SLUG Is Expressed in Endothelial Cells Lacking Primary Cilia to Promote Cellular Calcification

Gonzalo Sánchez-Duffhues, Amaya García de Vinuesa, Jan H. Lindeman, Adri Mulder-Stapel, Marco C. DeRuiter, Conny Van Munsteren, Marie-José Goumans, Beerend P. Hierck, Peter ten Dijke

Objective—Arterial calcification is considered a major cause of death and disabilities worldwide because the associated vascular remodeling leads to myocardial infarction, stroke, aneurysm, and pulmonary embolism. This process occurs via poorly understood mechanisms involving a variety of cell types, intracellular mediators, and extracellular cues within the vascular wall. An inverse correlation between endothelial primary cilia and vascular calcified areas has been described although the signaling mechanisms involved remain unknown. We aim to investigate the signaling pathways regulated by the primary cilium that modulate the contribution of endothelial cells to vascular calcification.

Approach and Results—We found that human and murine endothelial cells lacking primary cilia are prone to undergo mineralization in response to bone morphogenetic proteins stimulation in vitro. Using the Tg737cre;p63cre cilia-defective mouse model, we show that nonciliated aortic endothelial cells acquire the ability to transdifferentiate into mineralizing osteogenic cells, in a bone morphogenetic protein–dependent manner. We identify β-CATENIN–induced SLUG as a key transcription factor controlling this process. Moreover, we show that the endothelial expression of SLUG is restricted to atheroprone areas in the aorta of LDLR−/− mice. Finally, we demonstrate that SLUG and phospho-homolog of the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from gene sma for small body size)-1/5/8 expression increases in endothelial cells constituting the vasa vasaurs in the human aorta during the progression toward atherosclerosis.

Conclusions—We demonstrated that the lack of primary cilia sensitizes the endothelium to undergo bone morphogenetic protein–dependent-osteogenic differentiation. These data emphasize the role of the endothelial cells on the vascular calcification and uncovers SLUG as a key target in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2015;35:616-627. DOI: 10.1161/ATVBAHA.115.305268.)

Key Words: atherosclerosis ■ BMP receptor ■ cilia ■ transforming growth factors-β

As a common result of atherosclerosis or metabolic disorders (ie, diabetes mellitus and renal disease), vascular calcification constitutes a major health problem worldwide.1 Although the biomedical understanding of this disease has increased during the past years, the underlying molecular mechanisms need to be uncovered to develop novel effective approaches for its intervention.

To date it is well accepted that calcification of the atherosclerotic plaque occurs via mechanisms resembling the process of bone mineralization.2,3 This process, which mainly affects the intima layer, involves cross-talk between different cell types (osteoblast-like cells, vascular smooth muscle cells, pericytes, endothelial cells [ECs], inflammatory cells, etc), secreted cytokines, and growth factors, such as the bone morphogenetic proteins (BMPs).1 Interestingly, elevated expression levels of BMPs have been found in sites of vascular calcification,4,5 thereby promoting an osteogenic-like environment in the vascular wall. The BMP ligands (BMP-2/4, BMP-5/7/8, GDF-5/6/7 and BMP-9/10) signal via 2 types of serine/threonine kinase receptors,6,7 leading to the phosphorylation of homologs of the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from gene sma for small body size) (SMAD)-1/5/8, which associate with the common mediator SMAD (ie, SMAD4). SMAD complexes accumulate in the nucleus and regulate the expression of specific target genes, including osteogenic factors.8,9

Emerging evidence suggest that the osteogenic signals initiated in the tunica adventitia are transmitted to the calcifying tunica media via the vasa vasorum,10,11 promoting the recruitment of smooth muscle actin–positive cells. In the intima layer, these cells produce extracellular matrix molecules, including interstitial collagen, and form the calcified plaque. It has been recently proposed that ECs contribute to vascular calcification via a dedifferentiation process known as
endothelial-to-mesenchymal transition (EndoMT), resulting in osteogenic cells. In mice, it was reported that EndoMT leads to ≥10% of the smooth muscle cells in the neointima and affects ≤80% of luminal ECs. EndoMT is thought to be executed by a subset of transcription factors (including SLUG) potently regulated by inflammatory cytokines, WNT, NOTCH, and members of the transforming growth factor-β (TGF-β) superfamily, including the BMPs.

Despite a variety of systemic risk factors that have been suggested, the distribution of calcified lesions probably reflects differing hemodynamics along the vasculature.

In mice, plaques accumulate in the inner curvature of the aorta, at the intersections with other vessels (carotid and subclavian arteries) and the aortic side of the semilunar valves, where ECs are under disturbed flow. Interestingly, accumulation of ECs expressing primary cilia has been reported at the boundaries of the plaques, whereas ECs under shear stress (like in the outer curve of the aorta or on top of the atheroma) rapidly disassemble them. The endothelial primary cilium acts as a mechanosensor that allows the cell to respond more efficiently to environmental changes. It consists of a protrusion composed of a 9+0 bundle core of microtubule doublets that extends throughout the cell membrane into the extracellular space and binds to the cytoskeleton via the basal body. Its presence in vascular ECs has been extensively reported and recently it has been shown that it regulates EndoMT during cardiogenesis. The oak ridge polycystic kidney (ORPK) mouse model consists of the genetic disruption of the Polaris (IFT88, Tg737, and Polaris) gene, impairing the intraflagellar transport homolog 88 (IFT88) superfamily, including the BMPs.

