

Identification and Characterization of Aortic Valve Mesenchymal Progenitor Cells with Robust Osteogenic Calcification Potential

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Advanced valvular lesions often contain ectopic mesenchymal tissues, which may be elaborated by an unidentified multipotent progenitor subpopulation within the valve interstitium. The identity, frequency, and differentiation potential of the putative progenitor subpopulation are unknown. The objectives of this study were to determine whether valve interstitial cells (VICs) contain a subpopulation of multipotent mesenchymal progenitor cells, to measure the frequencies of the mesenchymal progenitors and osteoprogenitors, and to characterize the osteoprogenitor subpopulation because of its potential role in calcific aortic valve disease. The multilineage potential of freshly isolated and subcultured porcine aortic VICs was tested *in vitro*. Progenitor frequencies and self-renewal capacity were determined by limiting dilution and colony-forming unit assays. VICs were inducible to osteogenic, adipogenic, chondrogenic, and myofibroblastic lineages. Osteogenic differentiation was also observed *in situ* in sclerotic porcine leaflets. Primary VICs had strikingly high frequencies of mesenchymal progenitors ($48.0 \pm 5.7\%$) and osteoprogenitors ($44.1 \pm 12.0\%$). High frequencies were maintained for up to six population doublings, but decreased after nine population doublings to $28.2 \pm 9.9\%$ and $5.8 \pm 1.3\%$, for mesenchymal progenitors and osteoprogenitors, respectively. We further identified the putative osteoprogenitor subpopulation as morphologically distinct cells that occur at high frequency, self-renew, and elaborate bone matrix from single cells. These findings demonstrate that the aortic valve is rich in a mesenchymal progenitor cell population that has strong poten-

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Calcific aortic valve disease is characterized by thickening and calcification of the valve leaflets.¹ Ultimately, structural and compositional changes in the valve extracellular matrix can lead to stenotic valves that are dysfunctional and must be replaced surgically. Valve calcification occurs through multiple, non-mutually exclusive mechanisms.^{2,3} Calcium accumulation in dying cells and lipoprotein deposits contributes to valve matrix mineralization in many instances.^{2,4} This dystrophic process is often accompanied by the active formation of ectopic bone and other mesenchymal tissues. For example, lamellar bone with osteoblasts and osteocytes is observed in 11% to 13% of calcified human aortic valves.^{4,5} The marrow of this heterotopic bone contains adipose tissue.^{4,5} Chondrocytes, cartilage, and endochondral ossification have been observed in both human and mouse aortic valves.^{4,6} Late-stage diseased valves are frequently fibrotic with an abundance of myofibroblasts, often observed adjacent to calcified regions.^{7–9}

The source of ectopic mesenchymal cell types and tissues in cardiac valves is unknown. The interstitium of the normal aortic valve is populated primarily by fibroblasts and a small percentage of smooth muscle cells and myofibroblasts.^{7,8,10,11} While this cell population, collectively referred to as valve interstitial cells (VICs), is recognized to be heterogeneous,^{11–14} the identity and characteristics of various VIC subpopulations are generally unknown. In the vasculature, mesenchymal progen-

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itor subpopulations have been identified within smooth muscle cells¹⁵ and pericytes.^{16–20} These vascular progenitors are able to differentiate, with varying capacities, to multiple mesenchymal lineages including osteogenic, chondrogenic, adipogenic, and myogenic. Recently, Liu et al¹² hypothesized the presence of progenitor VIC and osteoblastic VIC subpopulations that may contribute to valve repair and calcification; however, the identity, frequency, and differentiation potential of such subpopulations remain unknown.

Under appropriate conditions, VICs form calcified nodules in culture.²¹ The mechanisms by which calcified nodules form are not clearly defined. In some cases, such as in response to transforming growth factor- β , calcification *in vitro* is rapid and dystrophic, driven by apoptosis of VICs and involving alkaline phosphatase activity.^{22,23} Apoptotic cells and transforming growth factor- β are also associated with dystrophic valve calcification *in vivo*.^{22–24} Conversely, evidence of bone-related matrix proteins,^{21,25,26} transcription factors,²⁶ and mineral²¹ in VIC cultures suggests that calcification can occur *in vitro* via an osteogenic process, which is consistent with evidence *in vivo*.^{4,5,27,28} However, even in VIC cultures that express bone markers, calcified nodules are reported to contain a core of dead cells,²¹ which is not observed in bone nodules formed by osteoprogenitors harvested from established sources.^{29–31} Therefore, the osteogenic potential of VICs has not been established definitively, and the identity and frequency of the putative osteoprogenitor subpopulation are not defined.

To determine whether the aortic valve contains a subpopulation of mesenchymal progenitor cells, we tested the multilineage potential of freshly isolated and subcultured porcine aortic VICs. We confirmed that VICs have osteogenic potential *in vitro* and *in vivo*, as well as adipogenic, chondrogenic, and myofibroblastic differentiation capacity. By limiting dilution and clonal analyses, we found a high frequency of mesenchymal progenitor cells with limited self-renewal capacity. Further, we identified a morphologically distinct subpopulation that is highly enriched for osteoprogenitors, occurs at high frequency, self-renews, and elaborates bone matrix from single cells. These novel findings suggest that the aortic valve is rich in a multipotent mesenchymal progenitor cell population that is distinct from other vascular progenitor cells and has the capacity to contribute to valve calcification.

Materials and Methods

Unless otherwise noted, all reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). Animal protocols were approved by the Animal Care Committee of the University of Toronto and conducted according to their guidelines.

VIC Isolation

Hearts were obtained from 8-month-old pigs from a local abattoir (Quality Meat Packers, Toronto, ON). For each experiment, VICs were isolated from a minimum of eight

hearts. Experiments were repeated at least three times for each study. Aortic valve leaflets were excised and rinsed in PBS containing 1% penicillin and streptomycin, and 1% amphotericin B. Rinsed leaflets were placed in collagenase (150 units/ml) in *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer with 0.36 mmol/L calcium chloride (pH = 7.4) for 20 minutes at 37°C and then 7 minutes in 0.125% trypsin with EDTA at 37°C. Leaflets were vortexed and lightly scraped on both surfaces to remove endothelial cells. Tissues were then minced and placed in collagenase (150 units/ml) in PBS for 2 hours at 37°C. After collagenase digestion, a cell suspension was obtained by removing undigested tissue pieces with a 70 μ m cell strainer. A volume of complete medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum [Hyclone, Logan, UT] and 1% penicillin and streptomycin) equal to the cell suspension volume was added and centrifuged at 284 \times *g*. Supernatant was removed and the cells were resuspended in complete medium. Viable cells were counted using a Vi-Cell cell viability analyzer (Beckman Coulter, Mississauga, ON).

