predominantly seen at cell membranes, where phosphorus, highly present in the phospholipids, binds with calcium that is passively diffusing into the dead cells.[169, 170] Subsequently, calcium phosphate mineral deposition proceeds along collagen and elastin fibers, independently of cellular components.[169, 171] For this reasons, the research on anticalcification treatments for

bioprostheses is focusing on agents able to remove or modified the phospholipids substrate that favour the initiation of mineral deposition.[170, 172, 173]

The increasing evidence about lipids accumulation and RAS activation inside the calcific valve offered the rationale for testing hypocholesterolemic therapies (namely statins) and RAS inhibitors (ACE-inhibitors and AT-1 receptor antagonists) as therapeutics strategies for slowing progression of valve disease. According to this hypothesis, some *in vitro* and animals studies with statins showed that these drugs could interfere with the acquisition of an osteoblast-like profile by VIC.[146, 174] Some retrospective clinical studies and few small-size prospective studies suggested that treatment with statins could slow the progression of valve disease.[175] More recently, we received the results of the SEAS trial, the first prospective study investigating whether a hypocholesterolemic treatment (simvastatin plus ezetimibe) was able to slow the progression of valve degeneration, among patients with mild-to-moderate asymptomatic aortic valve disease. This trial failed to demonstrate a significant effect of the treatment compared to placebo in preventing the need for cardiac valve surgery and the worsening of the peak aortic-jet velocity.[176] The lack of efficacy of the hypocholesterolemic treatment suggests that, once mineral deposition in the leaflet is initiated, the calcium accrual might occur independently from further cholesterol accumulation in the valve. At later stages epitaxial mineral deposition can also be independent of obVIC and inflammatory cells, suggesting that pro-inflammatory and phenotypical modulatory effects of lipids, if any, can be of importance at very early stages of valve disease (potentially during the transition from aortic sclerosis to aortic stenosis). The possible effect of ACE-inhibitors on valve calcific degeneration has been investigated in small population-based studies. One retrospective study showed an association between the use of ACE-inhibitors and a reduced progression of valve calcification.[177] This data

were not confirmed by another retrospective study which described a lack of correlation between treatment with ACE-inhibitors and the rate of calcific valve degeneration.[178] A clarification of the pathobiological mechanisms of valve calcification is needed to correctly interpret the contradictory results of these first clinical studies. It appears that a closer look to the cell types and pathways actively involved in valve disease progression should represent the basis for the development of innovative therapeutic strategies.

8. Aim of the study

This study aimed to identify the potential impact of phenotypic VIC heterogeneity on pathologic aortic valve calcification. The major goal was to the search in the valve for a subpopulation of cells with the capacity of differentiating into osteoblast-like cells, to describe their phenotypic profile and identify the molecular pathways driving calcium deposition. A clonal approach was employed to allow a better analysis on the potential contribution of single VIC subtypes to the calcific degeneration of the valve. We investigated whether VIC clones could differently respond to pathogenic factors (such as LPS and Pi) activating pathways and molecular patterns similar to those express by bone cells during ossification. The identification of this unique pro-calcific cell phenotype and elucidation of the mechanisms driving VIC phenotypic conversion into osteoblast-like cells may help to develop drugs specific for receptors/pathways involved in the inappropriate activation of the osteogenic program.

9. Methods

9.1 Bovine tissue collection and VIC isolation

Bovine hearts (15 months of age) were obtained from a local slaughterhouse within 15 minutes from slaughter. The whole hearts were immediately placed in DMEM (Sigma) plus 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma) and 1μg/ml Amphotericin B (Fungizone, Bristol-Mayer Squibb) and transferred in ice to the laboratory. Aortic valves were excised and the isolated leaflets were embedded in Tissue-Tek OCT Compound (Sakura, Japan), frozen in liquid nitrogen and stored at -80° for histological and immunocytochemical analysis. A non-coronary leaflet was divided in two parts; one third was cryopreserved as described above and the remaining part was used to obtain a primary culture of BVIC following an explant method. Endothelial cell lining of *fibrosa* and *ventricularis* layers were gently scraped out. The aortic leaflet was minced in fragments of about 2-3 mm^3, digested with type-I collagenase (125 units/ml; Sigma), elastase (8 units/ml; Fluka, Germany) and soybean trypsin inhibitor (0,375mg/ml; Sigma) for 30 minutes at 37°C. Fragments were collected in a petri dish (Falcon BD Bioscience, San Diego, CA) and cultured in DMEM containing 4.5 g/L glucose plus FBS 20%, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). BVIC spread from pre-digested fragments after 7-10 days. Collected cells were subcultured repeatedly and used as “uncloned” cell population or subjected to a procedure of cloning using a limited dilution technique. Clonal cell expansion and subcultures were made using the same medium described above and performed up to 13 passages.

9.2 Treatments of uncloned and cloned VIC

Uncloned and cloned cells (passages 4 to 11) were seeded at density of 10^4 cells/cm^2 in six-well plates and treated upon reaching confluence with 2.5 ml of medium (DMEM, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) supplemented with different combinations of LPS (E. Coli, Sigma) (100 ng/ml) and inorganic phosphate (Pi) (prepared using a solution of NaH_2PO_4 to obtain a final concentration of 2.4 mmol/L in the medium). The medium was changed every third day. To induce osteogenic differentiation selected clonal cells were plated as described and treated for 15 days with DMEM (10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) supplemented with 50 μg/ml ascorbic acid (Sigma), 10 nM dexamethasone (Sigma) and 10 mM β-glycerophosphate (Flucka).

9.3 VIC seeding on collagen scaffolds

BVIC clonal cells ($2x10^6$cells/cm^2) were statically seeded on bovine porous microfibrillar type-I collagen sponges (Davol, Cranston, USA) and cultured with DMEM containing 20% of FBS. After one week the cells were shifted to DMEM plus 5% FBS and treated for 12 days with different combination of Pi (2.4 mmol/L, final concentration) and LPS (100 ng/ml). At the end of the treatment the seeded sponges were embedded in OCT, frozen in liquid nitrogen and stored at -80°C. Cryosections (8 μm) were used for further histochemical analysis.

9.4 Immunocytochemical and histochemical studies

Characterization of BVIC was performed by immunofluorescence assays on cytocentrifuged preparation (Shadon cytospin 4 centrifuge, Thermo Fischer Scientific, Inc. Waltham, MA). Cytospins-obtained cells were fixed in 2% p-formaldehyde in PBS pH 7.2 and tested in

single or double immunofluorescence with the following antibodies: anti-SM α-actin (SMA; clone 1A4, Sigma), anti-MyHC-apla1 (type A non-muscle myosin heavy chains; NMM) anti-SM myosin (SMM), anti-CD29 (VMRD Inc., Pulmann, WA, USA), anti-von Willebrand Factor (vWF; Dako), anti-CD45 (Dako), anti-osteopontin (OPN; Abcam), anti-osteocalcin (OC; Abcam), anti-SM22 (Abcam, Cambridge UK), anti-3G5 (ATCC). The secondary antibodies were the AlexaFluor594-conjugated goat Fab' to mouse IgG (Molecular Probes), anti-rabbit TRITC conjugated goat IgG (Chemicon) and Cy2 conjugated goat Fab' to rabbit IgGs (Chemicon). Cells nuclei were identified by Hoechst staining (Hoechst 33258; Sigma). Distribution of antigens was studied using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and images were acquired by using a Leica DC300F digital videocamera (Leica, Wetzlar, Germany). The same primary antibodies were used to carry out an immunocytochemical study of intact bovine aortic leaflets. HRP-conjugated anti-mouse and anti-rabbit IgGs (Dako) were used as secondary antibodies and nuclei were counterstained with Mayer's hematoxylin. Images were acquired using a Leica DC300 digital videocamera (Leica). For both tissue and cell analysis, controls were performed by using non-immune IgGs instead of the primary antibodies. Calcification of the type-I collagen sponges was determined by Alizarin Red and von Kossa staining using standard protocols. Aptotosis was detected in adjacent sections by using the Apoptag kit (Chemicon), following manufacturer protocols. The amount of calcium deposition in the collagen scaffolds was quantified using a computer-driven system analysis (ImageJ, NIH). The extent of apoptosis was established by counting the number of cells showing positive fluorencesce. Experiments were done in triplicate and both calcium deposition and apoptosis were quantified in three different sections per samples. The

calcified area was expressed as percentage of total scaffold area and apoptosis as percentage of positive cells.

9.5 Calcium deposition assay

Cells were decalcified in 0.6 M HCl overnight (500 µl/well) and the calcium content in the supernatant was quantified colorimetrically using the o-cresolphtalein complexone method (Chema Diagnostica, Italy).

9.6 Alkaline phosphatase activity assay

After three washes with PBS the cells were solubilized with 500 µl/well of 1% Triton X-100 in 0.9% NaCl. The samples were centrifuged and the supernatants were collected to determine the ALP activity and the protein content. The enzyme activity was established using a kinetic assay (Chema Diagnostica, Italy) that measure p-nitrophenol production. The protein content was measured using a BCA protein assay kit (Pierce) and ALP activity data were normalized to the protein content.

9.7 Flow cytometry analysis

The expression of intracellular OC on BVIC was assessed by flow cytometric analysis using direct immunofluorescence assay. Briefly, the cells were fixed using Fix and Perm kit (Caltag) according to the manufacturer's instructions. After permeabilization and incubation at 37°C with PE-conjugated anti-OC mAb (R&D Systems) or the matched control mAb, cells were washed and analyzed. For flow cytometry analysis, 30,000 cells were acquired and scored using a FACSCalibur analyzer (Becton Dickinson, Sunnyvale, California). Data were processed using the Macintosh CELLQuest software program.

The threshold of positivity was set at the nonspecific binding observed in the presence of relative control isotype.

9.8 Proteomic analysis

Proteomics was conducted to evaluate modification in the pattern of protein expression by selected clonal cells, treated for 12 days with LPS or control. At the end of the treatments the cells were solubilised with a lysis buffer, composed of TRIS-HCl 12 mM, DTT 1 mM and a cocktail of protease inhibitors. The lysate was collected and submitted to freeze-thawing in liquid nitrogen and sonication in ice. Current analytical methods, performed using non-fractionated protein mixtures, would generate patterns that are too complex for proper interpretation. So, a sample pre-fractionation is accomplished by ultracentrifugation at 100000 RCF for 1 hour at 10°C. The cytosol-enriched protein fraction in the supernatant is then concentrated through ultrafiltration (Microcon-Amicon YM-3, Millipore Corporation) and diluted with a solution of urea 8 M, CHAPS 2% p/v, IPG buffer 0.5%, DTT 60 mM. Proteins are quantified by Bradford method and then separated using two sequential electrophoretic steps (2-DE). Firstly, isoelectric focusing is carried out on Immobilized pH Gradient (IPG) Strips using an EttanTM IPGphor Isoelectric Focusing Unit (Amersham Biosciences). The second dimension is carried out with an Ettan DALTsix Large Vertical Electrophoresis System (Amersham Biosciences), on polyacrylamide gels. The protein spots separated in the gels were stained with Colloidal Coomassie Brilliant Blue that guarantees a good sensitivity and it is compatible with subsequent mass spectrometry analysis for protein identification. The protein pattern was recorded as digitalized images using an Epson Expression 1680 Pro scanner with 16 bit dynamic range and 300 dpi resolution. The comparative analysis among gels, i.e. the comparison of the 2-DE protein

maps from different groups to discover differently expressed proteins, was performed using dedicated software (Proteomweaver, BioRad) for spot detection and comparison. Proteins found to be statistically different among groups were excised from gels, digested by trypsin and identified by mass spectrometry (MALDI-TOF/TOF). Typically, 200 shots were collected from each spot in data-dependent mode. The analyses were conducted using R-cyano-4-hydroxycinnamic acid (Sigma, Vallensbaek Strand, Denmark) (2.5 mg/mL in acetonitrile/0.1% formic acid 50/50) as matrix, mixing equal volumes of sample and matrix and spotting 1 μL of the mixture on a standard 96-well stainless steel MALDI target plate. The spectra were analyzed using Mascot engine search (Matrix Science, London, UK) and PIUMS (www.hh.se/staff/ bioinf). The search was done against the bovine session of the Swissprot database (http://www.expasy.org/; http://www.ebi.ac.uk/swissprot/index.html). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm, carbamidomethylcysteine as fixed modification, and oxidation of methionine as variable modification. The proteins were considered correctly identified when both softwares yielded the same identification with a p value <0.05 and when the coverage of the sequence was at least of 30%. Sequences not identified in the bovine sessions were analysed using the human database.

9.9 Western blotting analysis

Western blotting analysis was performed on the cell preparations set up to obtain ALP activity determination. Protein content was measured using a BCA protein assay kit (Pierce) and equal amounts of proteins loaded on gels were separated by 10% SDS-PAGE, followed by an electrophoretic transfer to a nitrocellulose membranes. The blotted proteins were reacted in sequence with anti-SMA antibody (Dako) followed by anti-mouse IgG

conjugated with HRP (Dako). Bound antibodies were detected with the enhanced chemiluminescence reagent kit (Pierce).

9.10 Measurement of reactive oxygen species production

Fluorescence reactive oxygen species (ROS) production was quantified by using the free radical probe TEMPO-9AC (4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-oxyl, Invitrogen). Cells were loaded with TEMPO-9- AC (10 μmol/l) for 10 min at 37°C in a physiological buffer after 9 days of treatment with LPS or control. After removing external TEMPO-9-AC, cells were visualized using an inverted Zeiss Axiovert 200 motorized microscope. Fluorescence was monitored by quantifying intensity changes over time at an excitation wavelength of 358 nm. The fluorescence rate was calculated as the slope of the linear least squares fitting of fluorescence intensity.

9.11 Statistical analysis

In vitro data are expressed as mean ±SD. Statistical analysis was performed by using Student's *t* test for the intra-group analysis and ANOVA followed by post-hoc Fisher's LSD test for the inter-group analysis. For proteomic analysis, after completion of spot matching, the normalized intensity values of individual protein spots were used to determine the differential protein level between groups. The statistical analysis was performed by using Student's *t* test. Significance was accepted at $p<0.05$.