We detected calcium deposits using alizarin red solution (ARS) staining in those human umbilical vein ECs characterized by a lower number of primary cilia, as indicated by acetylated α-TUBULIN staining (Figure 1A–1C). Moreover, the mouse EC lines MS-1, mouse embryonic ECs (MEEC), and 2H-11 and the bovine aortic ECs BAEC lacking primary cilia were strongly stained with ARS in response to BMP-6 (Figure 1D–1F). Interestingly, the mouse aortic ECs (MECs) with a high number of cells expressing primary cilia, as shown by acetylated α-TUBULIN staining, were poorly differentiated into osteogenic cells. In contrast, MECs isolated from the primary cilium defective Orpk−/− mice (Tg737<sup>orpk<sup>/orpk</sup></sup>) were highly responsive to BMP-6-induced mineralization. These data suggest that primary cilia influence the mineralizing response of ECs to BMP ligands in human and mice.

**BMP Activity Is Necessary for Nonciliated ECs to Undergo Mineralization**

To investigate how the primary cilium affects the BMP calcifying response of ECs, we compared MECs from wild-type and Orpk−/− mice. As indicated by alkaline phosphatase (ALP) staining (Figure 2A) and enzymatic assay (Figure IIA in the online-only Data Supplement), as well as ARS staining (Figure 2B; Figure IIB in the online-only Data Supplement), Tg737<sup>orpk<sup>/orpk</sup></sup> MECs underwent osteogenic differentiation in response to BMPs, whereas wild-type MECs did not express ALP or mineralized. Moreover, BMP-6 was the most potent ligand-inducing osteogenic differentiation of Tg737<sup>orpk<sup>/orpk</sup></sup> MECs, which correlated with a more sustained SMAD1/5/8 phosphorylation (Figure 2C).

Using the SMAD1–SMAD4–dependent transcriptional luciferase reporter plasmid, BRE-LUC, we determined that BMP/SMAD signaling induced by BMP-6 was not enhanced in nonciliated MECs, and the BMP activity necessary for nonciliated ECs to undergo mineralization.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement. Sample collection and handling were performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands, and the code of conduct of the Dutch Federation of Biomedical Scientific Societies.
type I receptor kinase inhibitor LDN-193189 successfully blocked BRE-LUC activation in both cell lines (Figure 2D). Similarly, SMAD1/5/8 was equally phosphorylated in response to 1-hour stimulation with BMP-6 (Figure 2E).