VIC Culture

VICs were routinely maintained in complete media at 37°C in a humidified incubator with 5% CO₂ in air. For passage-dependent experiments, VICs were seeded at 10,000 cells/cm² in tissue culture flasks in complete medium, which was changed every 2 days, until VICs were about 90% confluent. VICs were trypsinized with 0.125% trypsin with EDTA for experiments and further subculture. To induce osteogenic differentiation of primary and subcultured VICs, 10 mmol/L β -glycerophosphate, 10 μ g/ml ascorbic acid, and 10 nmol/L dexamethasone were added to the complete medium 2 days after VICs were plated, and this osteogenic medium was changed every 2 days for 21 days. To induce adipogenesis, primary VICs were cultured in adipogenic medium comprising complete medium, 33 μ mol/L biotin, 17 μ mol/L pantothenate, 5 μ mol/L rosiglitazone (Cayman Chemical, Ann Arbor, MI), 100 nmol/L bovine insulin, 1 μ mol/L dexamethasone, and 200 μ mol/L isobutyl methylxanthine. The adipogenic medium was changed three times a week until adipocytes were observed under a light microscope. To induce chondrogenesis, 2.5 \times 10⁵ of primary VICs were placed into a 15-ml tube and centrifuged at 284 \times *g* for 5 minutes to form a cell pellet. The media was removed and replaced with 0.5 ml chondrogenic medium comprised of complete medium and 10 ng/ml transforming growth factor- β 1 (Chemicon, Temecula, CA). The chondrogenic medium was replaced every 4 days for 21 days. To induce myofibroblastic differentiation, primary VICs were plated at 10,000 cells/cm² and grown in complete medium on glass coverslips for up to 6 days.

Alkaline Phosphatase and von Kossa Staining

To stain for alkaline phosphatase (ALP), VICs were fixed in 10% neutral-buffered formalin (NBF) and rinsed in distilled water. VICs were stained using Naphthol AS MX

phosphate as the substrate, fast red violet LB salt, and 0.2 M/L Tris hydrochloride (pH 8.3). The stained samples were rinsed with distilled water three times and examined under a light microscope. To show the presence of calcium salts, von Kossa staining was performed. Briefly, VICs were fixed in 10% NBF, rinsed in distilled water, and then treated with 2.5% silver nitrate solution under bright light.

Osteocalcin Immunohistochemical Staining

Immunohistochemical staining for osteocalcin was performed using Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA). Samples were fixed in 10% NBF and washed with 0.05% Tween 20 in PBS. Following fixation, samples were treated with 3% hydrogen peroxide in methanol at room temperature for 10 minutes, blocked with horse serum for 20 minutes at room temperature, and then incubated for 3 hours at room temperature with 20 μ g/ml mouse anti-bovine osteocalcin antibody (clone OCG4; Affinity BioReagents, Golden, CO) diluted in 0.3% Triton X-100 in PBS. Secondary biotinylated antibody and 3,3'-diaminobenzidine substrate were then applied. The stained samples were dehydrated in an ethanol gradient.

Apoptosis

Apoptotic cells were detected using the APOPercentage apoptosis assay kit (Bicolor Ltd, Carrickfergus, UK). Briefly, samples were stained with APOPercentage dye diluted 1:20 in complete medium for 30 minutes at 37°C. Apoptotic cells stained purple. As a positive control for apoptosis, samples were treated with 5 mmol/L hydrogen peroxide for 3.5 hours at 37°C before staining; intense staining was observed confirming the ability of this method to detect apoptosis in VICs.

Scanning Electron Microscopy

Samples were fixed in 10% NBF and dehydrated by a series of ethanol washes (30%, 50%, 70%, 95% and 100% ethanol). The samples were critical point dried with liquid carbon dioxide in a Polaron CPD7501, mounted on aluminum stubs, and sputter-coated with gold using a Polaron SC 515 Scanning Electron Microscopy Coating System. Coated samples were then examined using a scanning electron microscope (Model S-2500; Hitachi Instrument).

Transmission Electron Microscopy

Cultures were fixed in a Karnovsky style fixative (2.5% glutaraldehyde, 4% paraformaldehyde, 5 mmol/L calcium chloride in 0.1 M/L Sorenson's phosphate buffer at pH 7.0) for 1 hour, then washed with phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M/L phosphate buffer for 30 minutes at room temperature. The fixed samples were then washed with water, dehydrated in an ethanol gradient, embedded in Epon 812 resin, and heated for 48 hours at 60°C. The epoxy disk was re-

moved from the culture plate, re-embedded in Epon 812 and the area of interest was cut out with a fine saw and mounted onto a blank plastic block. Blocks were sectioned on a Reichart Ultracut E ultramicrotome. Sections were collected on copper grids and stained with saturated uranyl acetate for 10 to 15 minutes followed by Reynold's lead citrate for 10 to 15 minutes. Grids were then examined with a Hitachi H7000 transmission electron microscope operating at 75 kV. For electron diffraction studies, 200 nm thick sections were collected and examined unstained in an FEI (Philips) T20-Technai transmission electron microscope operating at 100 kV. Note that in thinner sections (~80 nm) the amount of mineral was not sufficient to produce a detectable diffraction pattern. Diffraction patterns were calibrated against a gold standard and measured in Adobe Photoshop.

Reverse Transcription-PCR

Total RNA was isolated from samples following standard protocols of the Micro RNeasy Kit (Qiagen, Mississauga, ON). Subsequently, mRNA from 500 ng of total RNA was reverse-transcribed using Superscript Reverse Transcriptase III (Invitrogen, Burlington, ON), and oligo-(dT)-12 to -18 primers (Invitrogen, Burlington, ON). Three-step PCR was then performed. Amplification of genes of interest (Table 1) was evaluated by agarose gel electrophoresis. Uninduced VICs (primary VICs cultured in complete medium for 2 to 4 days after cell isolation) were used as negative controls.