10. Results

10.1 Phenotypic cell profile of intact aortic valve leaflet

Immunocytochemical analysis of cryosections from intact aortic valve leaflets has confirmed the existence of a phenotypic heterogeneity among BVIC. In the *ventricularis* layer cells expressing SM lineage markers such as SMA, SM22 and SMM were more prevalent. These cells were negative for OC and some of them stained for OPN (Figure 5, panel A). Conversely, in the *spongiosa/fibrosa* layer the majority of cells stained positive for NMM but were negative for SMA and SMM. In these layers sparse cells were found positive for OC (Figure 5, panel B). Von Kossa staining showed no calcium deposition in the leaflets. To sum up, in the *ventricularis* BVIC expressed a myofibroblast/SM cell phenotype whereas in the *spongiosa/fibrosa* most BVIC can be identified as fibroblasts.

10.2 Isolation and characterization of BVIC clones

Uncloned BVIC growth in culture consistently showed heterogeneous morphologies, particularly evident at confluence. The majority of cells showed a cobblestone aspect whereas a minority of scattered BVIC displayed a spindle-shaped arrangement. Using a cloning procedure, we isolated 40 clones of BVIC. For preliminary analysis of the cells phenotypes we selected four clones, representative of the major cell morphologies found among the isolated clones, which displayed marked differences in growth pattern and spatial arrangement at confluence (Table 1). *Clone 1* exhibited high growth kinetic, cobble-stone morphology and was easily detached after trypsinization. These cells maintained a stable morphology over several passages and did not form nodules in long-term culture. *Clone 4* displayed a typical spindle-shaped morphology, slower growth rate and was more resistant

to detachment after exposure to trypsin. Upon reaching confluence, these cells easily aggregated forming ridges and rare nodules (Table 1, Figure 6).

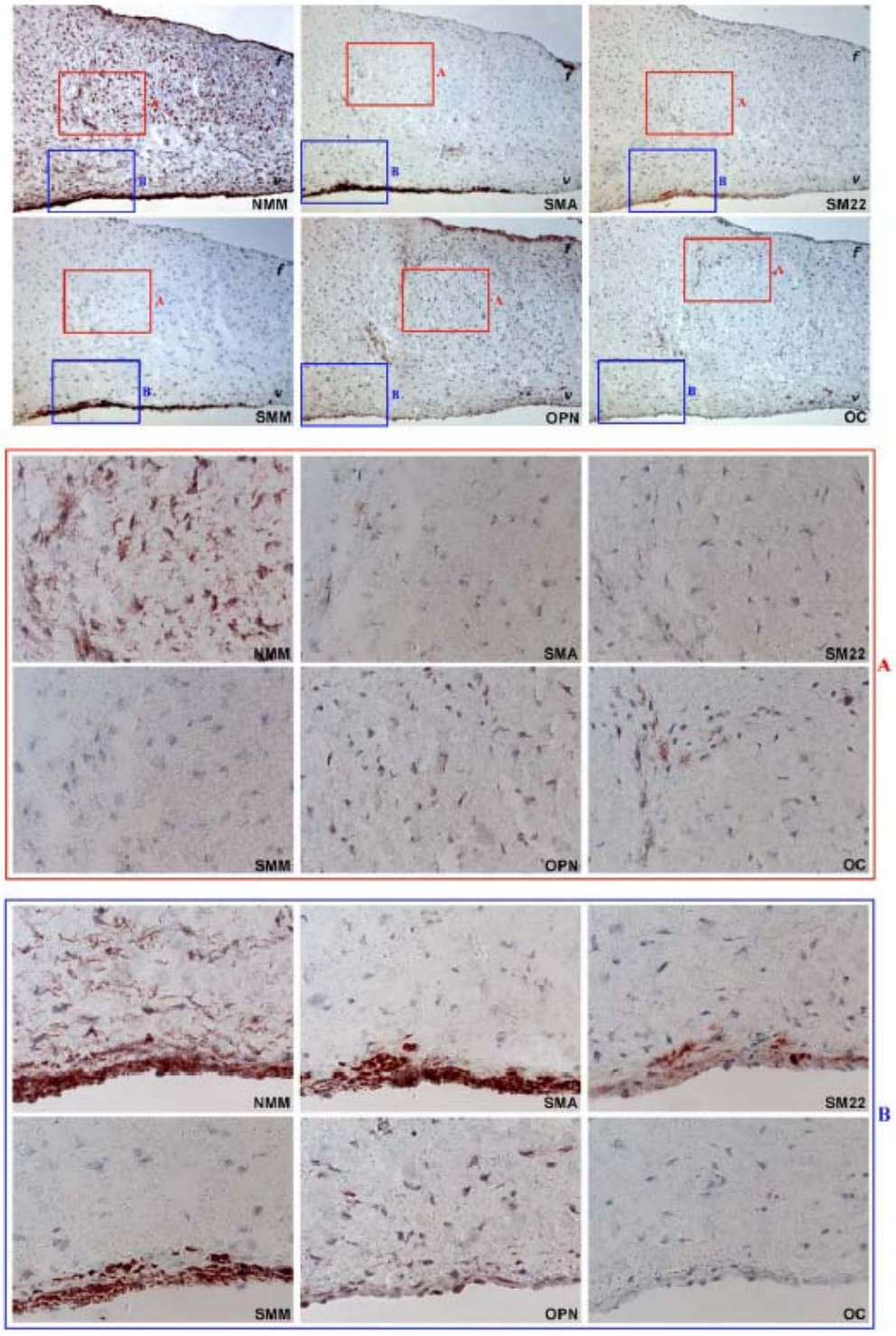

Figure 5. Immunohistochemical profile of BVIC in the bovine aortic valve leaflets. Immunohistochemical staining of the aortic valve leaflets showed that cells expressing SM cell lineage markers (SMA, SM22 and SMM) are mainly located in the *ventricularis* layer of the valve (v). Scattered BVIC, expressing OC but negative for SMA, SM22 and SMM, can be identified mainly in the *fibrosa/spongiosa* layer of the valve (f) (magnification 100X). Area bracketed in the boxes are shown at higher magnification (400X) in the A (*fibrosa/spongiosa*) and B (*ventricularis*) series of pictures.

As for *Clone 1*, *Clone 4* kept a quite stable morphology and growth behaviour after several passages (up to 13). *Clone 2* and *3* showed an intermediate phenotype compared to *Clone 1* and *4*. These cells did not maintain a stable morphology with subculturing and, after 8-9 passages, displayed some features of senescence (arrest of growth and change of morphology characterized by vacuolization and acquisition of flattened and enlarged shape).

Studies on cytocentrifugates showed that all the four clones were negative for endothelial (vWF) and the hematopoietic marker CD45 (Table 1). All of them stained positive for CD29 (mesenchymal cell surface marker) and NMM. Only rare cells from *Clone 1* were positive for SMA and none of them stained for SMM. All the cells from *Clone 4* expressed SMA and a high percentage were also positive for SMM (Table 1, Figure 7). *Clone 2* and *3* showed to be more heterogeneous than *Clone 1* and *4* in terms of SMA expression and did not stain for SMM. None of the cells from *Clone 1* was positive for OPN, whereas a faint staining was observed for OC. The latter was not expressed by *Clone 4*, which stained positive for OPN (Table 1 and Figure 7).

10.3 Endotoxin and phosphate effects on ALP activity expression and calcium deposition in uncloned BVIC

We then investigated the effect of LPS (100 ng/ml) and Pi (final concentration 2.4 mmol/L) in promoting ALP activity expression and calcium deposition in uncloned BVIC. Cells treated with LPS showed a progressive, time-dependent increase in ALP activity compared to controls and cells treated with Pi alone (Figure 8). Combined treatment for 9 days of BVIC with LPS plus Pi also induced an increase in ALP activity comparable to LPS alone. However, no further increase in ALP activity occurred after this time point in the cells treated with both LPS and Pi (Figure 8).

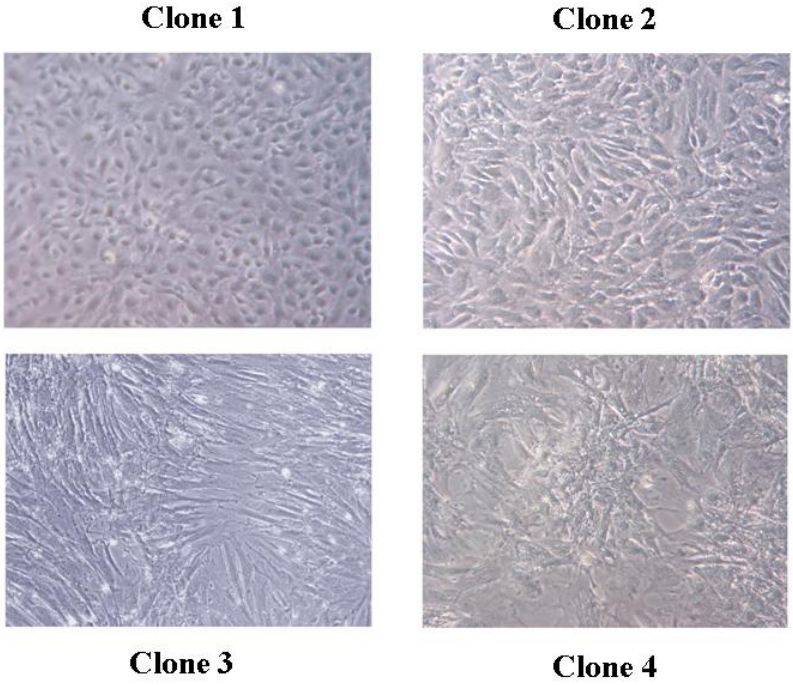

Figure 6. Phase contrast images of BVIC clones (magnification 200X).

Table 1. Morphological and immunophenotypic characterization of BVIC clones.

		Clone 1	*Clone 2*	*Clone 3*	*Clone 4*
Basic characteristics of BVIC clones	**Morphology at confluence**	Cobble-stone, small	Cobble-stone, large	Spindle-shaped, small	Spindle-shaped, large
	Proliferation pattern*	Fast (less than 13 hrs)	Slow (more than 36 hrs)	Slow (more than 36 hrs)	Slow (more than 24 hrs)
	Plastic adherence†	Rapidly detachable	Quite rapidly detachable	Quite strongly adherent	Strongly adherent
	Nodule formation	-	-	-	-/+
Immunophenotypic profile of BVIC clones	**CD45**	-	-	-	-
	CD29	+++	+++	+++	+++
	vWF	-	-	-	-
	3G5	-	-	-	-
	NMM	+++	+++	+++	+++
	SMA	-	+	++	+++
	SM22	-	-	++	+++
	SMM	-	-	-	++
	OPN	-	-	++	+++
	OC	+	+/-	-	-

*Proliferation pattern was evaluated as time to confluence after plating a density of 5000 cells/cm^2 on petri dishes (60 cm^2); †after trypsinization.

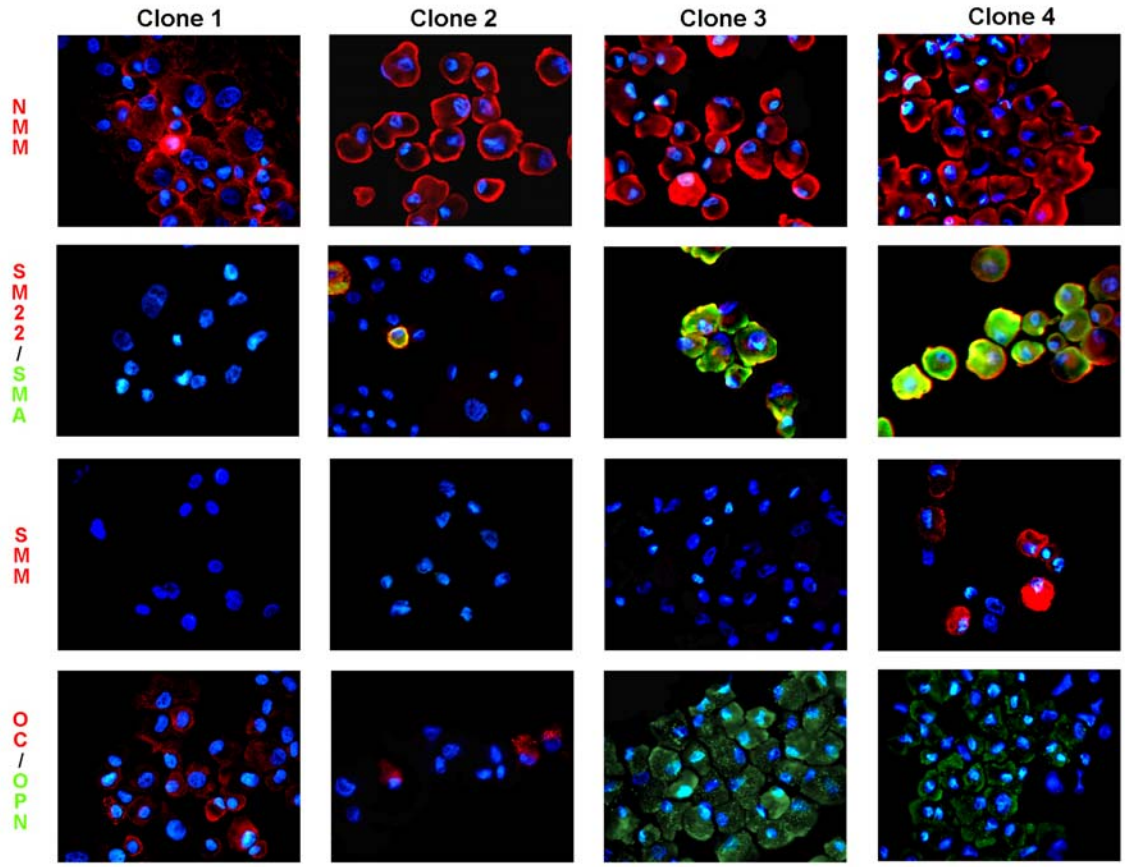

Figure 7. Immunophenotypic characterization of BVIC clones. *Clone 1* cells are characterized by a fibroblast-like phenotype (NMM positive, SMA negative, SM22 negative, SMM negative), express OC and are negative for OPN. *Clone 4* showed a differentiated SM cell profile (positive for all SM cell lineage markers) and reactivity for OPN but not for OC. *Clone 2* and *3* showed an intermediate phenotype (magnification 400X).