According to previous reports, Tg737<sup>orpk</sup>/orpk cells exhibited a higher basal phosphorylation of SMAD2. To investigate whether TGF-β signaling is responsible for the mineralizing phenotype of Tg737<sup>orpk</sup>/orpk cells, we cultured these cells in the...
Figure 2. Bone morphogenetic protein (BMP) signaling is required for nonciliated endothelial cells (ECs) to mineralize. A, Wild-type and Tg737orpk/orpk mouse aortic ECs (MECs) were cultured for 1 week in the presence of transforming growth factor (TGF)-β3 and BMP ligands at the indicated concentrations and stained for alkaline phosphatase (ALP) activity. B, Alizarin red staining (ARS) of wild-type and Tg737orpk/orpk MECs cultured for 2 weeks under osteogenic conditions (osteogenic medium [OM]) in the presence of BMP ligands. The BMP-2, BMP-6, and BMP-7 exclusively promoted the mineralization of nonciliated MECs. C, Western blot showing the effect of BMP ligands on homolog of the Drosophila protein, mothers against decapentaplegic (MAD) the Caenorhabditis elegans protein SMA (from gene sma for small body size) (SMAD)-1/5/8 phosphorylation in Tg737orpk/orpk MECs. D, BRE-LUC reporter assay in wild-type and Tg737orpk/orpk MECs. Serum starved ciliated and nonciliated cells were compared on their ability to induce BRE-reporter activity in response to BMP-6 (50 ng/mL; *P = 0.0382 and **P = 0.0025) and in the presence of the BMP type I receptor inhibitor LDN-193189 (*P = 0.0415 and ^P = 0.0020). Average of ≥3 independent assays ± SD is shown. E, Western blot analysis comparing the phosphorylation of SMAD1/5/8 and SMAD2 in Tg737orpk/orpk nonciliated cells. F, Western blot analysis comparing the phosphorylation of SMAD1/5/8 and SMAD2 in Tg737orpk/orpk nonciliated cells, cotreated with BMP-6 (50 ng/mL) or TGF-β3 (5 ng/mL) and SB-431542 (10 μmol/L) or LDN-193189 (120 nmol/L). G, Representative ARS pictures of Tg737orpk/orpk cells treated with BMP-6 (50 ng/mL) and SB-431542 (10 μmol/L) or the BMP-inhibitor LDN-193189 (120 nmol/L), in the presence of OM.
Figure 3. Disruption of the primary cillum primes mouse aortic endothelial cells (MECs) to undergo bone morphogenetic protein (BMP)-induced calcification. A, Representative pictures of alkaline phosphatase (ALP), alizarin red, and von Kossa staining performed on wild-type, Tg737<sup>orpk/orpk</sup> and rescued Tg737<sup>O/o</sup>-ift88 MECs stimulated with BMP-6 (50 ng/ml). B, The mRNA expression of the osteogenic genes *Runx2*, *Collagen-1α* (*Col1α*), and *Bone Sialoprotein* (*bsp*; ***P<0.0001), as well as the EC gene markers *Ve-cadherin* (**P<0.0001), Von Willebrand growth factor (*vwf*; ***P<0.0001), and *Pecam-1* (**P<0.0002), were quantified by quantitative polymerase chain reaction. Average of 3 independent assays ±SD is shown. C, Wild-type, Tg737<sup>orpk/orpk</sup>, and Tg737<sup>O/o</sup>-ift88 MECs were stained for acetylated α-TUBULIN to detect the primary cillum (white arrows). As previously indicated, the overexpression of IFT88 is sufficient to reassemble the primary cillum. Moreover, the knockdown of *ift88* was performed in wild-type MECs (sh *ift88*<sup>1</sup> and sh *ift88*<sup>2</sup>), which resulted in a reduction in the number of cells expressing primary cilia. D, ALP activity was measured in wild-type MECs and *ift88* knocked-down cells (**P=0.0014 and ***P<0.0001) and after stimulated for 1 week with BMP-6 (50 ng/ml; **P=0.0002 and **P=0.0040). E, Representative ARS pictures of wild-type MECs stably transduced with empty shRNA vectors, as well as 2 constructs targeting mouse *ift88*, stimulated with BMP-6 (50 ng/mL).
presence of the activin receptor–like kinase-4/5/7 kinase inhibitor SB-431542 or BMP type I receptor kinase inhibitor LDN-193189 for 5 days followed by BMP-6 or TGF-β stimulation for 2 weeks. Western blotting confirmed that SMAD1/5/8 and SMAD2 phosphorylation were effectively inhibited by LDN-193189 and SB-431542, respectively (Figure 2F). ARS staining showed that, although BMP type I receptor inhibition mitigates Tg737 orpk/orpk MECs mineralization, TGF-β type I receptor kinase activity is dispensable (Figure 2G; Figure IIC in the online-only Data Supplement). These findings suggest that BMP/SMAD signaling, but not TGF-β/SMAD signaling is required for the calcification of Tg737 orpk/orpk MECs.

Next, the specific role of the primary cilium disruption in MECs was studied. Tg737 orpk/orpk MECs stably overexpressing IFT88 (Tg737 orpk/if88 MECs) resembled the wild-type nonmineralizing phenotype in response to BMP-6 (Figure 3A), as analyzed by ALP, ARS, and von Kossa stainings. Moreover, Tg737 orpk/if88 MECs rescued the assembling of primary cilia (Figure 3C). Furthermore, quantitative polymerase chain reaction analysis showed that Tg737 orpk/if88 MECs resembled the wild-type phenotype characterized by low expression of the osteogenic genes Runx2, Col1α1 and Bsp, and high expression of the EC markers Ve-cadherin, Von Willebrand factor (vWF), and Pecam-1 (Figure 3B) compared with nonciliated Tg737 orpk MECs. Finally, increased ALP activity (Figure 3D) and mineralization activity (Figure 3E; Figure III A in the online-only Data Supplement) was observed in and if88 stably knocked down MECs in response to BMP-6. These results demonstrate an increased sensitivity of MECs lacking primary cilium to undergo BMP-mediated osteogenic differentiation.

**Transcription Factor SLUG Mediates the BMP-Induced Cell Mineralization in Embryonic MECs Lacking Primary Cilia**

To investigate the mechanism involved in the osteogenic differentiation of nonciliated ECs in response to BMPs, we analyzed the mRNA expression of the epithelial-to-mesenchymal transition markers Slug, Snail, Twist, and Sip-1 (Figure 4A). We found that all of them were significantly upregulated in nonciliated Tg737 orpk/orpk MECs in comparison with wild-type and Tg737 orpk/if88 MECs. Particularly, Slug mRNA was nearly absent in wild-type MECs and dramatically induced in mutant nonciliated MECs. This observation was confirmed at the protein level by Western blotting and immunofluorescent labeling using a Slug specific antibody (Figure 4B and 4C). Furthermore, stable if88 knockdown cells increased the expression of Slug (Figure III B in the online-only Data Supplement). Noteworthy, BMP-6 further enhanced Slug expression specifically in Tg737 orpk/orpk MECs (Figure 4D).

To demonstrate the involvement of Slug in BMP-6–induced calcification of MEC Tg737 orpk/orpk cells, we obtained stably knocked-down Slug cells (Figure IIIC in the online-only Data Supplement) and observed that their ability to mineralize in response to BMP-6 (Figure 4E; Figure IIID in the online-only Data Supplement) was impaired. Moreover, ectopic expression of Slug in wild-type MECs (Figure 4F) increased the endogenous expression of the osteogenic markers RunX2 (Figure 4G) and Osterix (Oxx; Figure 4H), as well as the ALP activity of wild-type MECs (Figure 4I). Finally, by cotransfection assays, we found that Slug overexpression directly upregulated the transcription of the luciferase reporters Collagen-1-α (Figure III E1) and Osteopontin (Figure III F in the online-only Data Supplement). Taken together, these results demonstrate that loss of the primary cilium promotes calcification of MECs in a Slug-dependent manner.