Immunostaining of Porcine Aortic Valve Leaflets

Aortic valve leaflets were obtained from normal pigs or hypercholesterolemic pigs fed an atherogenic diet (0.5% cholesterol, 10% lard, and 1.5% sodium cholate) for 6 months as described.³² This diet induces the formation of lesions in the fibrosa of the aortic valves that are rich in lipids and are calcified focally, similar to human lesions.³³ Leaflets were removed, fixed in 10% NBF, and paraffin-embedded. For immunostaining for Runx2 expression, sections were dewaxed, and rehydrated, and antigen retrieval was done with 0.1% (w/v) trypsin for 30 minutes at 37°C. Sections were blocked in 3% bovine serum albumin in PBS for 20 minutes at 37°C, and then incubated for three hours at room temperature on a shaker with polyclonal rabbit anti-mouse Runx2 (M-70) primary antibody (sc-10758; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) diluted 1:10 in 0.3% triton/PBS. Sections were washed four times with PBS/tween, blocked with 10% goat serum for 30 minutes at room temperature, and incubated with goat anti-rabbit IgG AlexaFluor 568 (Invitrogen, Burlington, ON; 1:100 dilution in 10% goat serum) for 30 minutes at room temperature. The cell nuclei were counterstained with Hoechst 33258.

Oil Red O Staining

VICs cultured in adipogenic media were fixed with 10% NBF, rinsed with PBS, and rinsed again with 20% isopro-

Table 1. Porcine PCR Primer Sequences

Gene	Accession number	Oligonucleotides	Product size (bp)
<i>Osteocalcin</i>	AW346755	5'-TCAACCCCGACTGCGACGAG-3' 5'-TTGGAGCAGCTGGGATGATGG-3'	204
<i>Osteonectin</i>	AW436132	5'-TCCGGATCTTTCCTTTGCTTTCTA-3' 5'-CCTTCACATCGTGGCAAGAGTTTG-3'	187
<i>Runx2</i>	TC248152*	5'-CCCTTGGTCTCCATTTCTCA-3' 5'-CCCAGACCTACCGAATCAGA-3'	100
<i>Adipocyte fatty acid-binding protein 2</i>	AF102872	5'-GGCCAAACCCAACTGA-3' 5'-GGGCGCCTCCATCTAAG-3'	167
<i>Peroxisome proliferator-activated receptor γ 2</i>	AF103946	5'-GCGCCCTGGCAAAGCACT-3' 5'-TCCACGGAGCGAAACTGACAC-3'	238
<i>Lipoprotein lipase</i>	AF102859	5'-GCAGGAAGTCTGACCAATAA-3' 5'-CTTACCAGCTGGTCCACAT-3'	294
<i>SRY-box containing gene 9</i>	AF029696	5'-ATCAGTACCCGCACCTGCAC-3' 5'-CTTGTAAATCCGGGTGGTCTT-3'	146
<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	AF017079	5'-TGTACCACCAACTGCTTGGC-3' 5'-GGCATGGACTGTGGTCATGAG-3'	122

*TIGR Tentative Consensus number.

panol. Oil Red O staining solution (0.6% Oil Red O, 59.4% isopropanol and 40% distilled and deionized water) was added to cover the entire culture. After 30 minutes, the stain solution was removed. The culture was then rinsed with 20% isopropanol and washed thoroughly with water.

Alcian Blue Staining

After 3 weeks of culture in chondrogenic media, cell pellets were fixed in 10% NBF, embedded in paraffin, sectioned, and stained with alcian blue 8GS (pH 2.5) to detect proteoglycans.

α -Smooth Muscle Actin Immunostaining

VICs grown in complete media on glass coverslips were fixed in 10% NBF and permeabilized with 0.1% Triton X-100. Following fixation, the cells were blocked with 3% bovine serum albumin in PBS for 20 minutes at 37°C, and then incubated overnight at 4°C with monoclonal mouse anti-human α -SMA antibody (clone 1A4) diluted 1:100 in 3% bovine serum albumin. The next day, cells were washed twice with PBS and blocked with 10% goat serum for 30 minutes at room temperature. AlexaFluor 488 goat anti-mouse IgG (Invitrogen) was then applied for 30 minutes at room temperature. The cell nuclei were counterstained with Hoechst 33258.

Colony Forming Unit-Fibroblast Assay

The frequency of mesenchymal progenitors was estimated using the colony forming unit (CFU)-F assay.³⁴ The mesenchymal progenitor frequency was measured for primary and passaged VICs. Viable VICs were seeded at 5, 10, 20, and 40 cells/well into 24-well tissue culture plates in complete medium. The plates were left undisturbed for 5 days, after which the medium was changed every 2 days. After 10 days of culture, cells were fixed and stained with 1% crystal violet in ethanol. The number of colonies, comprising more than 16 cells, was counted in each well. The average number of colo-

nies for each plating density was calculated and these points were plotted against the number of cells seeded. The plot was then fitted linearly and the slope of the line was taken as the mesenchymal progenitor frequency.

CFU-Osteoprogenitor Assay

The frequencies of osteoprogenitors (CFU-O) in primary isolated and passaged VICs were measured using standard methods based on the Poisson's distribution.^{30,35} Viable VICs were seeded at 5, 10, 20, 50, and 100 cells/well into 96-well plates. The plates were left undisturbed for 3 days and then osteogenic media was added and changed every 2 days for 21 days. The cultures were then fixed in 10% NBF and stained for alkaline phosphatase (ALP). Each well was screened for the presence or absence of ALP-positive bone nodules. The fraction of wells without ALP-positive nodules for each cell plating density was calculated. Based on a Poisson's distribution, the negative natural logarithm of the fraction of wells without nodules (ie, $-\ln$ (fraction of wells without nodules)) is the expected number of osteoprogenitors for a given plating density.³⁵ The expected number of osteoprogenitors was calculated for each plating density and these points were plotted against the number of cells plated per well. The plot was then fitted linearly and the slope of the line was taken as the frequency of osteoprogenitors.

Statistical Analysis

Data are reported as mean \pm SE. Analysis of variance and Fisher's least significant difference (LSD) method were used to test for significant differences in multiple groups comparisons and pair-wise comparisons, respectively.