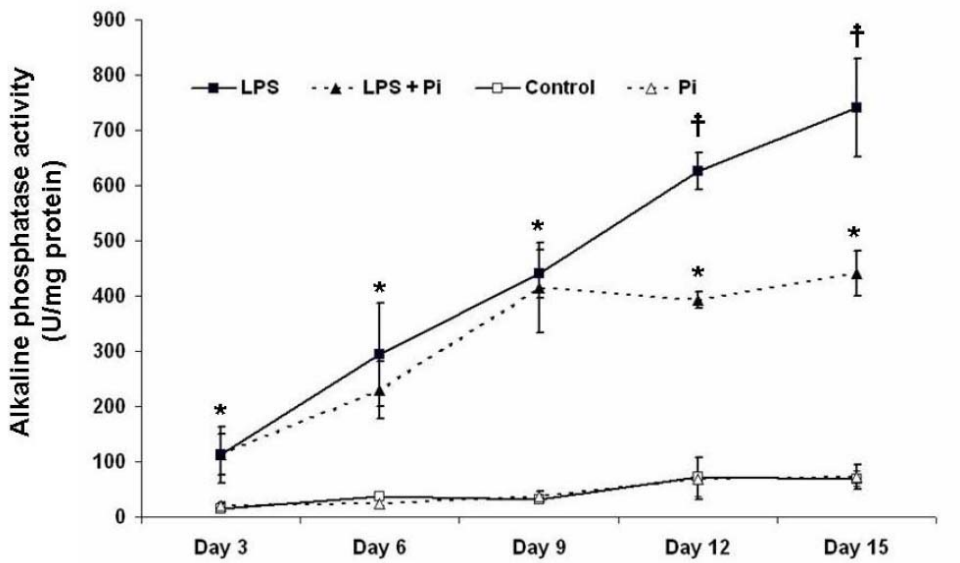

Figure 8. ALP activity and calcium deposition in uncloned BVIC treated with endotoxin and phosphate. LPS (100 ng/ml) and LPS plus Pi (2.4 mmol/L) induced a time-dependent increase of ALP activity in uncloned BVIC. ALP activity was quantified in triplicate in the whole cells extracts and normalized for the protein content (*p<0.05 *vs* control and Pi alone, †p<0.05 *vs* the other groups).

Effect of LPS on ALP activity is dose-dependent, as enzyme activity being detectable also in cells treated with 1 ng/ml of LPS (Figure 9).

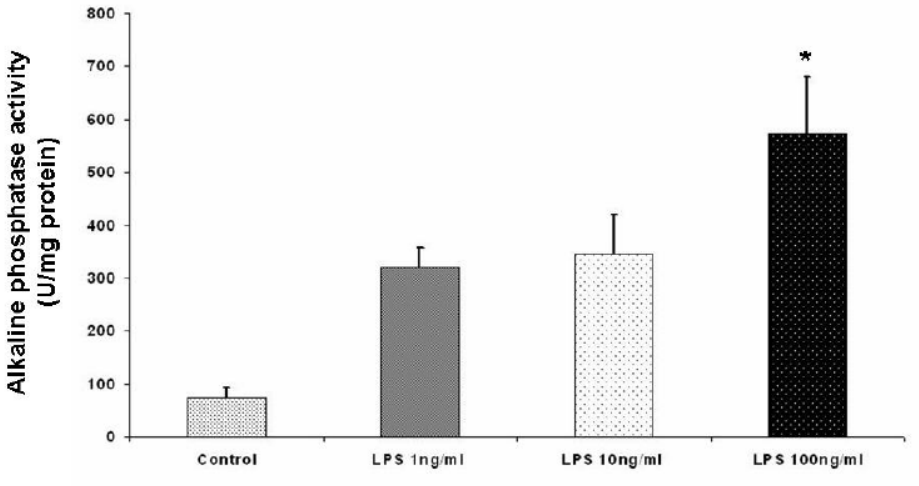

Figure 9. Dose-response effect of LPS on ALP activity expression by uncloned BVIC. LPS induced a dose-dependent increase of ALP activity in uncloned BVIC treated for 12 days (*$p<0.05$). ALP activity was quantified in triplicate in the whole cells extracts and normalized for the protein content.

Interestingly, addition of Pi to the culture medium was able to promote matrix calcification only in cells treated with LPS. No calcium deposition was observed in the cells treated with Pi alone or LPS alone (Figure 10). Calcification appeared *in vitro* as dark granules disperse in the matrix and could be observed from 12 days of treatments onward. In both short- and long-term cultures we did not find the formation of mineralized nodules.

10.4 Selective response of BVIC clones to endotoxin and phosphate treatment

We then asked whether the isolated BVIC *Clones 1-4* display a diverse response to LPS stimulation in terms of ALP activity expression. We observed that the basal level of ALP activity was higher in the *Clone 1* compared to other clones and that after 12 days of treatment with LPS (100 ng/ml) only cells from this clone exhibited a relevant increase in ALP activity (Figure 11). A modest augment in enzymatic activity was also detected in *Clone 3* and *Clone 4*. None of the clones showed mineralization of the matrix after

treatment with LPS and supplementing the culture medium with Pi (2.4 mmol/L), even in long-term cultures (21 days) (Figure 12).

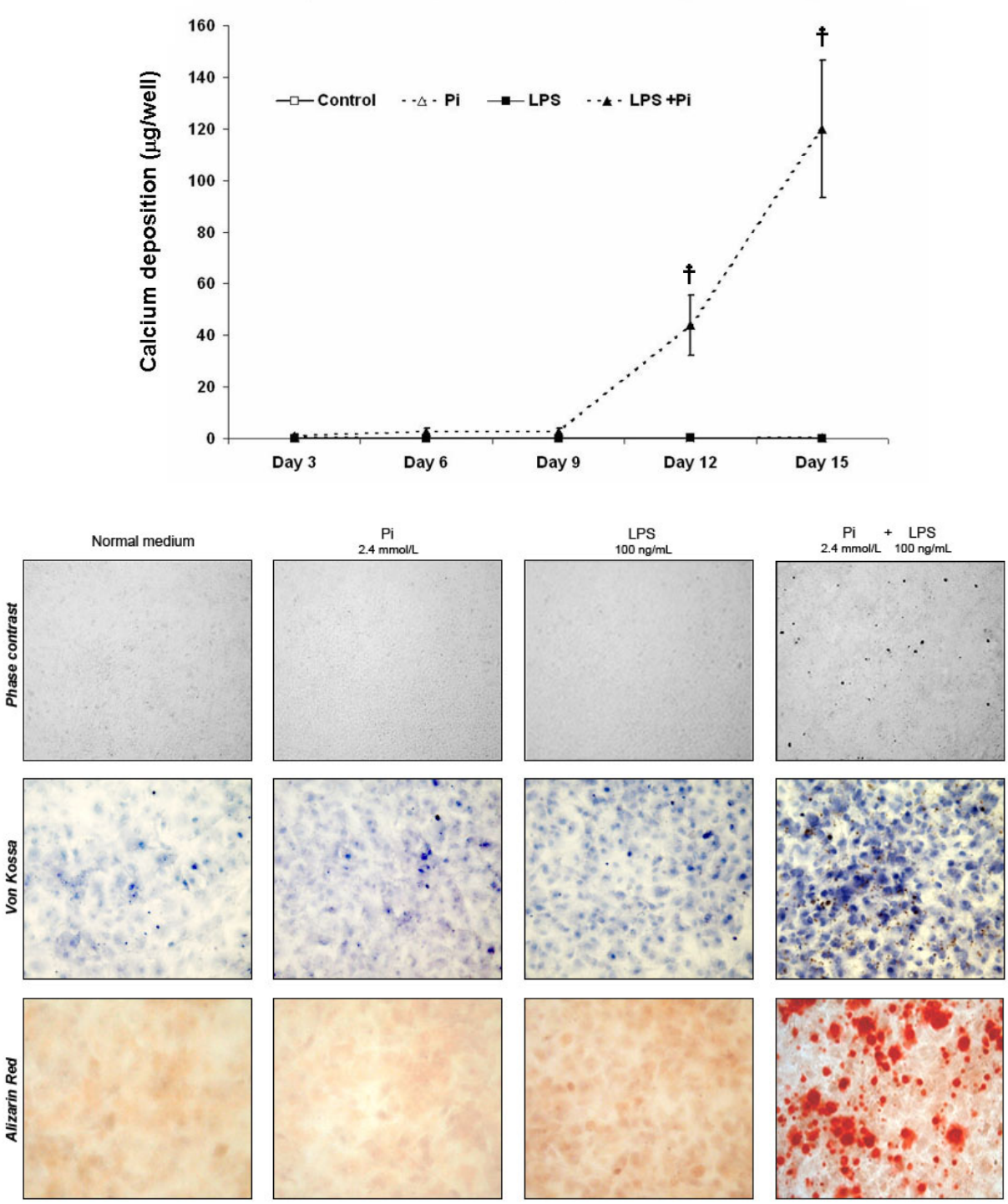

Figure 10. Calcium deposition in uncloned BVIC treated with endotoxin and phosphate. Treatment for 15 days of uncloned BVIC with a combination of LPS (100 ng/ml) and Pi (2.4 mmol/L) promoted a significant increase of calcium deposition in the plates. Calcium was quantified in triplicate and express as µg/well (†$p<0.05$ *vs* the other groups) (upper panel). Calcification could be observed as scattered granules deposits only in BVIC treated with LPS (100 ng/ml) and Pi (2.4 mmol/L) (phase-contrast panels magnification 50X, von Kossa and Alizarin Red staining panels magnification 200X) (lower panel).

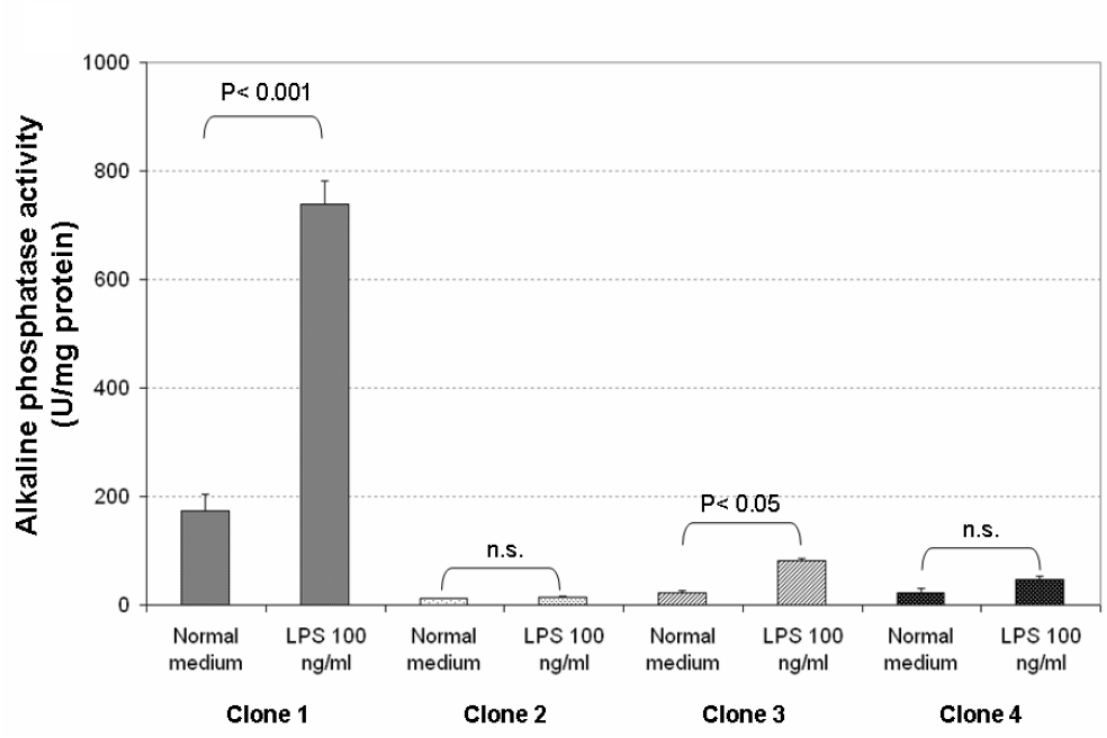

Figure 11. ALP activity in BVIC clones treated with endotoxin and phosphate. BVIC clones were treated for 12 days with LPS (100 ng/ml). ALP activity was quantified in triplicate in the whole cells extracts and normalized for the protein content. Compared to control, *Clone 1* showed a four-fold increase of ALP activity. A modest increase in the enzyme activity could be also documented in *Clone 3* and *4*.

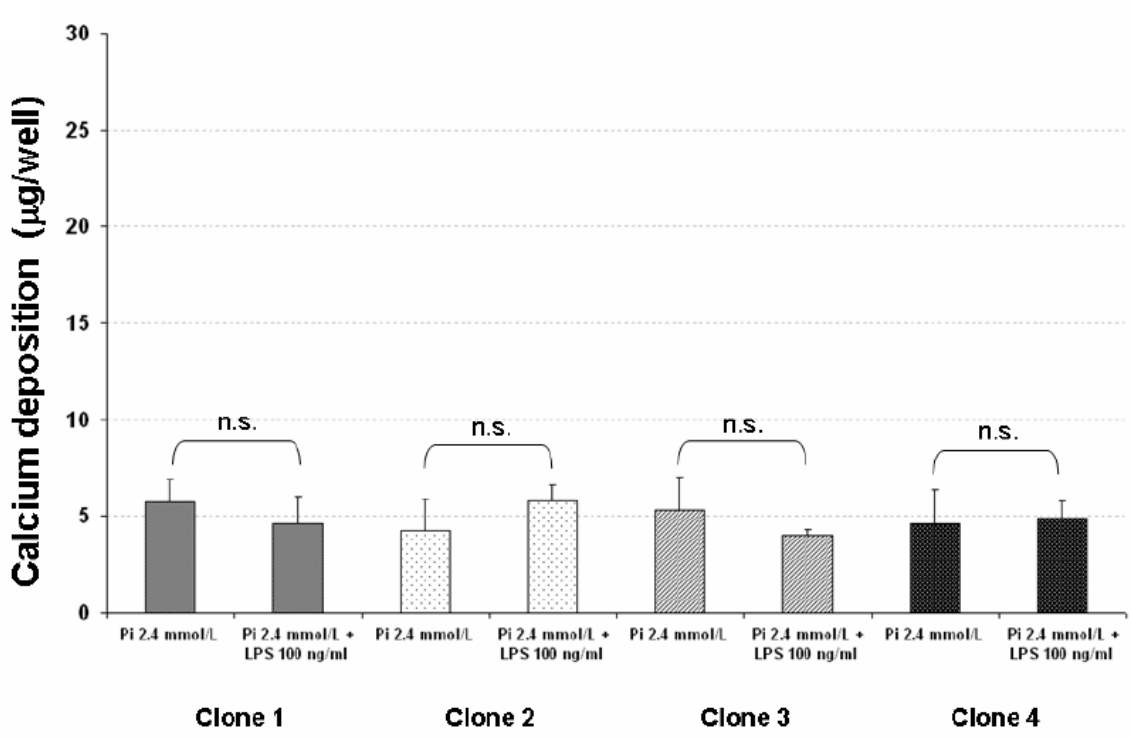

Figure 12. Calcium deposition in BVIC clones treated with endotoxin and phosphate. BVIC clones were treated for 21 days with Pi (2.4 mmol/L) or Pi plus LPS (100 ng/ml). For all the clones no calcium deposition could be observed in the plates. Calcium was quantified in triplicate and express as µg/well.