**Increased Expression of β-CATENIN in Nonciliated MECs Contributes to Upregulate Slug Expression**

It has been demonstrated that many signaling pathways (including WNT) contribute to vascular calcification although the cross talk pathways remain unknown. To decipher the mechanism by which the primary cilium modulates the expression of Slug, we compared the expression of β-CATENIN between wild-type and Tg737 orpk/orpk MECs. As Figure 5A and 5B shows, nonciliated MECs displayed a higher cytosolic and nuclear β-CATENIN expression, which coincides with increased Slug expression. Moreover, increased expression of β-CATENIN was also observed in if88 knockdown cells (Figure IIIB in the online-only Data Supplement). We found that Tg737 orpk/orpk MECs activated more potently the TCF4/β-CATENIN reporter construct (BAT-LUC; Figure 5C), as well as the Slug promoter luciferase reporter plasmid (Figure 5D), which is consistent with our previous results (Figure 4A). In addition, cotransfection of a dominant negative TCF4AN expression vector, which disrupts the TCF4/β-CATENIN interaction, reduced the reporter activity in nonciliated cells (Figure 5E), whereas wild-type TCF4 increased it. Furthermore, we showed that siRNA-mediated transient knockdown of β-CATENIN in Tg737 orpk/orpk MECs downregulated theSlug-LUC reporter plasmid activity (Figure 5F), as well as BMP-6–induced ALP activity (Figure 5G and 5H). All together, our findings demonstrate that disruption of the primary CIL LiUM in MECs provokes high expression of β-CATENIN, inducing the transcription of Slug. These results suggest that primary cilium may act as signaling complexes to integrate β-CATENIN and BMP signal transduction pathways in MECs to regulate their osteogenic differentiation.

**Slug Expression Is Associated With Atherogenic Regions in Mice and Humans**

Recently, an active role of the endothelium on the formation of the atherosclerotic plaque and the calcification of the vessels has been demonstrated. To investigate whether the absence of primary cilium sensitizes the endothelium to an SLUG-dependent transformation and later calcification, we studied the aorta of LDLR−/− mice fed with a Western-type diet for 20 weeks. This treatment leads to neointima formation and allows us to investigate whether Slug contributes to the dedifferentiation of ECs before calcification.

Immunofluorescent analysis on paraffin sections (n=3) using antibodies against PECAM-1 and acetylated α-TUBULIN allowed us to identify areas undergoing neointima formation (atheroprote area), as well as nonaffected areas of the endothelium (Figure 6A). We detected PECAM-1 and primary cilium in nonaffected areas along the aorta (Figure 6B), whereas they
were nearly absent in atheroprone areas (Figure 6C and 6D). Moreover, immunohistochemical analysis on consecutive sections revealed that SLUG was expressed in clusters of ECs lining the neointima (Figure 6C), whereas it was absent in healthy endothelium (Figure 6B and 6E), thus supporting our in vitro results.

Figure 4. Lack of primary ciliary-induced SLUG is necessary for bone morphogenetic protein (BMP)-6–mediated mineralization of mouse aortic endothelial cells (MECs). A, quantitative polymerase chain reaction (qPCR) expression analysis of the endothelial-to-mesenchymal transition mediators Slug (**P<0.0010 and P<0.0001), Snail (**P<0.0011), Twist (**P<0.0001) and Sip-1 (**P<0.0001 and *P<0.0357) in wild-type, Tg737orpk/orpk and Tg737orpk/ift88 MECs in basal conditions. Average of ≥3 independent assays ±SD is shown. B, Increased protein expression of SLUG in Tg737orpk/orpk cells was confirmed by Western blotting, compared with wild-type and Tg737orpk/ift88 MECs. C, Wild-type, Tg737orpk/orpk and Tg737orpk/ift88 MECs were immunostained for acetylated α-TUBULIN (red channel) to detect the primary cilium (white arrows), and SLUG (green channel). D, Expression analysis by qPCR of Snail and Slug in wild-type and Tg737orpk/ift88 MECs stimulated for 2 weeks with or without BMP-6 (50 ng/mL) in osteogenic medium. BMP-6 increased Slug (**P<0.0014) and downregulated Snail (**P<0.0009) in nonciliated cells. BMP-6 downregulated Slug (**P<0.0006) in wild-type MECs. E, Representative pictures of ARS staining on Tg737orpk/orpk MECs knocked-down for Slug and stimulated with BMP-6 (50 ng/mL). F, G, and H, Slug (**P<0.0026), Runx2 (**P<0.0185), and Osterix (Osx; ***P<0.0001), respectively, qPCR analysis of wild-type MECs stably transduced with lentivirus expressing an empty vector or murine Slug, respectively. Average of ≥3 independent assays ±SD is shown. I, ALP assay in lentiviral transduced wild-type MECs overexpressing Slug and incubated with BMP-6 (50 ng/mL; ***P<0.0001). Average of ≥3 independent assays ±SD is shown.
Next, we studied the aorta of human specimens during the progression of atherosclerosis. Using immunofluorescent labeling to stain sections of human atherosclerotic aortae (Figure 6F), we detected luminal ECs assembling primary cilia (Figure 6G), in contrast to ECs constituting the vasa vasorum, where no primary cilia were visible (Figure 6H).