Results

Osteogenic Differentiation of VICs

The majority of primary VICs had elongated fibroblast-like morphologies at confluence and rarely formed distinct

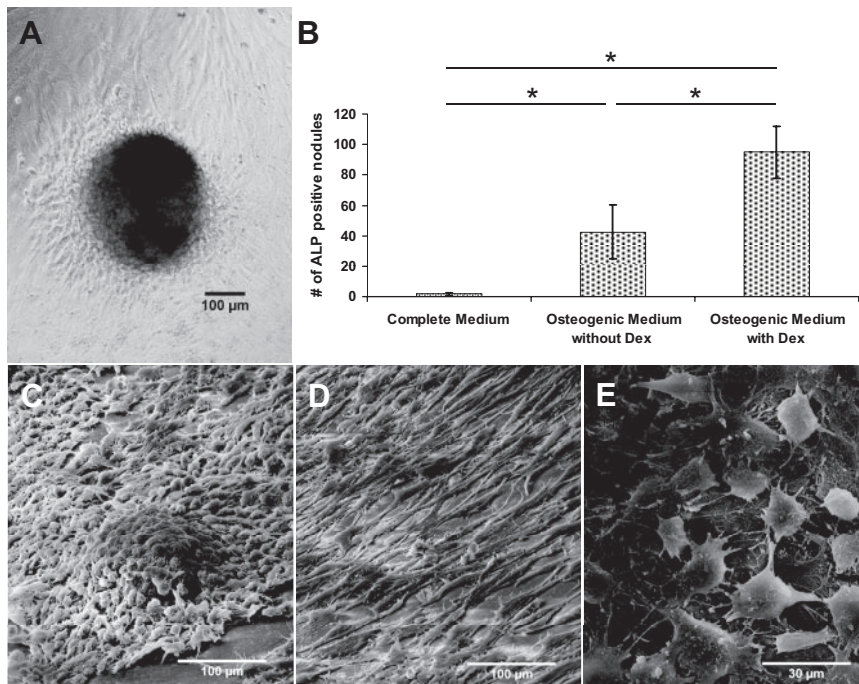


Figure 1. VIC nodule formation *in vitro* is potentiated by osteogenic supplements. **A:** Phase contrast micrograph of a VIC nodule. **B:** After 1 week of culture, ALP-positive nodules formed most frequently in osteogenic medium (complete medium with 10 mmol/L β -glycerophosphate and 10 μ g/ml ascorbic acid) supplemented with 10 nmol/L dexamethasone (Dex). Data are presented as mean \pm SE for $n = 3$ independent samples. $P < 0.05$ by Fisher LSD. Additional experiments showed similar results. **C:** Cells associated with the nodules exhibited cuboidal, osteoblast-like morphologies. **D:** In contrast, cells in the non-nodule forming areas were elongated and fibroblastic. **E:** Cells beneath the nodules appeared viable and well-adhered to synthesized extracellular matrix.

nodules spontaneously in growth media, as described previously.^{13,21} The addition of osteogenic supplements (10 mmol/L β -glycerophosphate, 10 μ g/ml ascorbic acid, and 10 nmol/L dexamethasone) to the culture medium increased both the rate of nodule formation and the number of nodules (Figure 1, A and B). Dexamethasone potentiated nodule formation, as rapid and robust nodule formation was not observed without it. Cells associated within and surrounding the nodules exhibited cuboidal, osteoblast-like morphologies (Figure 1C), whereas cells in the non-nodule forming areas were elongated and fibroblastic (Figure 1D). Cells beneath the nodules appeared viable and well-adhered to synthesized extracellular matrix (Figure 1E).

After 3 weeks of culture in osteogenic media, the nodules were mineralized as indicated by positive von Kossa staining (Figure 2A). Transmission electron microscopy of the cross section of the nodules showed mineral that was in some cases closely apposed to intact, seemingly viable cells, suggesting cell-mediated calcification (Figure 2B). Electron diffraction patterns from mineral deposits in 200-nm thick sections of the nodules showed between two to six rings (Figure 2C) with d-spacings consistent with those of hydroxyapatite (JCPDS 9-0432). Consistent with this being an osteogenic process, the nodules stained positive for ALP activity (Figure 2D) and osteocalcin (Figure 2E); the expression of both markers was predominant in the nodules. Gene expression analysis by PCR showed positive expression for several bone markers including osteocalcin, osteonectin, and runt-related transcription factor 2 (Runx2) (Figure 2G). Cells within the nodules did not take up the APOPercentage dye, indicating that they were not apoptotic (Figure 2F). Together, these data confirm that VICs are capable of osteogenic differentiation and demonstrate that the

nodules formed under these culture conditions mineralize through an osteogenic, not dystrophic, calcification process.

We also examined normal and sclerotic porcine leaflets for osteogenic cells *in situ*. In normal leaflets, VICs immunostained for Runx2 were not evident (inset of Figure 2H). However in sclerotic valves from hypercholesterolemic pigs, there were abundant Runx2-positive cells localized primarily to the lesions (Figure 2H), confirming the osteogenic differentiation potential of interstitial cells *in situ*.

Multilineage Potential of VICs

We next determined whether VICs are able to differentiate to other mesenchymal lineages. To assess adipogenic potential, primary VICs were plated at 20,000 cells/cm² and grown in adipogenic media for two to three weeks. Adipocytes were readily identified morphologically by lipid droplets in the cytoplasm, which was confirmed by Oil Red O staining (Figure 3A). Gene expression analysis by PCR also showed positive expression of several adipogenic markers, including adipocyte fatty acid-binding protein 2, lipoprotein lipase, and peroxisome proliferator-activated receptor γ 2 (Figure 3E). To assess chondrogenic potential, primary VICs were pelleted and grown in chondrogenic media for three weeks. The pellets stained positive for alcian blue (Figure 3B), indicating the presence of proteoglycans. Gene expression analysis by PCR showed that cells in the pellets also expressed chondrogenic transcription factor SRY-box containing gene 9 (Figure 3F). To confirm myofibroblastic potential,^{7,8,11,13,36} primary VICs were plated on tissue culture plastic at 10,000 cells/cm² and grown for up to six