Further studies were performed only on *Clone 1* and *4* that maintain stable growth pattern without evidence for cellular senescence. As far as the immunophenotypic profile in these clones is concerned, *Clone 1* expressed low levels of SMA when tested in immunofluorescence assays (Table 1) but Western blotting tests revealed that this clone showed an increase in SMA expression with subculturing (Figure 13A). However, the concomitant treatment of *Clone 1* with LPS prevented the accumulation of SMA. Conversely, endotoxin treatment of *Clone 4* was not able to exert such an inhibition (Figure 13A). Flow cytometry tests confirmed that OC was expressed at a low level by *Clone 1* (about 50% of the cells) and almost absent in *Clone 4*. Interestingly, treatment of *Clone 1* cells with LPS for 12 days increased OC expression (about 80% of the cells) whereas this treatment had no effect on *Clone 4* (Figure 13B).

Since *Clone 1* expressed high level of ALP activity and an increased expression of OC we investigated whether this clone could be considered as an osteoblast precursor and retaining the potential to differentiate in osteoblast-like cells following treatment with osteogenic medium. However, after 15 days of treatment of this clone with an osteogenic inducing medium (a combination of ascorbic acid, dexamethasone and β-glycerophosphate), no effect was observed on both ALP activity expression and matrix mineralization (Figure 14).

We then tested whether *Clone 4* could influence the functional properties of *Clone 1* via a co-culture experiment. Hence, these two clones were admixed in a 1:1 cell ratio and the combined culture treated again with a combination of LPS and Pi. After 12 days of treatment, calcium deposition could be observed in the co-culture setting, but not in the original clones, cultured separately (Figure 15A). At the end of the treatment period, cells in co-cultures showed a peculiar appearance and spatial arrangement characterized by some

cells (resembling those of *Clone 4*) giving rise to ridges and rare nodules which surrounded "islets" of cells (resembling the cobble-stone morphology of *Clone 1*) still showing a monolayer placement. In this particular setting calcium deposition seemed to be localized to the "ridges" (Figure 15B).

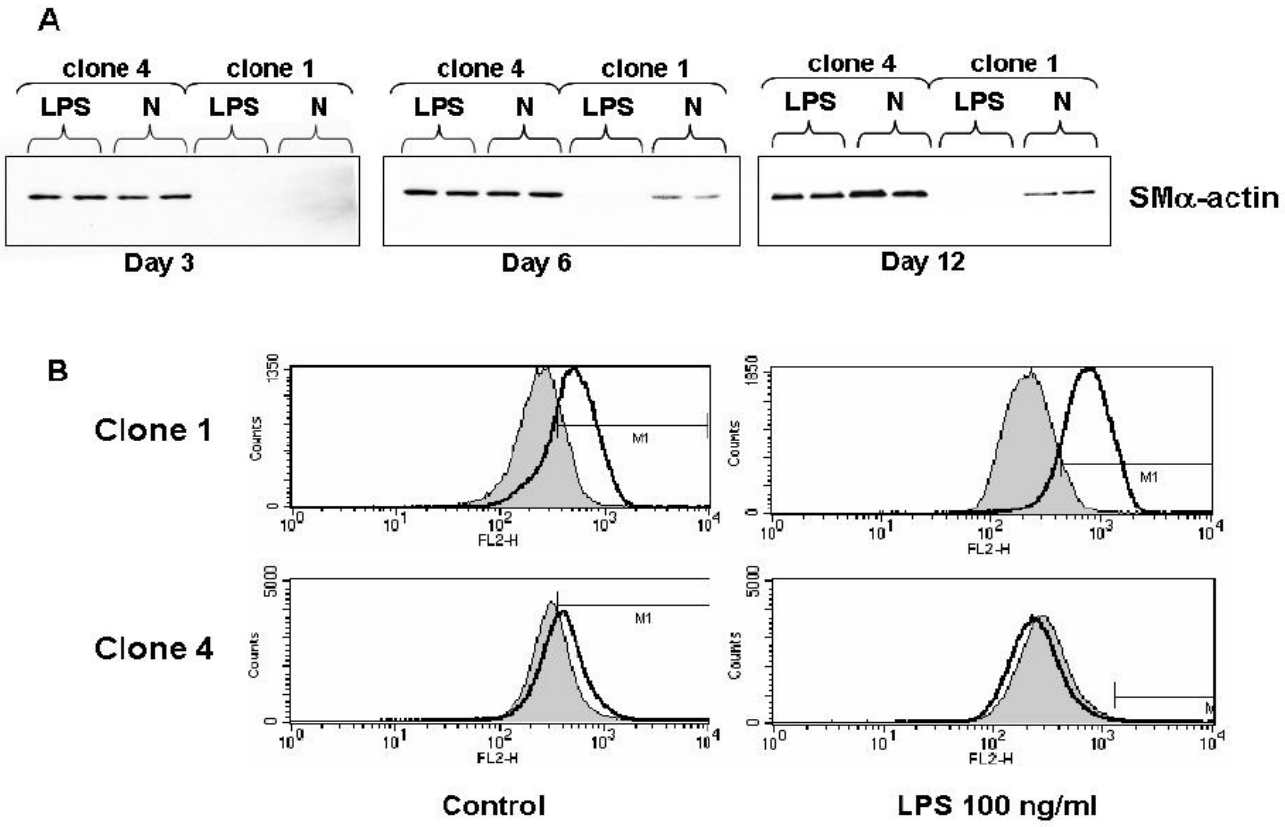

Figure 13. Differential expression of SM α-actin and osteocalcin by BVIC clones after endotoxin treatment. Western blotting analysis demonstrated that *Clone 1* accumulated SM α–actin (SMA) with culturing (N = normal medium). Treatment with LPS (100 ng/ml) prevented SMA expression. No effect of endotoxin on SMA expression could be observed in *Clone 4* (experiments are shown in duplicate). (B) Flow cytometry analysis demonstrated that *Clone 1* treated for 12 days with LPS (100 ng/ml) increased the expression of OC. The same treatment had no effect on *Clone 4* (black lines indicate OC expression; solid fill-ins indicate relative control isotype).

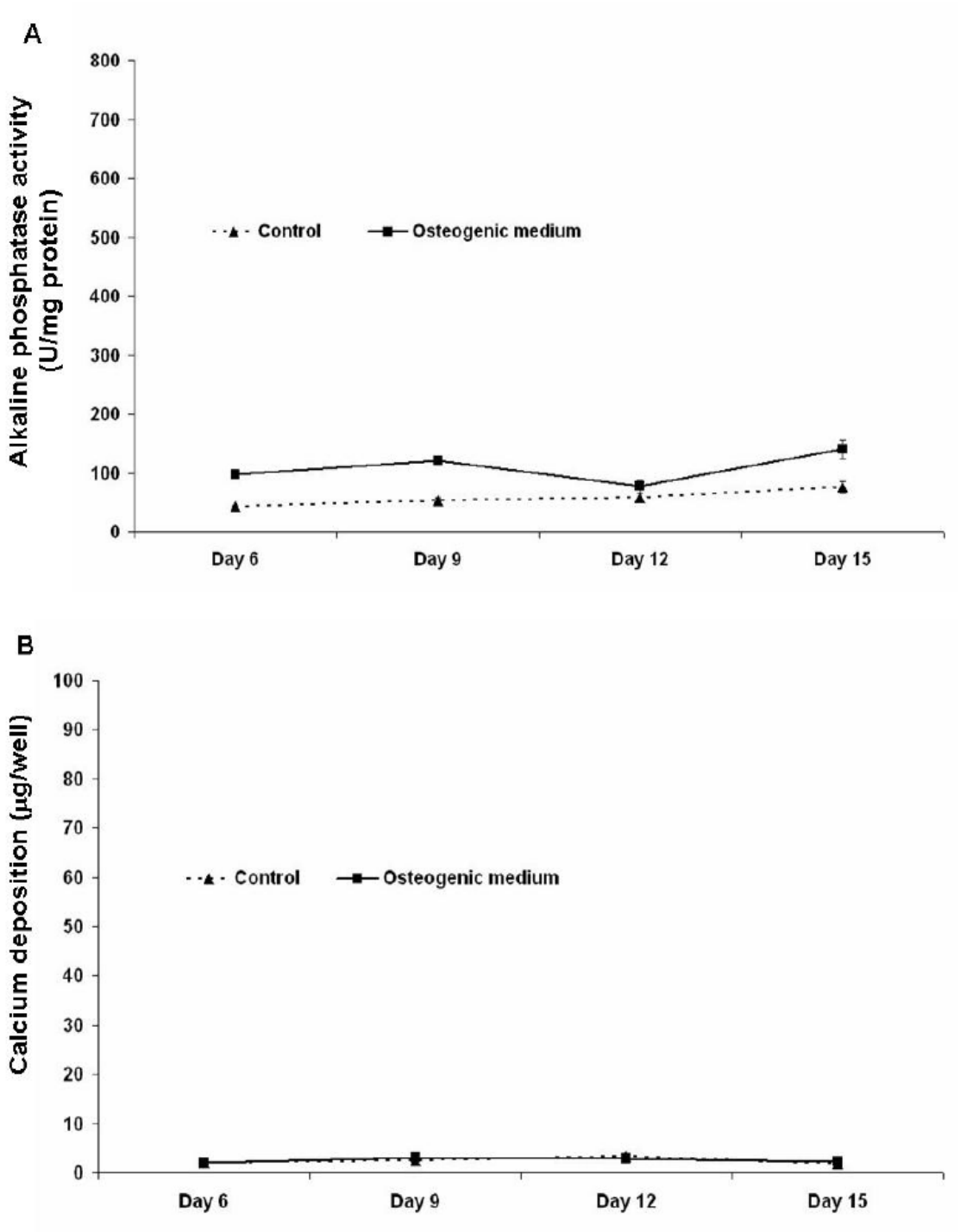

Figure 14. ALP activity and calcium deposition in *Clone 1* cells treated with osteogenic inducing medium. *Clone 1* cells were treated for 15 days with a combination of 50µg/ml ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate (osteogenic medium). We did not observed a significant increase over time of ALP activity expression (A) and calcium deposition (B) in the cells treated with the osteogenic medium compared to control.

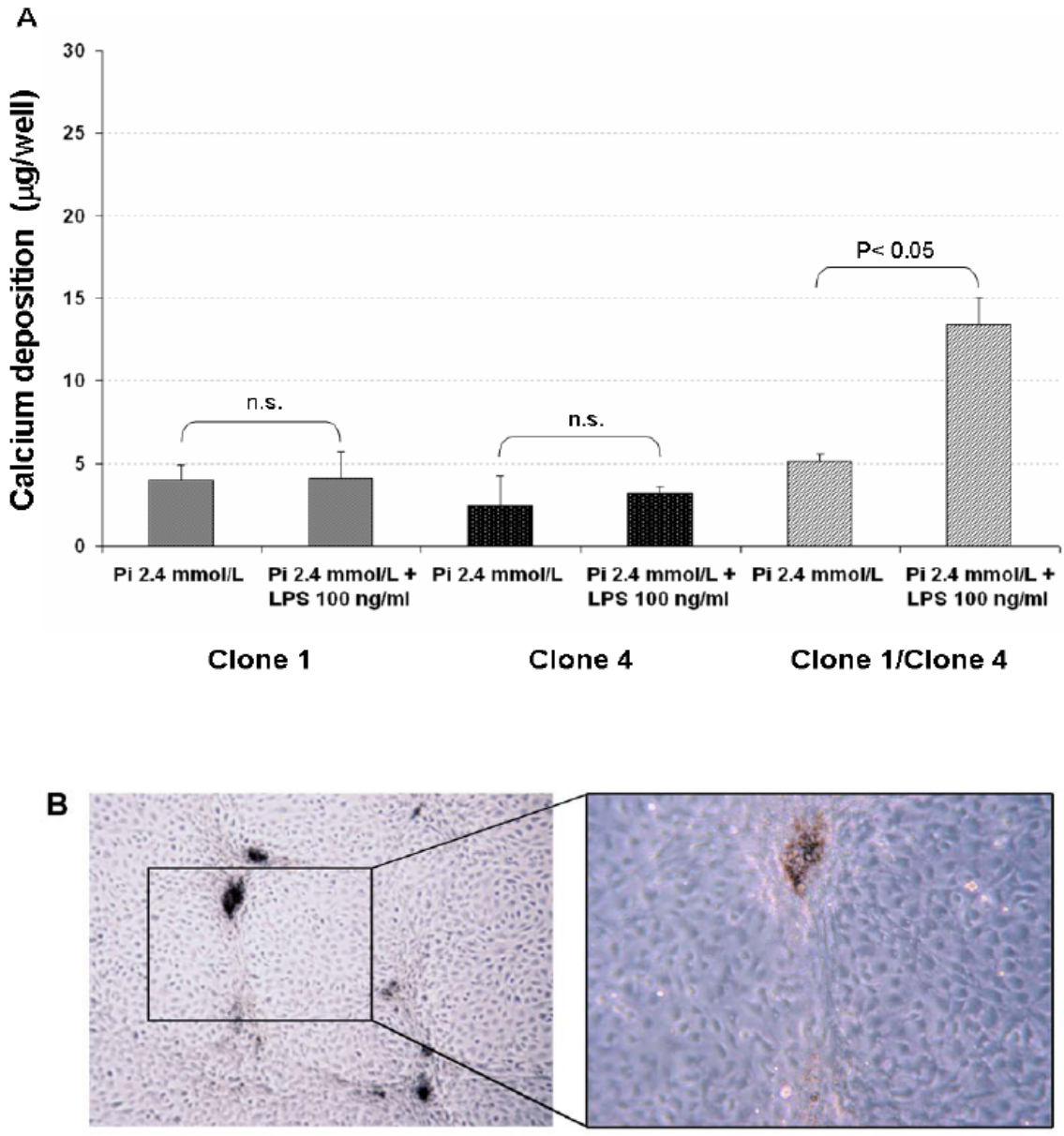

Figure 15. Calcium deposition was observed in co-cultures of specific BVIC clones. (A) *Clone 1* and *4* were cultured alone or placed in co-culture and treated for 12 days with Pi (2.4 mmol/L) or LPS (100 ng/ml) plus Pi. Only cells in co-cultures treated with the combination of LPS and Pi showed a significant increase of calcium deposition. (B) Phase-contrast images showing the peculiar arrangement observed in co-cultures of *Clone 4* and *1* after treatment for 12 days with LPS and Pi (magnification 100X, inset 200X).