Interestingly, invasion of the human aorta by microcapillaries has been correlated in time with the formation of the plaque and the recruitment of mesenchymal-like cells to the necrotic core. Therefore, according to our in vitro results, ECs in the vasa vasorum may be sensitized to BMP-induced osteogenic differentiation. Interestingly, these capillaries have shown to provide the atherosclerotic site with mesenchymal-like cells with osteogenic potential in humans. To determine whether

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**Figure 5.** Elevated expression of β-CATENIN in nonciliated cells induces SLUG expression in mouse aortic endothelial cells (MECs). 

A, Expression analysis of SLUG and β-CATENIN by Western blotting in cytosolic (CE) and nuclear (NE) extracts of wild-type and Tg737/orpk MECs. B, Immunofluorescent staining of β-CATENIN (red channel) and SLUG (green channel) on wild-type and Tg737/orpk MECs. Nuclei were stained with DAPI (blue channel). C, BAT-LUC reporter assay on wild-type and Tg737/orpk MECs. BAT-LUC activity was normalized using the TCF4-defective Fop-flash plasmid (***$P=0.006$). Average of ≥3 independent assays ±SD is shown. D, Slug promotor-luciferase reporter assay comparing the transcriptional activity in wild-type and Tg737/orpk MECs (***$P=0.0002$). Average of ≥3 independent assays ±SD is shown. E, Cotransfection of Slug promoter with a TCF4 expressing vector or a dominant negative counterpart (TCF4-N), in Tg737/orpk MECs (**$P=0.006$). Average of ≥3 independent assays ±SD is shown. F, Tg737/orpk MECs were cotransfected with siRNAs targeting β-catenin or scrambled siRNAs, in the presence of the Slug reporter gene (***$P<0.001$), and luciferase activity was quantified. Average of ≥3 independent assays ±SD is shown. G, Tg737/orpk MECs were transfected with siRNAs targeting β-catenin or scrambled siRNAs, stimulated with BMP-6 (50 ng/mL) and stained for alkaline phosphatase (ALP). A representative picture is shown. H, Tg737/orpk MECs were transfected with siRNAs targeting β-catenin or scrambled siRNAs, stimulated with BMP-6 (50 ng/mL) and lysates were used in ALP enzymatic assay (***$P<0.001$). Average of ≥3 independent assays ±SD is shown.
ECs constituting the vasa vasorum may act as a source of mesenchymal-like cells through EndoMT, we stained paraffin sections from the aortae of healthy donors (ie, without signs of atherosclerosis), as well as early fibroatheroma and fibrotic calcified plaques (n=4) specimens, according to the classification proposed by Virmani et al, using antibodies for phospho-SMAD1/5/8 and SLUG (Figure 6I and 6J). Semiquantitative analysis revealed increased SLUG expression in ECs at the early stages of the atheroma formation (early fibroatheroma; Figure 6K), before vessel calcification.
A similar pattern was followed by phospho-SMAD1/5/8 staining (Figure 6L), which indicated that active BMP signaling is occurring in these particular ECs. These findings support our in vitro data and the fact that BMPs induce osteogenic differentiation in nonciliated ECs in a SLUG-dependent manner.

**Discussion**

In this study, we demonstrated that lack of primary cilia primes human and murine ECs to undergo a BMP-dependent-osteogenic differentiation. In vitro we showed that the absence of cilia leads to an increase in the expression of EndoMT mediators and, in particular, SLUG. Moreover, we showed that the primary cilium modulates the expression of β-CATENIN, which regulates the transcription of Slug. Furthermore, we confirmed our findings in an in vivo model using LDLR−/− mice and showed that SLUG is expressed in the regions of the aorta undergoing neointima formation, where PECAM−/−positive cells and primary cilia are not detected. Finally, we demonstrated that the expression of SLUG and phospho-SMAD1/5/8 in ECs from the vasa vasorum in the human aorta is increased during the progression of atherosclerosis, suggesting that our findings may also occur in a pathological context. These data highlight the physiological importance of the primary cilium mediating the cross-talk between TGF-β/ BMP and β-CATENIN signaling pathways in vascular calcification and highlights SLUG as a possible therapeutic target.