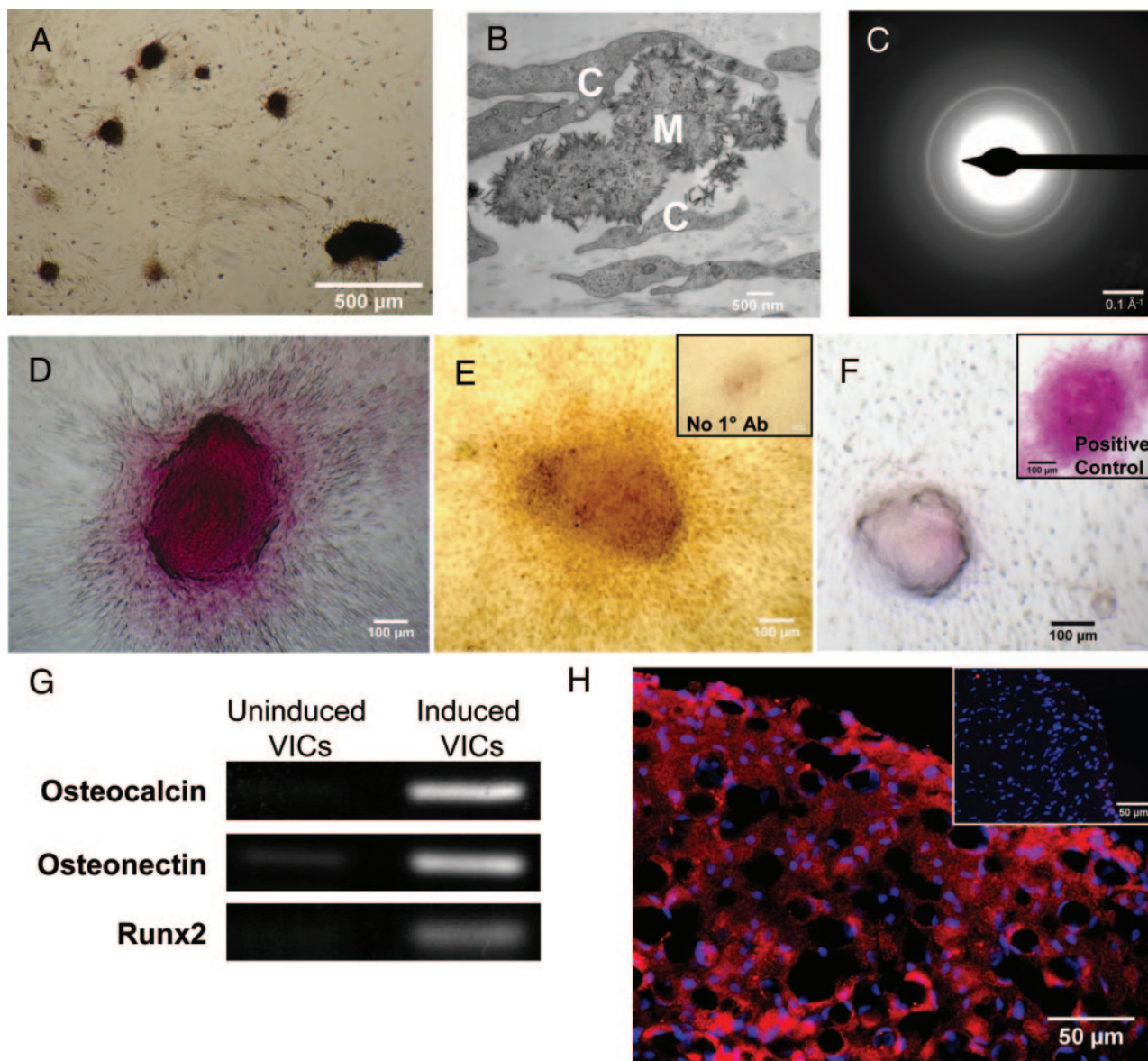
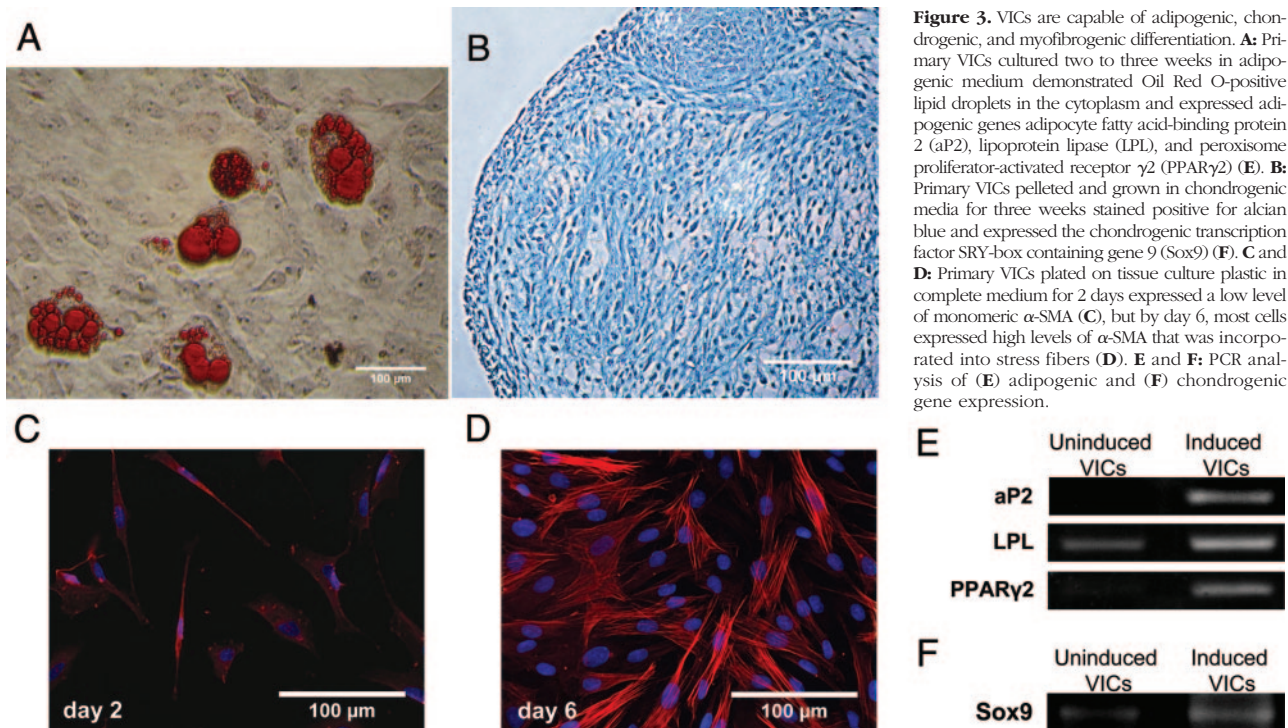