10.5 Calcification of collagen scaffolds by BVIC clones

Despite the increase of ALP activity and OC after treatment with LPS, cells of *Clone 1* were not able to induce calcium deposition when grown on the plastic substrate. We hypothesized that the limited ability of this clone to promote calcium deposition could be

related to its peculiar growth pattern, adhesion properties and, perhaps, reduced production of collagenous extracellular matrix. To test this hypothesis, cells from *Clone 1* and *4* were seeded on bovine microfibrillar type-I collagen sponges and treated again for 12 days with Pi (2.4 mmol/L) or Pi plus LPS (100 ng/ml). Histological analysis of the collagen scaffolds showed that both cells types populated the sponges and that the treatments induced calcification of the matrix. Interestingly, using this peculiar 3D-setting and in the presence of Pi alone, cells of *Clone 1* were able to promote collagen scaffolds calcification. Supplementation with LPS induced an even higher mineralization of the matrix compared to Pi alone. Modest calcium deposition in the collagen scaffolds seeded with *Clone 4* was observed after treatment with Pi and LPS (Figure 16A,B). Collagen sponges without cells but treated for 12 days with LPS and Pi did not give calcification.

We also studied the activation of apoptotic pathway in the two clones cultured in the collagen sponges. A high degree of apoptosis was observed in the calcified collagen patches seeded with *Clone 1* cells and treated with the combination of LPS plus Pi. A lower level of apoptotic degeneration was observed in the same clonal cells treated with Pi alone. Apoptosis was almost absent in untreated *Clone 1* cells and in all the sponges seeded with *Clone 4* cells (Figure 17).

10.6 Modification in cytosolic protein profile in clonal cells acquiring a pro-calcific phenotype

Proteomic analysis was conducted on the cytosolic fraction of proteins obtained from *Clone 1* cells treated for 12 days with LPS or control. Modification in the cytosolic protein profiles of *Clone 1* cells was analyzed using two-dimensional gel electrophoresis (2-DE) coupled to mass spectroscopy.

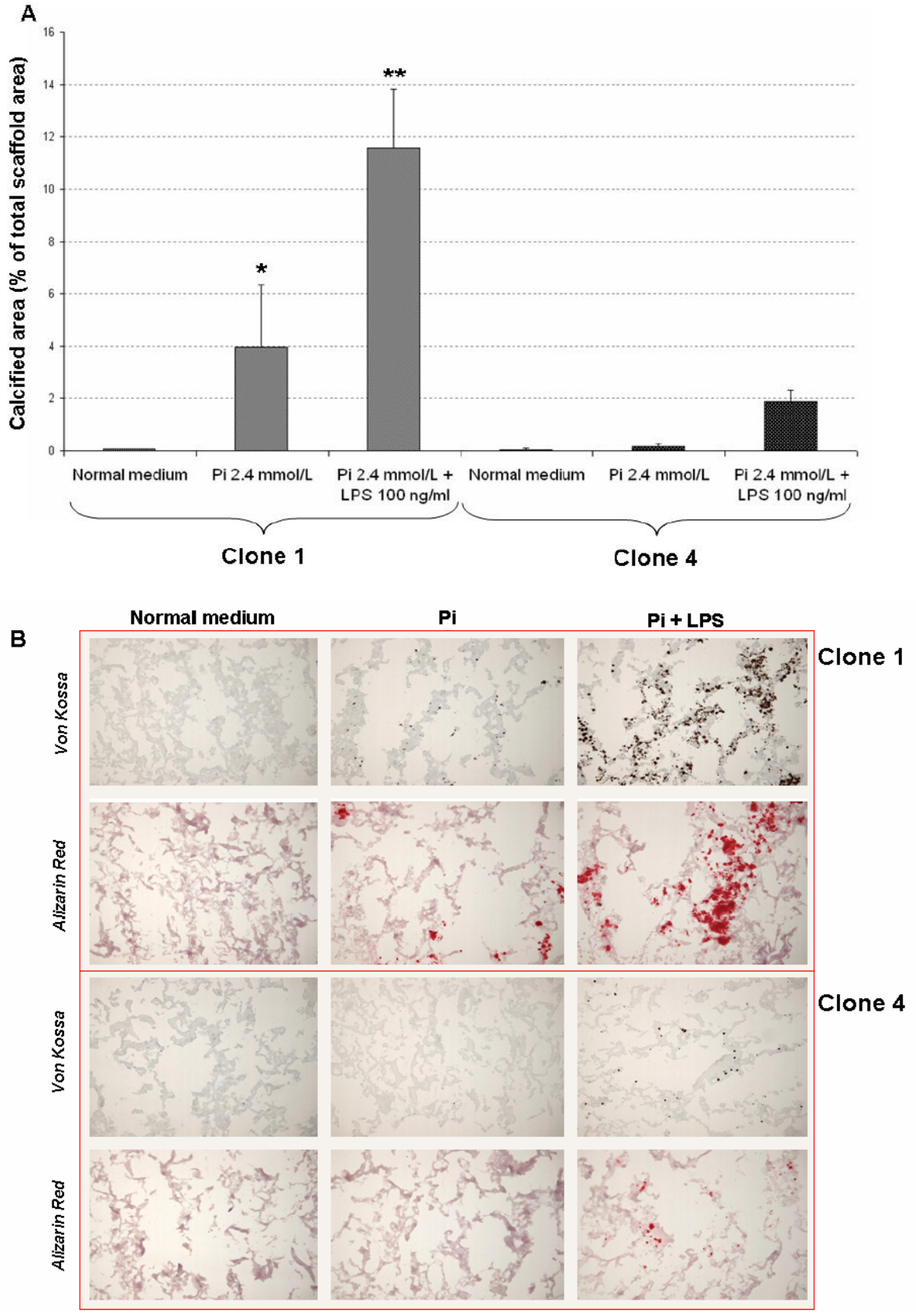

Figure 16. BVIC clones promoted calcification of type-I collagen scaffolds when treated with phosphate and endotoxin. *Clone 1* and *4* were seeded on microfibrillar type-I collagen sponges and treated for 12 days with Pi (2.4 mmol/L) or Pi plus LPS (100 ng/ml). Calcium deposition was demonstrated by von Kossa and Alizarin Red staining (B). Quantification of von Kossa-positive scaffold area is expressed as percentage of total scaffold area (*$p<0.05$, **$p<0.001$) (A) (magnification 100X).

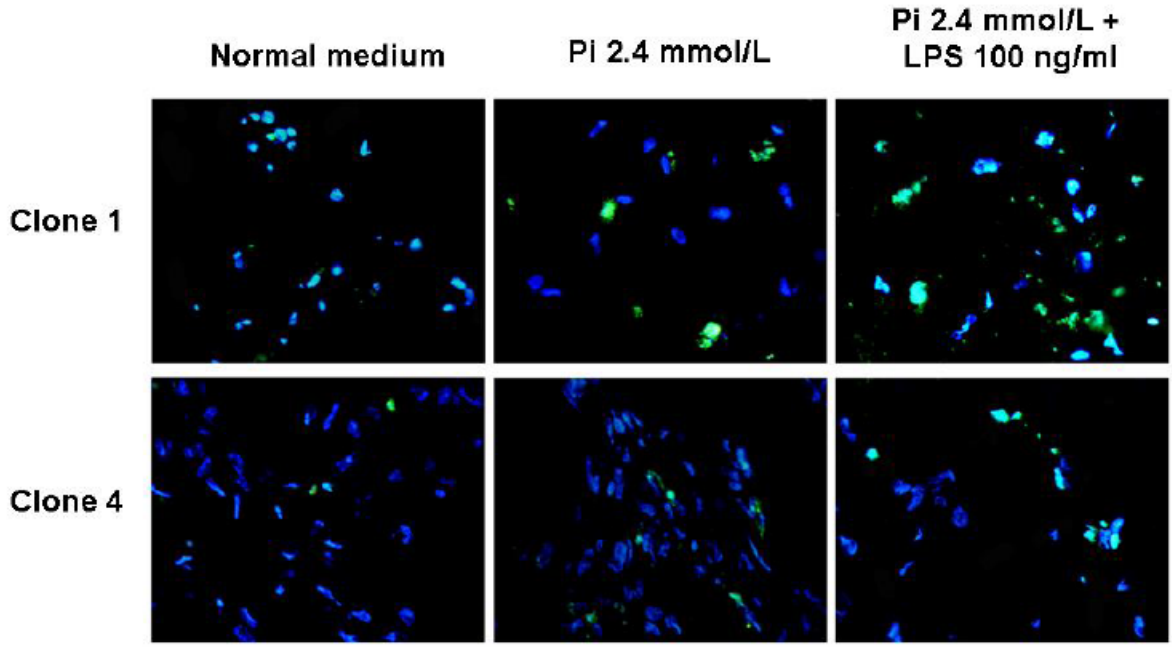

Figure 17. Apoptosis of clonal cells seeded in type-I collagen sponges. Apoptosis was evaluated in clonal cells seeded in type-I collagen sponges. A high degree of apoptosis was observed in the sponges seeded with *Clone 1* cells and treated with Pi (2.4 mmol/L) alone (about 50% of the cells) or the combination of LPS (100 ng/ml) plus Pi (about 90% of the cells). Apoptosis was almost absent in untreated *Clone 1* cells and in all the sponges seeded with *Clone 4* cells. Nuclei were counterstained with Hoechst.

A total of 161 proteins were separated by 2-DE and statistically analyzed. Significant modification (considered as both $p<0.05$ and at least 1.5-fold change) was observed in 54 spots, which were then subjected to mass spectroscopy analysis (Figure 18).

The latter allow the identification of 34 unique proteins belonging to structural, enzymatic, and regulatory proteins. Some spots represented different isoforms of the same molecule, probably due to post-translational modifications. The proteins were identified and classified according to Swissprot database (http://www.expasy.org). Major changes were observed among the expression levels of proteins involved in: i) protein assembly (8 spots, with 7 unique proteins including Hsp60, HspB6, Hsp70), ii) energy metabolism (12 spots, with 3 unique proteins, namely phosphoglycerate kinase-1, alpha enolase, triosephosphate isomerase), iii) cytoskeletal organization (10 spots, with 7 unique proteins, including vimentin, β-actin, α-actinin-4, fascin, profilin-1, macrophage-capping protein, DRP-2), iv)

cell redox homeostasis (11 spots, with 7 unique proteins, including superoxide dismutase [Cu-Zn] {SOD [Cu-Zn]}, thioredoxin, dimethylarginine dimethylaminohydrolase [DDAH]), v) protein metabolism and transport (7 spots, with 6 unique proteins, including calumenin, transitional endoplasmatic reticulum ATPase, cytosol aminopeptidase, fatty acid-binding protein, ubiquitin carboxyl-terminal hydrolase isozyme L1), vi) nucleic acid metabolism (2 spots, with 2 unique proteins, namely heterogeneous nuclear ribonucleoprotein K, elongation factor 1-β) and signal transduction (4 spots, with 2 unique proteins, namely reticulocalbin and AHNAK). See the Appendix for detailed description of the proteins references, function and quantitative analysis results.

Of note, we documented a significant decrease in the level of antioxidant proteins such as SOD [Cu-Zn] and thioredoxin. These modifications were also accompanied by reduced levels of DDAH-1 and -2, an enzyme involved in intracellular metabolism of asymmetric dimethylarginine (ADMA), a known inhibitor of NOS (Appendix and Figure 19). These findings are in line with previous reports suggesting that dysregulation of ROS production and NO bioactivity could be involved in the pathogenesis of aortic valve calcific degeneration.[157]

10.7 Reactive oxygen species production in pro-calcific clonal cells

Based on the proteomic analysis results showing a reduced expression of antioxidant proteins (such as SOD[Cu-Zn] and thioredoxin), we investigated whether the acquisition of the pro-calcific profile by *Clone 1* was accompanied by an increase in ROS production. As predicted, after treating the *Clone 1* cells with LPS for 9 days we could observe a significant increase in ROS release by the cells (Figure 20).