We have shown that the calcifying phenotype of human and murine ECs in response to BMPs is tightly determined by the presence of the primary cilium. To date, it is considered that the expression of the primary cilium is affected by shear stress, which induces EndoMT in nonciliated MECS via a mechanism dependent on activin receptor–like kinase-5 signaling. Nevertheless, in our report, preincubation of the Tg737opk/opk cells with an activin receptor–like kinase-4/5 inhibitor failed to abolish the BMP-triggered calcification of MECS, whereas treatment with a BMP type I receptor kinase inhibitor completely blocked the mineralization. These results underline BMP signaling as an ultimate regulator of endothelial mineralization and, indeed, are consistent with the development of BMP inhibitors to block vascular calcification,59 rather than fibroblast growth factor and TGF-β inhibitors,50,51 which may interfere with early EndoMT and not with calcification itself. Moreover, investigating other mechanisms (for example, in addition to shear stress or inflammation) that regulate the expression and function of the primary cilium might contribute not only to identify new cardiovascular risk factors but also to find new druggable targets to prevent arterial calcification.

Importantly, despite the fact that the Tg737opk/opk cells may have undergone a dedifferentiation process during the early development of the mice, the restoration of the primary cilium by overexpressing ifi88 rescued the wild-type phenotype. Moreover, the stable knockdown of ifi88 in wild-type cells could partially mimic the Tg737opk/opk cells phenotype, characterized by high SLUG and β-CATENIN expression, together with the ability to mineralize in response to BMPs. Although the ifi88 knockdown efficiency was considerable, still some ciliated cells could be observed in the cultures, which results in a heterogeneous population, probably less sensitive to BMP-induced mineralization than the Tg737opk/opk cell culture.

Previous reports have proven the direct participation of ECs in the neointima formation using lineage tracing.16,52 To gain insights into the mechanisms regulating this process, we found that the expression of primary cilia in the aorta of Western-type diet fed LDLR−/− mice was restricted to nonaffected areas, characterized by PECAM−/−expressing ECs. On the contrary, primary cilia or PECAM-1 were not observed in ECs at SLUG-positive regions, suggesting a loss of the endothelial phenotype. In accordance with this, Tg737opk/opk MECs expressed low levels of endothelial markers (including Pecam-1) and they were increased in ciliated wild-type and Tg737opk/ift88 MECs.

As it has been indicated elsewhere, experimental atherosclerosis in animal models does not mimic the human condition entirely.53 In particular, mice do not exhibit resident smooth muscle cells in the intima layer, whereas human atheroprotein regions contain a considerable population of these cells. Moreover, during human atherogenesis, a massive population of smooth muscle actin–positive cells migrate through the vasa vasorum into the intima, where they proliferate and differentiate into osteogenic cells in response to growth factors. To investigate whether the ECs from the vasa vasorum themselves may contribute to this osteoprogenitor population, we demonstrated that SLUG is nearly absent in healthy individuals and its expression in the ECs from the vasa vasorum increases at the early stages of the atheroma formation, following the same expression pattern as phospho-SMAD1/5/8. Because primary cilia were not detected in capillary ECs, our findings suggest that the expression of SLUG in ECs forming the vasa vasorum is highly sensitive to BMP stimulation and supports the role of capillaries as suppliers of cells with an osteogenic potential. Therefore, our results contribute to uncover the exact origin of mesenchymal-like cells in vivo in the context of human pathologies associated to vascular calcification.

In summary, we propose a model (Figure I in the online-only Data Supplement) where BMP signaling activation is necessary, but not sufficient, to induce the transdifferentiation of ECs into osteoblast-like cells. The osteogenic effect of BMPs is dramatically enhanced in the absence of the endothelial primary cilia. This mechanism is strongly dependent on the transcription factor SLUG, which promotes the secretion of a bone matrix and leads to the calcification and destruction of the vessel. An interesting area of future research will be to analyze whether inhibition of BMP or WNT signaling, as well as direct targeting of SLUG, in animal models limits the vascular calcification in the atherosclerotic plaque.

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Disclosures

None.

References


Vascular calcification is a multifactorial disorder leading to severe damage of the blood vessels. The molecular mechanisms underlying its progression are still unknown. Recently, it has been shown that endothelial cells directly participate in vascular calcification via a mechanism called endothelial-to-mesenchymal transition. In this study, we propose that primary cilia affects the differentiation of human and murine endothelial cells into osteogenic cells. This organelle plays a key role on the integration of different signaling pathways and its disruption causes an increase in the transcription factor β-CATENIN that induces the expression of Slug. Noteworthy, bone morphogenetic proteins potently induced the osteogenic transformation of endothelial cells lacking primary cilia, and downregulation of Slug was sufficient to abrogate endothelial calcification in response to bone morphogenetic proteins. Finally, we showed that SLUG is increased in the endothelium of LDLR−/− mice and in the onset of human atherosclerosis, where it correlates with increased bone morphogenetic protein signaling. Therefore, our results point not only at SLUG itself but also cytokines and growth factors modulating its expression (such as bone morphogenetic proteins) as biomarkers and therapeutic targets in vascular calcification.
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MATERIALS AND METHODS