Figure 2. Mineralized nodules are bone-like. **A:** Nodules stained positive for von Kossa, indicating the presence of calcium salts within the nodules. **B:** Transmission electron microscopy of the cross section of a nodule showing mineral closely apposed to intact, viable cells. M: mineral; C: cells. **C:** Electron diffraction patterns from mineral deposits showed between two to six rings with d-spacings consistent with those of hydroxyapatite. **D** and **E:** Consistent with this being an osteogenic process, the nodules stained positive for ALP activity (**D**) and osteocalcin (**E**; **inset** is negative control with no primary antibody added). **F:** Cells within the nodules did not take up the APOPercentage dye, indicating that they were not apoptotic (positive control is shown in the **inset**). **G:** Gene expression analysis by PCR showed positive expression of osteocalcin, osteonectin, and Runx2 in induced VICs. **H:** A large proportion of VICs within lesions in the fibrosa of sclerotic leaflets expressed Runx2 (**inset** is a healthy valve immunostained for Runx2).

days in complete medium. Two days after plating, a low level of monomeric α -SMA expression was detected in the cytoplasm of the cells (Figure 3C). By day six, many cells expressed high levels of α -SMA that was incorporated into stress fibers (Figure 3D), indicative of a myofibroblast phenotype.³⁷ Additionally, these cells expressed vimentin, but not desmin, smoothelin, or smooth muscle myosin (data not shown), indicating that they were not smooth muscle cells. We showed previously that VICs also undergo myofibroblast differentiation *in situ* in

diseased valves.³⁸ To assess myogenic potential, VICs were cultured in a variety of myogenic induction media.³⁹ However, no multinucleated myotubes were observed after up to three weeks of culture. Instead, VICs in myogenic induction media formed nodules similar to those in osteogenic media after one week. Based on these data, we conclude that VICs include a subpopulation of mesenchymal progenitor cells with differentiation potential to osteogenic, adipogenic, chondrogenic, and myofibroblastic lineages.



High Frequencies of Mesenchymal Progenitors and Osteoprogenitors in VICs

To determine the frequency of the putative mesenchymal progenitor subpopulation, we measured the mesenchymal progenitor frequency of primary and subcultured VICs from three independent isolations (each from eight pigs). The mesenchymal progenitor frequency of primary VICs was $48.0 \pm 5.7\%$ and was maintained at about 50% for up to six population doublings (Figure 4A). After nine population doublings, the frequency dropped significantly to $28.2 \pm 9.9\%$ ($p < 0.05$ compared with frequencies at earlier passages). The mesenchymal progenitor frequency was independent of the plating density, as the average number of colonies versus the number of cells plated was linearly related in all cases (Figure 4B).

Because of its potential role in valve calcification, we characterized the osteoprogenitor subpopulation more thoroughly. We determined the frequency of osteoprogenitors in primary and subcultured VICs from three independent isolations. Primary VICs grown in osteogenic media had an osteoprogenitor frequency of $44.1 \pm 12.0\%$ (Figure 5A). The osteoprogenitor frequency was maintained after one passage (~ 3 population doublings), and then decreased to $14.8 \pm 11.3\%$ at passage two (~ 6 population doublings) and $5.8 \pm 1.3\%$ at passage three (~ 9 population doublings) ($p < 0.05$ compared with frequencies of primary and passage one cells). The expected number of osteoprogenitors and the number of cells seeded were linearly related, indicating that the osteoprogenitor frequency measured was independent of the plating density (Figure 5B).

A Morphologically-Distinct Colony Enriched for Osteoprogenitors

We found single VICs were able to form four types of colonies, each with distinct cell morphologies (Figure 6A-D). These colonies did not express CD31, indicating

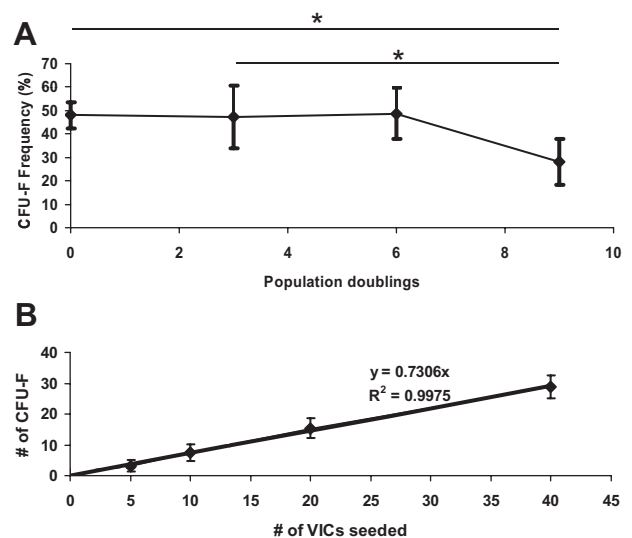


Figure 4. Mesenchymal progenitor frequencies in primary and subcultured VICs. **A:** The mesenchymal progenitor frequency of primary VICs was $48.0 \pm 5.7\%$ and was maintained at about 50% for up to six population doublings. After nine population doublings, the frequency dropped to $28.2 \pm 9.9\%$. $*P < 0.05$ by Fisher's LSD. **B:** The CFU-F frequency was independent of the plating density. Data in (**A**) and (**B**) are presented as mean \pm SE for $n = 3$ independent experiments. Representative data from one experiment with primary VICs are shown in (**B**).