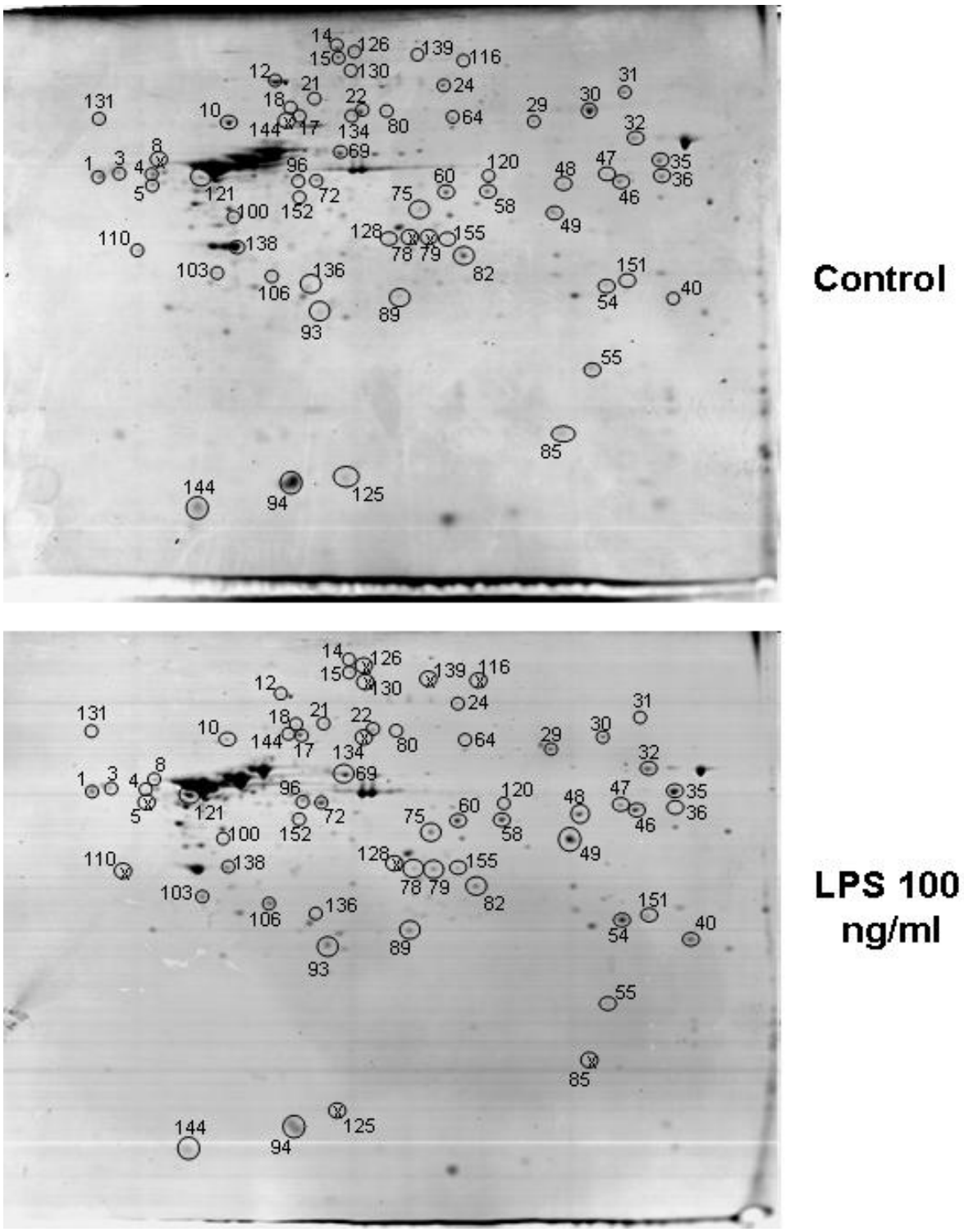

Figure 18. Representative 2-DE maps of Clone 1 cytosolic proteins after treatment with LPS or control. Clone 1 cells were treated for 12 days with LPS (100 ng/ml) or control. Two-dimensional gel electrophoresis (2-DE) of the cytosolic proteins fraction allows the separation of 161 proteins. Spot numbers reported in figure correspond to proteins significantly altered among groups and subsequently identified by mass spectroscopy analysis.

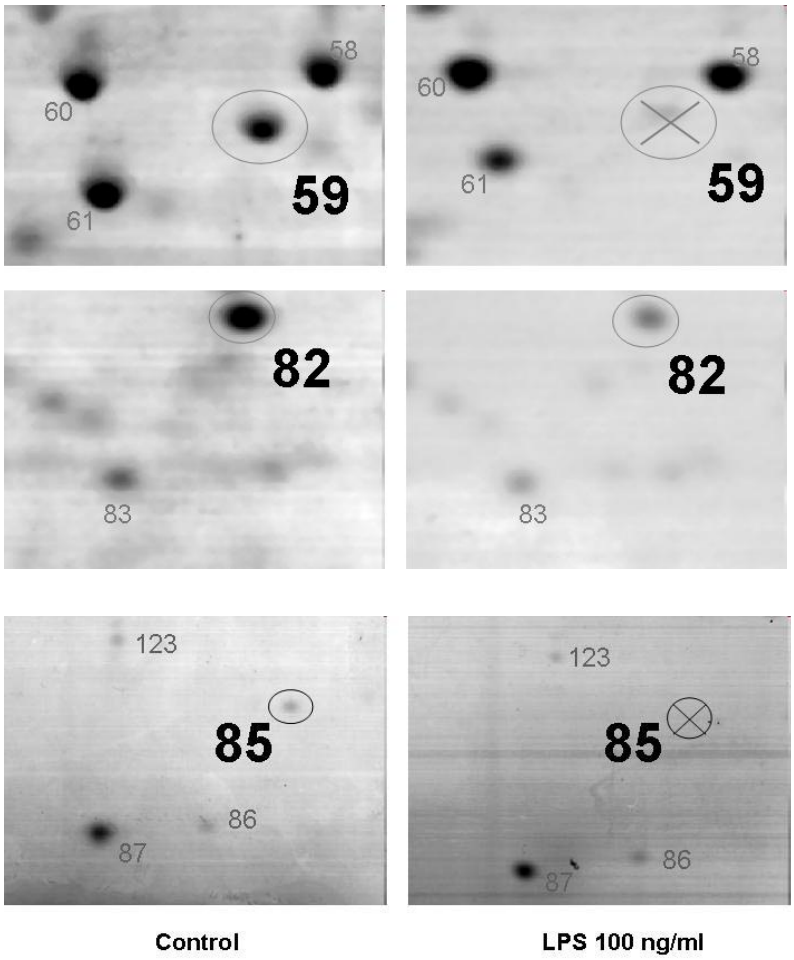

Figure 19. 2-DE gel images of selected spots. Spot n° 59: DDAH-1; spot n°82 DDAH-2; spot n° 85 SOD [Cu-Zn].

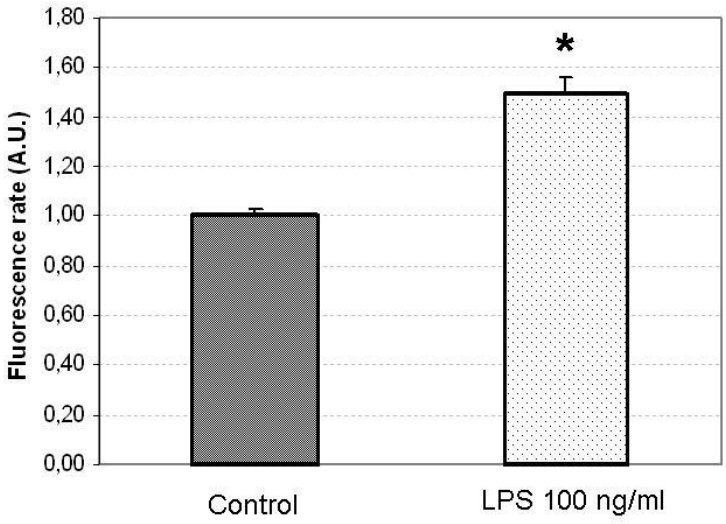

Figure 20. Reactive oxygen species production by Clone 1 cells treated with endotoxin. Clone 1 cells were treated for 9 days with LPS 100 ng/ml or control. Fluorescence ROS production was quantified by using the free radical probe TEMPO-9AC. Fluorescence was monitored by quantifying intensity modification over time and expressed as arbitrary units (A.U.) relative change to control (*$p<0.05$).

11. Discussion

In the present study, we have shown that BVIC derived from bovine aortic valve leaflets display different clonogenic features and susceptibility to *in vitro* calcification induced by endotoxin and phosphate treatment. The inherent phenotypic heterogeneity of primary cultures makes difficult to dissect the specific contribution of each cell subset, if any, to the calcification process. Thus, cloning is the procedure of choice to study the specific structural and functional properties of cells within a giving tissue and investigate their potential role in some pathological conditions (see, for example the study about the relative contribution of different arterial SMC subpopulations in vascular disease).[68, 179] The existence of cells with a calcifying potential in the cardiac valves has already been demonstrated *in vitro.* Using an uncloned population of human and canine VIC, Mohler et al. reported on the formation of calcified nodules after three to four weeks of culture. The nodules contained a central core of calcium, stained for BMP-2 and expressed ALP.[147] In our study we expanded this original observation taking into account that BVIC, similarly to other non-bovine VIC, is *in vivo* comprised of a heterogeneous cell population as identified by an appropriate immunophenotypic score (Table 1 and Figure 7). In concordance with data reported for other species, bovine show in their aortic valve leaflets a heterogeneous population of fibroblasts/myofibroblasts/SMC specifically distributed among the three valve layers.[51, 52] *In vitro* treatment of uncloned population of BVIC with endotoxin and Pi was able to induce the expression of ALP activity (a widely used marker for osteoblast-like differentiation), and promote calcium deposition. Isolation of BVIC clones has targeted clones with a peculiar propensity for an inducible calcification pattern and this has been attained *via* endotoxin treatment. In particular, this promoter increases ALP activity and OC expression in *Clone 1*, the one characterized by a fibroblast-like phenotype. Thus, it is likely

that in the uncloned BVIC population cells with this morphological and immunophenotypic profile play a major role in augmenting ALP activity. On the other hand, BVIC clones showing expression of markers of SMC lineage (such as SMA, SM22 and SMM) appear to have a reduced ability of up-regulating ALP and OC after stimulation with endotoxin. Interestingly, *in vivo* BVIC with an immunophenotypic pattern similar to *Clone 1* are mainly localized to the *spongiosa/fibrosa* layer, whereas BVIC similar to *Clone 4* (SM-like phenotype) predominate in the *ventricularis* layer. Although an in vivo direct spatial correlation with *in vitro* cell phenotypes cannot, at the present, be drawn is a matter of fact that these layers are characterized by a different propensity to undergo calcific degeneration. In fact, the *spongiosa/fibrosa* is prone to form calcified nodules, whereas the *ventricularis* is more calcium deposition-resistant.[139] Thus, our data suggest that the inherent biological properties of BVIC in *ventricularis* vs *fibrosa/spongiosa* could play a role in the pathobiology of aortic valve calcification. Previous demonstration of a different expression of calcification-related genes between the VEC populating the two sides of leaflet is in keeping with this hypothesis.[63]

Current theories on the cellular involvement in aortic valve calcification include: i) osteogenic differentiation of mesenchymal progenitor cells resident in the valve leaflet or arising from the bloodstream, ii) phenotypic transition of resident VIC toward a chondro/osteogenic phenotype, and iii) apoptotic degeneration of cells resident/recruited in the pathologic tissue. However, the relative contribution of these mechanisms in promoting valve calcific degeneration is not defined yet. Our clones were not grown using culture procedures for mesenchymal stem cells, and *Clone 1,* the one that exhibits the higher plasticity in term of SM (SMA) and osteoblast (ALP, OC) marker expression, do not "fully" acquire an osteoblast profile when treated with a specific osteogenic differentiation medium.

In his elegant paper Murshed et al. demonstrated that phosphate levels, ALP expression and the presence of type-I collagen represent the major determinants of ECM mineralization.[180] ALP and type-I collagen can be found in non-bone tissues but they are co-expressed only by osteoblasts in the bone and odontoblasts in the teeth. We identified *Clone 1* as the cell type expressing the higher ALP level following treatment with endotoxin but this response, though necessary, is not sufficient to promote calcium deposition in plastics by increasing Pi levels (even in long-term cultures). However, when these cells are seeded on type-I collagen scaffolds, induction of ALP activity and high Pi level induced calcification of matrix. This finding suggests that *Clone 1* is characterized by a limited ability to produce, at least when grown on plastics, the collagenous matrix needed for calcium deposition. Besides confirming the role played by these factors in driving the calcium deposition, our data demonstrate that a specific valve cell type reveals a unique pro-calcific profile under pathological conditions.

Nodule formation is often seen in cell culture models used to study mechanisms of vascular calcification. Canine, sheep and human aortic VIC cultures yield *in vitro* nodules, as well.[131, 147] However calcification of these VIC has not been studied using clonal cell populations. A clonal study was instead performed by Demer's group to identify the presence in the bovine arterial wall of calcifying valve cells (CVC), a specific subset of SMC that spontaneously calcifies *in vitro,* forms nodules and retains a mesenchymal multilineage potential (chondrogenic, leiomyogenic, stromogenic).[68, 94] CVC are characterized by the expression of 3G5, a surface ganglioside that is also express by pericytes.[68, 181] Differently from CVC, *Clone 1* cells even if express high level of ALP activity, are negative for 3G5 and do not form nodules even in a long-term culture. Interestingly, we observed that when *Clone 4,* characterized by low level of ALP but ability

to form some nodules, was added to *Clone 1*, cells that morphologically resemble *Clone 1* surround cells that grow in multilayer or nodules. In this setting calcium deposition is favoured in the nodule region (see Figure 15). We can speculate that the multilayered growth pattern of *Clone 4* can favour the deposition of collagenous matrix used by the *Clone 1* cells to complete the mineralization process (as seen when cells are seeded on the collagen sponges). In line with this scenario, previous studies that used uncloned valve cells have identified cells expressing ALP and OC also in area surrounding calcified nodules or sparse in the culture.[131, 147, 181]

The possible relevance of Pi during valve calcification is highlighted by previous *in vitro* studies that used phosphate donors (such as ß-glycerolphosphate) to promote calcium deposition by VIC.[152] During cell-driven calcification of extracellular matrix, Pi can favour the phenotypic acquisition of an osteoblast-like profile[76] and promote the activation of apoptotic program.[182] In our model, Pi alone (2.4 mmol/L final concentration) was not able to promote calcium deposition when the cells were grown on plastics. However, when *Clone 1* was seeded on collagen scaffolds the treatment with Pi was sufficient to induce the calcification of the matrix, accompanied by apoptosis of clonal cells. Apoptotic cell death can also explain the reduced ALP activity expression observed in uncloned cells treated with LPS plus Pi, compared to LPS alone (see Figure 8). The beginning of calcium deposition observed in the mixed population after 12 days is mirrored by apoptotic cell death, mainly confined to the cells expressing ALP. Previous studies carried out in both VIC and SMC have demonstrated that blocking apoptosis pathways can abate the *in vitro* process of calcification,[131, 182] suggesting that apoptosis could represent the final differentiation stage of cells acquiring a pro-calcific profile (as it happens at the end of chondrocyte maturation). Apoptosis cannot be considered *per se* a trigger for calcific degeneration of

vascular tissues as suggested by Clarke et al. who showed that the chronic induction of apoptosis in vascular SMC was not accompanied *in vivo* by calcium deposition in the arterial wall.[132] Nevertheless, increase in calcium deposition can be observed when apoptosis is induced in the context of atherosclerotic lesions, suggesting that the micro-environment and cell phenotypic modifications are the major determinants for the induction of calcium deposition.[132] The interplay among Pi, activation of apoptotic pathways and matrix mineralization in our valve cells certainly deserves a further study.