Cell culture and reagents

Generation of mouse embryonic wild type (WT) endothelial cells (EC) and mouse embryonic EC (MEC) with a mutation in the Tg737 gene (IFT88Tg737RPW or Tg737orpk/orpk) from the Oak Ridge Polycystic Kidney mouse has been previously described 1, as well as rescued Tg737orpk/orpk.ift88 (Tg737o/o.ift88) cells 2. Cells were passaged twice a week and maintained on 1% w/v gelatin (Merck Chemicals B. V., Amsterdam, The Netherlands) in advanced DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with 4.5 g/L D-glucose (Invitrogen, Breda, the Netherlands), 110 mg/L sodium pyruvate (Invitrogen, Breda, the Netherlands), non-essential amino acids (Invitrogen, Breda, the Netherlands), 2% (v/v) heat inactivated Fetal Bovine Serum (Sigma-Aldrich Chemie, Steinheim, Germany), 0.5% (v/v) antibiotic/antimycotic solution (Invitrogen, Breda, the Netherlands), 1% (v/v) insulin, transferin, selenium supplement (Invitrogen, Breda, the Netherlands), and 2mM L-glutamine (Invitrogen, Breda, the Netherlands). Human umbilical vein endothelial cells (HUVECs) were isolated as described elsewhere 3 and cultured in EBM-2 medium (Lonza, Basel, Switzerland) with 10% FBS on 1% w/v gelatin. Donor #5 was a single donor population purchased from Promocell (Heidelberg, Germany) and donor #8 is a pool from multiple donors purchased from Lonza (Basel, Switzerland). Murine pancreatic (MS-1)4, lymphoid (2H-11)5 and embryonic (MEEC)6 and bovine aortic endothelial cells (BAEC)7 have been described previously and were cultured in DMEM medium (Invitrogen, Breda, the Netherlands) supplemented with 10% FBS on 1% w/v gelatin.

BMPs were purchased from R&D systems (Abingdon, UK), and BMP-6/7 were generously provided by Dr. S. Vukicevic. TGF-β3 was a kind gift from Dr. K. Iwata. The BMP type I inhibitor LDN-193189 was purchased from Axon Medchem (Groningen, The Netherlands). The ALK4/5 inhibitor SB-431542 was acquired from Tocris (Bristol, UK). The NO donor S-Nitroso-N-acetylpenicillamine (#487910) and the eNOS inhibitor Nitro-L-arginine Methyl Hester Hydrochloride (#483125) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). The ROS scavengers Tempol (#176141), Tiron (#172553) and N-acetyl-cysteine (#9165) were acquired from Sigma Aldrich (Steinheim, Germany).


ALP assays

For alkaline phosphatase (ALP) staining assay, 5 x 10^4 Wild type, Tg737orpk/orpk or Tg737o/o.ift88 MECs were seeded into 24-well plates and incubated in growth medium
supplemented with indicated BMP/ TGF-β for 7 days. The medium was refreshed every 3 days. Afterwards cells were fixed with 3.7% formaldehyde and ALP activity was observed by histochemical examination after incubation with Naphtol AS-MX phosphate and fast blue salt at room temperature. Representative pictures were obtained using a Leica DMIL LED microscope with 10 times magnification. For quantification, cells were washed twice with phosphate buffered saline (PBS) and frozen for 1 hour at -80 ºC. Next, cells were lysed in ice with Triton X-100 buffer for 1 hour. Lysates were incubated at room temperature with a 4-Nitrophenyl phosphate disodium salt solution (PNPP) solution till it turns yellow and absorbance was measured at 405 nm.

Mineralization assays

For mineralization assays, 5 x 10^4 cells were seeded into 24-well plates and incubated in osteogenic medium containing 10^{-8} mol/L dexamethasone, 0.2 mmol/L ascorbic acid and 10 mmol/L β-glycerolphosphate in the presence of BMP/TGF-β ligands for 14 or 21 days. The medium was refreshed every 4 days. Afterwards cells were washed twice with PBS and fixed with 3.7% formaldehyde for 5 minutes. Next, cells were washed twice with distilled water and measurement of calcium deposition was performed by Alizarin Red Staining (ARS), as previously described. Precipitates originated from 3 independent ARS assays were dissolved using 10% cetylpyridinium chloride and absorbance was measured at 570 nm. In addition, von Kossa staining was performed by incubating the fixed cells in a 1% silver nitrate solution for 1 hour under ultraviolet light. Next, the cells were rinsed twice with distilled water and washed with 5% sodium thiosulfate for 5 minutes. After washing twice with distilled water, counterstaining was performed with Nuclear Fast Red solution (Sigma-Aldrich Chemie, Steinheim, Germany). Representative pictures were obtained using a Leica DMIL LED microscope with 10 times magnification.