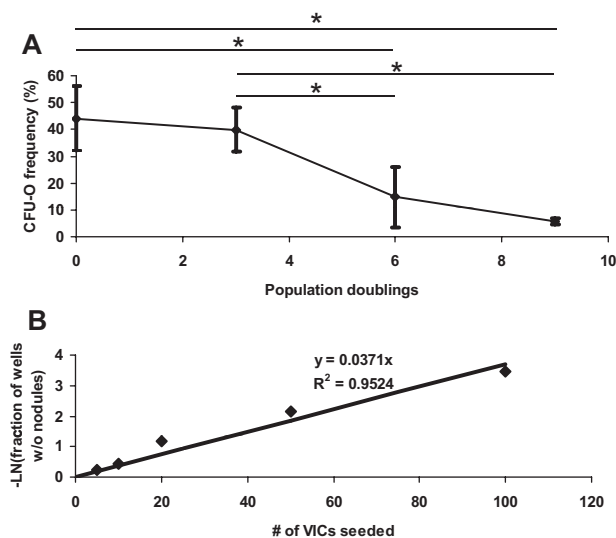


Figure 5. Osteoprogenitor frequencies in primary and subcultured VICs. **A:** Primary VICs had an osteoprogenitor frequency of $44.1 \pm 12.0\%$ that decreased to $5.8 \pm 1.3\%$ at passage three (~nine population doublings). * $P < 0.05$ by Fisher's LSD. **B:** The osteoprogenitor frequency was independent of the plating density. Data in (A) and (B) are presented as mean \pm SE for $n = 3$ independent experiments. Representative data from one experiment with P3 VICs are shown in (B).

that they were not contaminating endothelial cells. All colonies expressed α -SMA when grown in culture (data not shown), indicating that they had myofibrogenic potential. In primary VICs, $64.8 \pm 5.5\%$ of colonies were comprised of tight, swirling, fibroblast-like cells; $11.9 \pm 9.0\%$ were comprised of loosely-packed fibroblast-like cells; $10.0 \pm 5.4\%$ were made up of small, flat cells; and $13.3 \pm 7.0\%$ were made up of large, flat cells (Figure 6E). Interestingly, the relative proportion of these colony types changed with subculture. After three passages (~9 doublings), there was a significant decrease in the appearance of the tight, swirling fibroblast and loosely-packed fibroblast colonies. This was accompanied by a significant increase in colonies made up of large, flat cells.

The close correspondence between the decrease in the osteoprogenitor frequency and the frequency of the tight, swirling colonies with subculture led us to assess the osteogenic potential of this colony type compared with the others. VICs were seeded at 0.2 cells/well in 96-well plates and cultured in complete growth medium. After 14 days of culture, each well was examined under a phase contrast microscope and wells that had only one colony were identified and marked as a tight, swirling colony (S-type, Figure 6A) or as non-swirling colony (N-type). These colonies derived from single cells were then cultured in osteogenic media for an additional three weeks. The marked wells were stained for ALP and the number of ALP-positive nodules in each well was counted. Strikingly, 13 out of 13 S-type wells had ALP-positive nodules; multiple nodules formed from single cells were observed in nine of the 13 wells (the number of nodules per well ranged from one to 27, with a mean of 6.2). In contrast, only two of ten N-type wells had ALP positive nodules. This experiment was repeated multiple times with primary VICs from separate isolations, and the

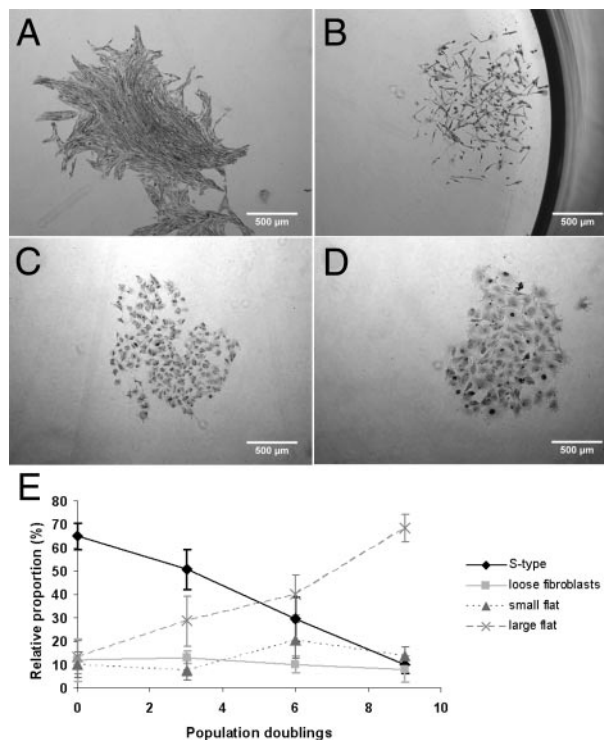


Figure 6. VICs contain a morphologically distinct subpopulation that is enriched for osteoprogenitors. **A–D:** Based on cell morphology, four major types of colonies were observed: (A) tight, swirling, fibroblast-like cells (S-type); (B) loosely-packed fibroblast-like cells (loose fibroblasts); (C) small, flat cells; and (D) large, flat cells. **E:** The relative proportions of each type of colony changed with subculture. In particular, the frequency of S-type colonies decreased significantly. This was accompanied by a significant increase in colonies made up of large, flat cells. The S-type colonies were enriched for osteoprogenitor cells (see text). Data are presented as mean \pm SE for $n = 3$ independent experiments.

same phenomenon was observed each time: in all cases, almost all S-type colonies formed ALP-positive nodules whereas few, if any, N-type colonies formed ALP-positive nodules. The presence of multiple nodules in S-type colonies derived from a single cell suggested these cells were capable of self-renewal. To confirm this was the case, we examined the osteogenic potential of subcultured cells from S-type colonies. Cells from a single S-type colony were trypsinized, diluted in complete medium, and then plated into another 96-well plate. 72.5% of the daughter colonies formed by S-type cells were also S-type. After culturing in osteogenic media for 21 days, ALP positive nodules were observed in these daughter colonies.

Discussion

Calcific aortic valve disease is now recognized as an active, multifaceted pathobiological process.⁴⁰ While some of the mechanisms involved in valve disease are similar to those in atherosclerosis, recent observations suggest anatomical, cellular, and genetic mechanisms that are unique to the aortic valve.^{12,40,41} Accordingly, an improved understanding of the pathogenesis of calcific aortic valve disease requires better definition of these unique features, including the characteristics of the cell

populations involved in the disease. In the present study, we identified for the first time a mesenchymal progenitor cell population in the aortic valve using rigorous quantitative assays established in the mesenchymal stem cell field. These cells can differentiate to the osteogenic, adipogenic, chondrogenic, and myofibroblast lineages, occur at high frequencies in the porcine valve, and contain a large subpopulation of morphologically distinct cells that self renew and elaborate bone matrix from single cells. The demonstration and characterization of a mesenchymal progenitor subpopulation is an important addition to our limited understanding of the unique characteristics of valve cells and their phenotypic heterogeneity, and of the cell populations that putatively contribute to calcific aortic valve disease.