Proteomic analysis allowed the identification of several *Clone 1* cytosolic proteins showing significant modification in the expression level after treatment with endotoxin. This includes proteins involved in cytosolic structural, metabolic and signaling functions, such as: i) chaperone-protein folding, ii) protein metabolism and transport, iii) cell redox homeostasis, iv) energy metabolism, and v) cytoskeletal organization. These results suggest that the acquisition of a pro-calcific profile by *Clone 1* cells is characterized by profound modification in functional and structural properties of the cells. Interestingly, among the proteins showing significant downregulation after treatment with LPS, we identified antioxidants molecules, such SOD[Cu-Zn] and thioredoxin. On these bases, we tested the ROS release by the *Clone 1* cells treated again with LPS, and observed a significant ROS production by the stimulated clonal cells. As mentioned above, increased ROS generation and reduced antioxidant mechanisms (including SOD[Cu-Zn]) have been recently shown inside human calcified valves.[157] Thus, our data suggest that VIC cells acquiring the pro-calcific profile could play a key role in generating the redox status unbalance observed in the calcified leaflets. ROS are also considered potential mediators for the acquisition of an osteoblast-like profile by vascular cells.[158, 160] Further studies are needed to clarify whether blocking ROS generation could hamper the pro-calficic differentiation of *Clone 1* cells.

ADMA is an endogenous, competitive inhibitor of NOS, which activity is essential to maintain endothelium-dependent vasodilatation by NO.[183] Several epidemiological studies showed that ADMA circulating levels are powerful independent predictors of cardiovascular mortality, probably due to the inhibitory effects on NOS activity.[184] Intracellular production of ADMA is derived from breakdown of protein containing methylated arginine residues, which are largely found in the nucleolus and are involved in RNA processing and transcriptional control. ADMA may be metabolized by DDAH or excreted in the urine. DDAH is found in every cell type and its reduced activity has been implicated in the impaired endothelial function observed in vascular disease.[185] Our observation of reduced DDAH expression in *Clone 1* cells acquiring a pro-calcific phenotype suggests a potential role for ADMA in modulating NOS bioactivity and cell redox homeostasis. In line with this hypothesis, NOS uncoupling have been recently implicated in the ROS generation documented inside human calcific valves,[157] while ADMA circulating levels have been linked to the presence of aortic stenosis.[186] Further studies will clarify the potential link between ADMA metabolism and the acquisition of the pro-calcific profile by *Clone 1* cells.

Human VIC express functional toll-like receptor (TLR) 2 and 4 and acquire an osteoblast-like phenotype following stimulation with LPS.[150] Indeed, we suggest that endotoxin, and probably other activators of TLR pathway, can be listed as promoters of valve calcification. Some studies hypothesized that microorganisms could promote calcific valve degeneration by inducing recurrent/low-grade endocarditis.[9, 153] Our data showing that LPS favour the acquisition of a pro-calcific profile by BVIC is in line with this hypothesis and open a new scenario on the role of microorganisms as possible players of valve calcification. We are currently investigating whether the phenotypical changes in *Clone 1*

treated with LPS are a direct consequence of TLR-pathway modulation of calcification-related genes or can be attributed to an autocrine effect by secreted inflammatory molecules (such as TNFα and ILs).

All these data have been obtained using bovine VIC. How these results can be transferred to human valve pathology? It is a matter of fact that, in the last few years, the bovine system has been preferentially used for investigating mechanisms of vascular calcification. CVC identified by Demer[68, 90, 92, 94] and pericytes[95, 96, 181] described by Canfield are both derived from bovine tissues (aorta and retina respectively). It cannot be excluded that some inherent specie-specific properties of bovine cells could explain the higher propensity towards calcification observed using these cell types. In addition, *in vitro* cultures of bovine cells are usually easier in term of cells viability and proliferative capacity, thus allowing the execution of clonal studies (as performed in our study). As mentioned above, a possible pro-calcific effect of LPS treatment in human VIC has been recently demonstrated by Meng et al.,[150] confirming our findings. However, these authors reported an increase in BMP-2 and Runx2 in VIC treated with LPS, without mentioning potential effects on ALP activity and calcium deposition. Even if ALP is under control of Runx2, it cannot be excluded that the human VIC are endowed, at least *in vitro*, with a lower ability of producing ALP, which is followed by reduced mineralizing capacity. On the contrary, ALP can be easily induced in bovine cells, as observed with CVC, pericytes and *Clone 1*. The higher *in vitro* production of ALP by these cells could furnish a valid explanation for the higher propensity towards calcium deposition observed using the bovine cellular system. Nevertheless, the identification of factors and pathways promoting the acquisition of the pro-calcific profile by the bovine cells can be of interest for selecting mechanisms deserving further investigation in humans. One of the major limits of our *in*

vitro model is the lack of information about the relative contribution to valve calcification by ECM remodeling, cellular remnants/debris and lipid insulation, all of which are observed in human pathological valve.

In conclusion, we observed that BVIC clonal subpopulations exhibit different calcifying potential in response to pathogenic factors. Specifically we identified a peculiar subpopulation of BVIC characterized by a fibroblast-like phenotype that express osteoblast-like markers and promote collagen-matrix calcification in response to endotoxin and elevated phosphate levels. Elucidation of the mechanisms, receptors and pathways involved in the BVIC acquisition of a pro-calcific profile may help to develop specific treatment strategies.

12. References

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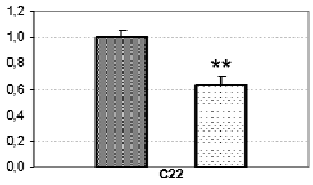
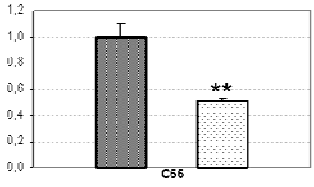
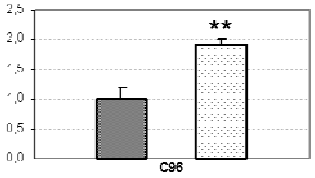
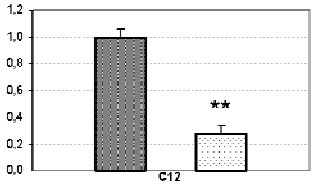
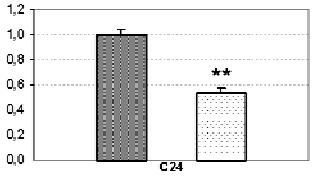
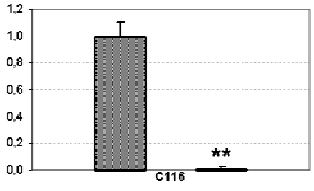
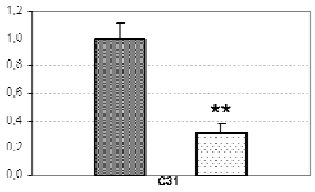
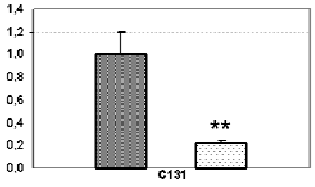
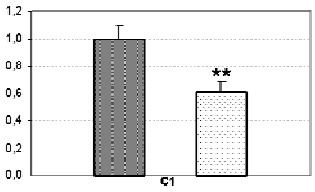
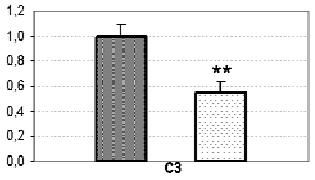
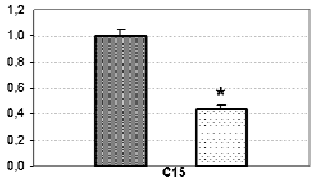
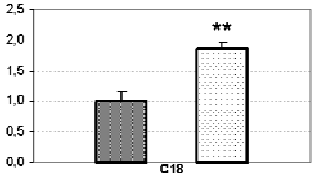
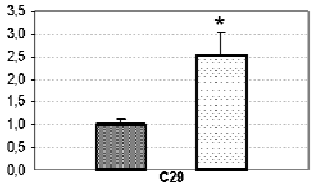
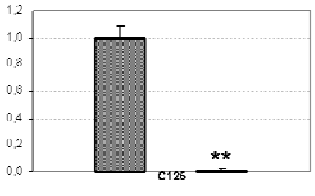
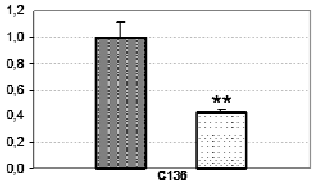
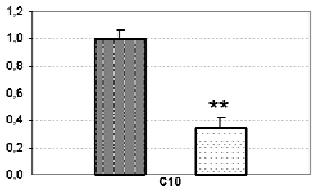
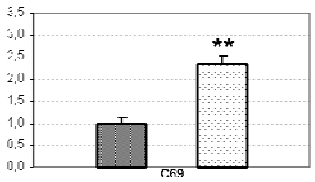
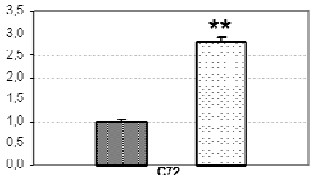
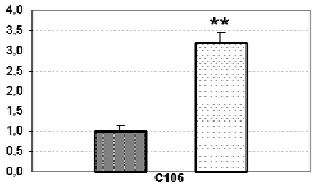
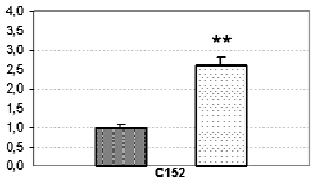
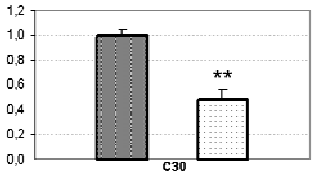
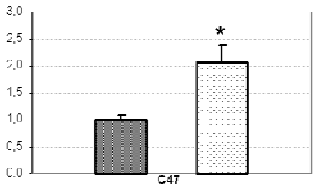
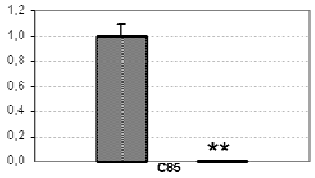
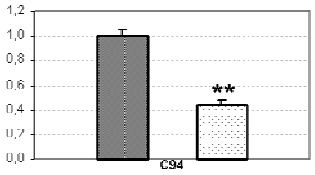
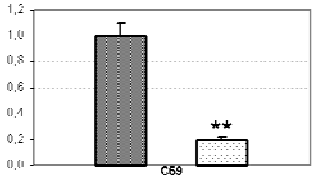
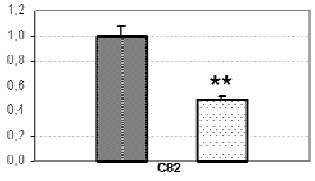
Appendix

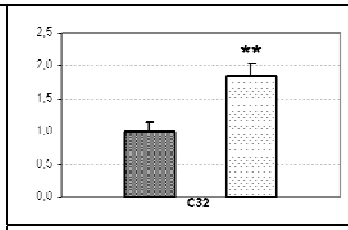
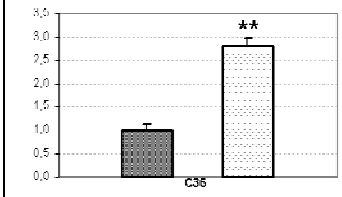
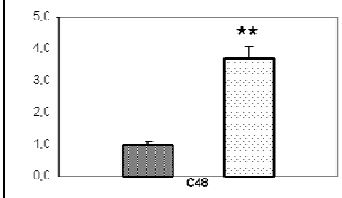
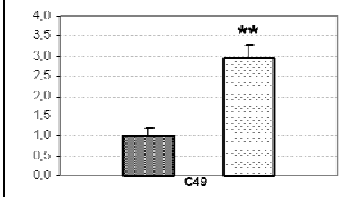
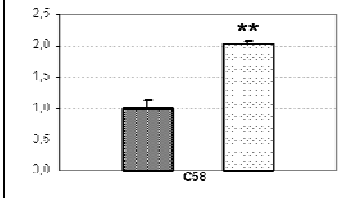
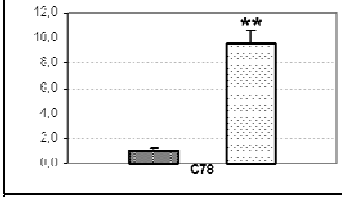
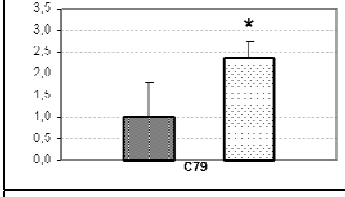
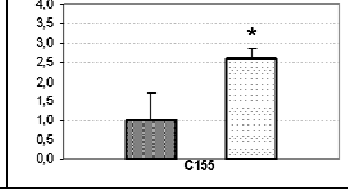
Cytosolic proteins from Clone 1 cells showing significant changes after 12 days of treatment with LPS (white columns) or control (gray columns) (*p<0.05; **p<0.01).