The origin of mesenchymal progenitor cells in the valve is unknown. Adult valve interstitial cells are derived from epicardially derived mesenchymal cells that invade the endocardial cushions⁴² and from cells derived from the embryonic epicardium.⁴³ Recent reports indicate that circulating hematopoietic stem cells also contribute to adult VIC populations.⁴¹ Notably, adult hematopoietic stem cells have multilineage differential potential to nonhematopoietic lineages, including myofibroblastic⁴⁴ and osteogenic.⁴⁵ However, the frequency of hematopoietic or other marrow-derived cells in healthy adult valves is unknown. In sclerotic human valves, less than 8% of VICs are marrow-derived dendritic or endothelial progenitor cells,⁴⁶ which is far less than the progenitor frequencies we measured in primary VICs from young adult porcine aortic valves that showed no apparent signs of disease.³³ Most likely, the valve progenitor population represents a combination of resident and circulating progenitors. The high progenitor frequency we observed here suggests that many resident VICs maintain progenitor characteristics postnatally and/or the valve is a preferred site for recruitment of circulating progenitors. A large population of valve progenitors may be necessary to maintain and repair the aortic valve, which is subjected to a mechanically demanding and potentially damaging environment.⁴⁷ While the frequency of valve progenitor cells is likely species-, age-, and culture condition-dependent, as is the case for other tissue-specific mesenchymal progenitors,⁴⁸ it is notable that the mesenchymal progenitor and osteoprogenitor frequencies we measured here in standard growth and osteogenic culture conditions are among the highest reported for mesenchymal progenitor cells.^{34,48}

Regardless of their origin, valve mesenchymal progenitor cells appear to be distinct from the mesenchymal progenitor populations identified in vascular smooth muscle cells and pericytes. The differentiation potential we observed for VICs differs from that reported for calcifying vascular cells, a subpopulation of vascular smooth muscle cells that have myogenic but not adipogenic potential.¹⁵ The differentiation repertoire of pericytes is similar to that of VICs,^{16–20} but pericytes are defined by their anatomical location around microvessels⁴⁹ and often express α -SMA. In contrast, the healthy aortic valve is avascular and is populated primarily by fibroblasts that do not express α -SMA or other smooth muscle markers.^{8,50}

Thus, while there are similarities between valvular and vascular progenitor populations, valve progenitor cells appear phenotypically and functionally distinct. These distinctions may have important implications for therapies that target osteogenic calcification mechanisms in the valve versus the vasculature.

The fact that VICs contain a large proportion of osteoprogenitors, and their osteogenic potential is demonstrated both *in vitro* and *in vivo*, is important to understanding the mechanisms of calcific aortic disease. Similar to vascular calcification,³ valvular calcification likely occurs through multiple mechanisms leading to dystrophic calcification associated with apoptosis^{2,22,24} and the formation of bone tissue via osteogenesis.^{4–6,27,28} Presumably the osteoprogenitor subpopulation contributes to the latter process, and therefore may be a preferred target for therapies aimed at preventing or arresting bone formation in the valve. Cell culture systems used to study the mechanisms of valvular calcification recapitulate aspects of both dystrophic and osteogenic calcification.^{21–23,25,26} In both cases, VICs form nodules that stain positive for ALP activity and mineral by alizarin red or von Kossa. However, there are significant differences in nodule microstructure, cell viability within nodules,^{22,23} and phenotypic profiles of cells associated with nodules formed by dystrophic versus osteogenic processes (unpublished observations, C.Y.Y.Y., C.A.S), suggesting that different mechanisms are responsible for their initiation and progression. Here we demonstrated that a subpopulation of early passage VICs has osteogenic potential and forms nodules with molecular, protein, cellular, and mineral features consistent with those formed by osteoblasts *in vitro*.^{29,51} However, the osteogenic potential of cultured VICs dropped significantly after approximately six to nine population doublings. Thus, in systems that use subcultured VICs, care must be taken when interpreting the mechanisms responsible for calcification and nodule formation. The decrease in progenitor frequencies with increasing cell doublings may have been due in part to expansion of the cells on stiff tissue culture polystyrene, as matrix stiffness alone can modulate lineage commitment of mesenchymal progenitors, even in standard growth medium.⁵² We have observed that commitment of primary VICs to the myofibroblast³⁸ and osteoblast (unpublished observations, C.Y.Y.Y., C.A.S.) lineages is sensitive to matrix stiffness. The sensitivity of VICs to matrix stiffness also suggests that their differential potential might not be limited to that described in this study. For example, more compliant substrates might be required, along with appropriate chemical stimuli, to induce commitment to myogenic and neurogenic lineages.⁵²

Aortic VICs are typically described as a heterogeneous population of fibroblasts, smooth muscle cells, and myofibroblasts.^{7,8,10,11} The discovery of a mesenchymal progenitor cell within the interstitium further defines the heterogeneity of VICs. Notably, we observed that the putative progenitor cells (ie, the VICs that were capable of forming colonies) were themselves heterogeneous in terms of their morphology and differentiation potential *in vitro*. In particular, we identified a subpopulation that

formed tight, swirling (S-type) colonies, was highly enriched for osteoprogenitors, and was capable of limited self-renewal *in vitro*. This was the most common subpopulation among the primary progenitor cells, but its frequency dropped with passaging in growth media. The loss of this subpopulation with subculture correlated well with the reduction in osteoprogenitors, consistent with the S-type cells being enriched for osteoprogenitors. The ability to isolate relatively large numbers of identifiable primary valve osteoprogenitor cells before their differentiation provides the unique opportunity to discover definitive biomarkers beyond morphology that may identify these cells prospectively, rather than by retrospective colony-based assays. This is important, because while the retrospective colony-based assays we used are rigorous, well-established, and relevant clinically, they do not fully characterize the *in vivo* potential of specific subpopulations.⁵³ Identification of progenitor-specific biomarkers will enable identification of valve progenitors *in situ* and more effective study of their fundamental characteristics and role in valve (patho)biology. Because of their capacity to contribute to ectopic mesenchymal tissue formation and calcification in the aortic valve, it will be important to determine the factors that dysregulate valve mesenchymal progenitor cell differentiation and the signaling mechanisms involved, as these cells may be preferred targets for therapeutic strategies.

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