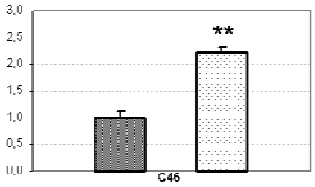
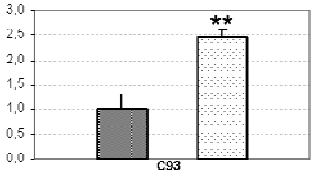
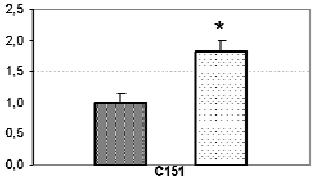
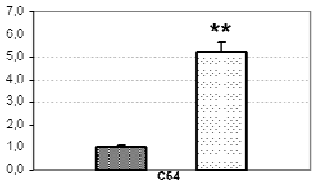
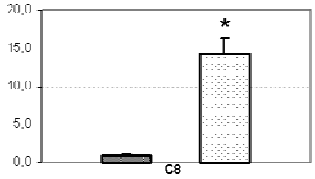
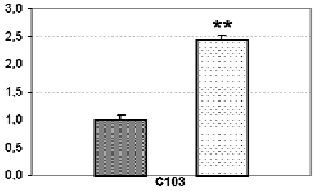
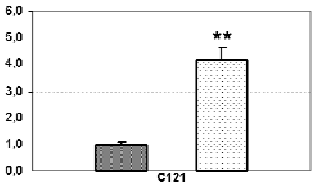
Spot	Protein name (alternative name)	Swissprot code / Gene name	Organism	Quantitative analysis (fold-change compared to control)	Function
CHAPERONE- PROTEIN FOLDING					
C22	**60kDa Heat shock protein** (Hsp60) (60 kDa chaperonin) (Chaperonin 60) (CPN60) (Mitochondrial matrix protein P1)	P31081 HSPD1	Bos taurus		Implicated in mitochondrial protein import and macromolecular assembly. May facilitate the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.
C55	**Heat shock protein beta-6** (HspB6) (Heat shock 20 kDa-like protein p20)	Q148F8 HSPB6	Bos taurus		HspB6 belong to a highly conserved family of stress response proteins. These proteins function primarily as molecular chaperones, facilitating the folding of other cellular proteins, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways. HspB6 is also associated with actin and modulates smooth muscle relaxation.
C96	**70 kDa Heat shock protein 1B** (HSP70.2)	Q27965 HSPA1B	Bos taurus		In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles.
C12	**78 kDa glucose-regulated protein** (GRP 78) (Heat shock 70 kDa protein 5)	Q0VCX2 HSPA5	Bos taurus		Probably plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
C24	**Stress-70 protein** (75 kDa glucose-regulated protein) (GRP 75) (Heat shock 70 kDa protein 9)	Q3ZCH0 HSPA9	Bos taurus		Mitochondrial protein implicated in the control of cell proliferation and cellular aging. May also act as a chaperone.
C116					

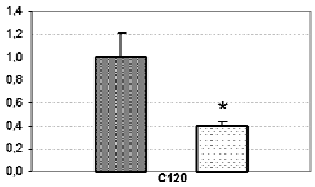
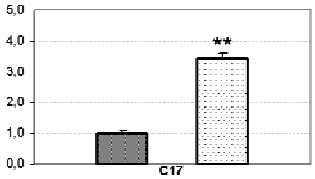
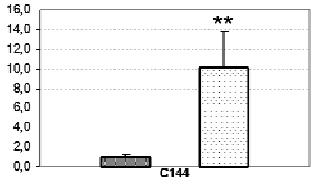
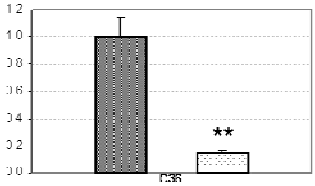
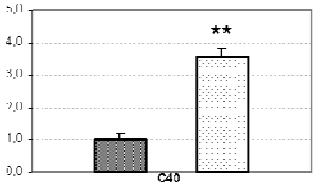
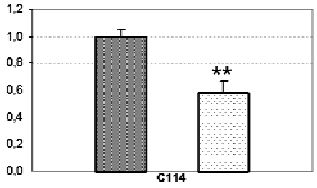
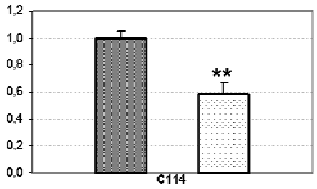
C31	**Stress-induced-phosphoprotein 1** (STI1) (Hsc70/Hsp90-organizing protein) (Hop)	Q3ZBZ8 STIP1	Bos taurus		Mediates the association of the molecular chaperones HSC70 and HSP90 (HSPCA and HSPCB).
C131	**Calreticulin** (CRP55) (Calregulin) (HACBP)	P52193 CALR	Bos taurus		Molecular calcium binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglucosylated glycoproteins that are synthesized in the ER. Interacts with the DNA-binding domain of NR3C1 and mediates its nuclear export.
PROTEIN METABOLISM AND TRANSPORT					
C1	**Calumenin**	Q3T0K1 CALU	Bos taurus		Involved in regulation of vitamin K-dependent carboxylation of multiple aminoterminal glutamate residues. Seems to inhibit γ-carboxylase GGCX. Binds 7 calcium ions with a low affinity.
C3	**Calumenin** isoform 1 (Crocalbin) (IEF SSP 9302)	O43852-1 CALU	Homo sapiens		Involved in regulation of vitamin K-dependent carboxylation of multiple aminoterminal glutamate residues. Seems to inhibit γ-carboxylase GGCX. Binds 7 calcium ions with a low affinity.
C15	**Transitional endoplasmic reticulum ATPase** (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin-containing protein) (VCP)	Q3ZBT1 VCP	Bos taurus		Necessary for the fragmentation of Golgi stacks during mitosis and for their reassembly after mitosis. Involved in the formation of the transitional endoplasmic reticulum (tER).
C18					
C29	**Cytosol aminopeptidase, isoform 1** (Leucyl aminopeptidase) (Leucine aminopeptidase3) (LAP) (Proline aminopeptidase) (Prolyl aminopeptidase) (Peptidase S)	P00727-1 LAP3	Bos taurus		Presumably involved in the processing and regular turnover of intracellular proteins. Catalyzes the removal of unsubstituted N-terminal amino acids from various peptides.

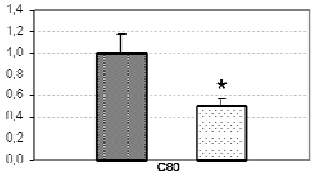
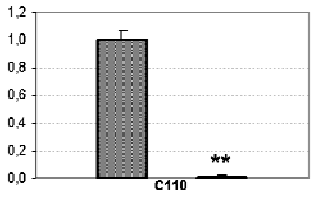
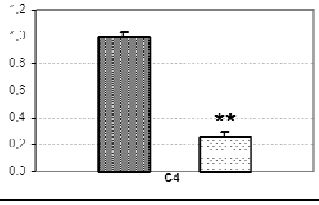
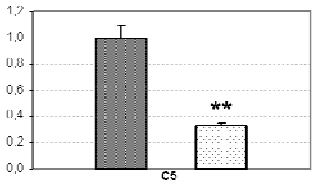
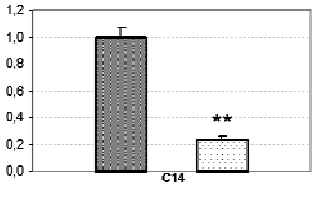
C125	**Fatty acid-binding protein** (AFABP) (Fatty acid-binding protein 4) (Adipocyte lipid-binding protein) (ALBP)	P48035 FABP4	Bos taurus		Lipid transport protein in adipocytes. Binds both long chain fatty acids and retinoic acid. Delivers long-chain fatty acids and retinoic acid to their cognate receptors in the nucleus. Interacts with PPARγ.
C136	**Ubiquitin carboxyl-terminal hydrolase isozyme L1** (UCH-L1) (Ubiquitin thioesterase L1) (Neuron cytoplasmic protein 9.5) (PGP 9.5)	P23356 UCHL1	Bos taurus		Ubiquitin-protein hydrolase involved both in the processing of ubiquitin precursors and of ubiquitinated proteins. This enzyme is a thiol protease that recognizes and hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. Also binds to free monoubiquitin and may prevent its degradation in lysosomes. The homodimer may have ATP-independent ubiquitin ligase activity.
CELL REDOX HOMEOSTASIS					
C10	**Protein disulfide-isomerase** (Prolyl 4-hydroxylase subunit beta) (Cellular thyroid hormone-binding protein)	P05307 P4HB	Bos taurus		PDI is a member of the thioredoxin superfamily, a large family of dithiol/disulfide oxidoreductases. Contains two thioredoxin domains. This multifunctional protein catalyzes the formation, breakage and rearrangement of disulfide bonds. At the cell surface, seems to act as a reductase that cleaves disulfide bonds of proteins attached to the cell. May therefore cause structural modifications of exofacial proteins. Inside the cell, seems to form/rearrange disulfide bonds of nascent proteins. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, facilitates aggregation (antichaperone). May be involved with other chaperones in the structural modification of the TG precursor in hormone biogenesis. Also acts a structural subunit of various enzymes such as prolyl 4-hydroxylase and microsomal triacylglycerol transfer protein MTTP.
C69					
C72					
C106					
C152					

C30	**Protein disulfide-isomerase A3** (Disulfide isomerase ER-60) (ERP60) (58 kDa microsomal protein) (p58) (ERp57)	P38657 PDIA3	Bos taurus		Catalyzes the rearrangement of -S-S- bonds in proteins. Contains two thioredoxin domains.
C47	**Protein disulfide-isomerase A6** (Thioredoxin domain-containing protein 7)	Q15084 PDIA6	Homo sapiens		Catalyzes the rearrangement of -S-S- bonds in proteins. Contains two thioredoxin domains.
C85	**Superoxide dismutase [Cu-Zn]**	P00442 SOD1	Bos taurus		Destroys radicals which are normally produced within the cells and which are toxic to biological systems.
C94	**Thioredoxin** (Trx)	O97680 TNX	Bos taurus		Participates in various redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions.
C59	**N(G),N(G)-dimethylarginine dimethylaminohydrolase 1** (Dimethylarginine dimethylaminohydrolase 1) (DDAH-1) (DDAHI) (Dimethylargininase-1)	P56965 DDAH1	Bos taurus		Hydrolyzes N(G),N(G)-dimethyl-L-arginine (ADMA) and N(G)-monomethyl-L-arginine (MMA) which act as inhibitors of NOS. Has therefore a role in nitric oxide generation.
C82	**N(G),N(G)-dimethylarginine dimethylaminohydrolase 2** (Dimethylarginine dimethylaminohydrolase 2) (DDAH-2) (DDAHII) (Dimethylargininase-2)	Q3SX44 DDAH2	Bos taurus		Hydrolyzes N(G),N(G)-dimethyl-L-arginine (ADMA) and N(G)-monomethyl-L-arginine (MMA) which act as inhibitors of NOS. Has therefore a role in nitric oxide generation.

ENERGY METABOLISM					
C32	**Alpha enolase** (2-phospho-D-glycerate hydro-lyase) (Non- neural enolase) (Phosphopyruvate hydratase) (Enolase 1) (HAP47)	Q9XSJ4 ENO1	Bos taurus		Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons.
C35					
C48					
C49					
C58					
C78					
C79					
C155					

C46	**Phosphoglycerate kinase 1**	Q3T0P6 PGK1	Bos taurus		Glycolytic enzyme that transfers a phosphate group from 1,3-biphosphoglycerate to ADP, forming ATP and 3-Phosphoglycerate.
C93					
C151					
C54	**Triosephosphate isomerase** (Triose-phosphate isomerase) (TIM)	Q5E956 TPI1	Bos taurus		Glycolytic enzyme that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Involved in other pathways as fatty acid biosynthesis, gluconeogenesis, lipid synthesis.
CYTOSKELETON AND RELATED PROTEINS					
C8	**Vimentin**	P48616 VIM	Bos taurus		Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells.
C103					
C121					

C120	**Actin, cytoplasmic 1** (Beta-actin)	P60712 ACTB	Bos taurus		Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
C17	**Alpha-actinin-4** (Non-muscle alpha-actinin 4) (F-actin cross-linking protein)	A5D7D1 ACTN4	Bos taurus		F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures. This is a bundling protein.
C144					
C36	**Macrophage-capping protein** (Actin regulatory protein CAP-G)	Q865V6 CAPG	Bos taurus		Calcium-sensitive protein which reversibly blocks the barbed ends of actin filaments but does not sever preformed actin filaments. May play an important role in macrophage function. May play a role in regulating cytoplasmic and/or nuclear structures through potential interactions with actin. May bind DNA.
C40	**Fascin** (Singed-like protein) (55 kDa actin-bundling protein)	Q16658 FSCN1	Homo sapiens		Organizes filamentous actin into bundles with a minimum of 4.1:1 actin/fascin ratio. Probably involved in the assembly of actin filament bundles present in microspikes, membrane ruffles, and stress fibers.
C114	**Profilin-1** (Profilin I)	P02584 PFN1	Bos taurus		Binds to actin and affects the structure of the cytoskeleton. At high concentrations, profilin prevents the polymerization of actin, whereas it enhances it at low concentrations. By binding to PIP2, it inhibits the formation of IP3 and DG.
C64	**Dihydropyrimidinase-related protein 2** (DRP-2) (Neural-specific protein NSP60)	O02675 DPYSL2	Bos taurus		Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, neuronal growth cone collapse and cell migration.

NUCLEIC ACID METABOLISM					
C80	**Heterogeneous nuclear ribonucleoprotein K** (hnRNP K)	Q3T0D0 HNRNPK	Bos taurus		One of the major pre-mRNA-binding proteins. Binds tenaciously to poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences. Can also bind poly(C) single-stranded DNA.
C110	**Elongation factor 1-beta** (EF-1-beta)	Q5E983 EEF1B	Bos taurus		EF-1-β and EF-1-Δ stimulate the exchange of GDP bound to EF-1-alpha to GTP.
SIGNAL TRANSDUCTION					
C4	**Reticulocalbin-1**	Q15293 RCN1	Homo sapiens		May regulate calcium-dependent activities in the endoplasmic reticulum lumen or post-ER compartment.
C5					
C14	**Neuroblast differentiation-associated protein AHNAK** (Desmoyokin)	Q09666 AHNAK	Homo sapiens		Function unknown. May be required for neuronal cell differentiation.
C139