Western Blotting

MEC cells were seeded into 6-well plates and cultured till they reached confluence. Next, cells were stimulated as indicated and then washed with cold PBS. All centrifugations were carried out in a table-top centrifuge at 4°C. The proteins extracted in 100 µL of chilled lysis buffer (20 mmol/L Hapes pH 8.0, 10 mmol/L KCl, 0.15 mmol/L EGTA, 0.15 mmol/L EDTA, 0.5 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L DTT, protease inhibitor cocktail (Roche Diagnostics, Almere, The Netherlands) and 1 mmol/L Phenylmethanesulfonfyl fluoride (PMSF) containing 0.5% Nonidet P-40 (NP-40). For nuclear and cytoplasmic fractioning, the cells were washed twice with cold PBS and lysed in 75 µL of buffer A (20 mmol/L Hapes pH 8, 10 mmol/L KCl, 0.15 mmol/L EGTA, 0.15 mmol/L EDTA, 0.15 mmol/L spermidine, 0.15 mmol/L spermine, 0.5 mmol/L Na_3VO_4, 5 mM NaF, 1 mmol/L DTT, protease inhibitor cocktail (Roche Diagnostics, Almere, The Netherlands), 1 mmol/L PMSF, y 0.1% NP-40) for 10 minutes in ice. Next 15 µL of Sucrose Buffer (50 mmol/L Hapes pH 7, 0.25 mmol/L EDTA, 10 mmol/L KCl and 70% sucrose) were added and the samples were centrifuged at 5 x 10^3 rpm. The supernatant was considered the cytosolic fraction and quantified as indicated below. The resulting pellet was washed in Buffer B (20 mmol/L Hapes pH 8, 50 mmol/L NaCl, 0.15 mmol/L EGTA, 0.25 mmol/L EDTA, 1.5 mmol/L DTT, 1.5 mmol/L MgCl_2, 25% glycerol, protease inhibitor cocktail (Roche Diagnostics, Almere, The Netherlands) and 1 mmol/L PMSF) and spun down for 5 minutes at 5 x 10^3 rpm. To collect the nuclear fraction, the obtained pellet was resuspended in 100 µL Buffer C (Buffer B with 400 mmol/L NaCl) and incubated in ice for 30 minutes. Finally, the samples were centrifuged for 5 minutes at maximum speed and the supernatant was saved and quantified.
Protein concentration was determined by the DC-Protein assay (Bio-Rad, Veenendaal, The Netherlands) and 30 µg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; in ice) for 1 hour. Blots were blocked in Tris-Buffered Saline solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 ºC, and immune-detection of specific proteins was carried out with primary antibodies (see above) followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit (GE Healthcare, Eindhoven, The Netherlands) secondary antibodies using an ECL system (Fisher Scientific, Landsmeer, The Netherlands).

**Quantitative Real Time RT-PCR (qPCR)**

Total RNA extraction was performed using NucleoSpin RNA II (Machery Nagel, Düren, Germany). 500 ng of RNA were retro-transcribed using RevertAid First Strand cDNA Synthesis Kits (Fisher Scientific, Landsmeer, The Netherlands), and real-time reverse transcription-PCR experiments were performed using SYBR Green (Bio-Rad, Veenendaal, The Netherlands) and a Bio-Rad CFX Connect device.

**mRunX2** forward, 5′-GAATGCTTCATTGCCTCCTAC-3′
mRunX2 reverse, 5′-GTGACCTGCAGAGATTAACC-3′
mCol1a1 forward, 5′-GCTTGGAGAAACTTTGCTT-3′
mCol1a1 reverse, 5′-GCACGGAAACTCCAGCTGAT-3′
mBSP forward, 5′-AGGAACTGACCAGTGGG-3′
mBSP reverse, 5′-ACTCAACGTTGCTGTTTTTT-3′
mVECAD forward, 5′-AGCCAGACTCGGACT-3′
mVECAD reverse, 5′-TCGGAAAGTTGCGCTCTGT-3′
mCD31 forward, 5′-CTCCAACAGAGCCAGA-3′
mCD31 reverse, 5′-GACCACTCCAAATGACAACCA-3′
mVWF forward, 5′-CTACCTAGAACGCGAGGCT-3′
mVWF reverse, 5′-CATCGATTCTGGCCGCAAG-3′
mGAPDH forward, 5′-TGCGAAAGTTGGAGTTGCC-3′
mGAPDH reverse, 5′-AAGATGGGTGATGGCGCTCC-3′
mSlug forward, 5′-CACATTGCAACACATATTGC-3′
mSlug reverse, 5′-TGTGCCCTCAGGTTGACTCT-3′
mSnail forward, 5′-TCCAAACCCACTGGGATAGA-3′
mSnail reverse, 5′-TTGGGCTTGATGGGACGACAT-3′
mTwist forward, 5′-CGGGTCATGGCTAAGCT-3′
mTwist reverse, 5′-CAGCTTCGGATCTGAAGTC-3′
mSip1 forward, 5′-ATGGCAACACATGGGTGAG-3′
mSip1 reverse, 5′-ATTGGACTCTGACAGATGGTGT-3′
mIft88 forward, 5′-GCAATGGGAGCGAGGACAG-3′
mIft88 reverse, 5′-AAGACGCTTCGATCACAGG-3′

Transfections, luciferase assays and DNA constructs.

For luciferase reporter assays, cells were seeded in 24-well plates and transfected with DharmaFECT Duo (Fisher Scientific, Landsmeer, The Netherlands), following the recommendations of the manufacturer. 48 hours after transfection the cells were harvested and lysed. Luciferase activity was measured using the luciferase reporter assay system from Promega (Leiden, The Netherlands) by a Perkin Elmer luminometer Victor3 1420. Each transfection mixture was equalized with empty vector when necessary and every experiment was performed in triplicate. The BRE-Luc reporter has been reported elsewhere. Collagen-1-alpha-Luc (COL1A-LUC) and Osteopontin-213-Luc (OPN-LUC) reporters were purchased from Addgene.
The mSlug-Luc was obtained by cloning into a pGL3-Luc vector (Promega, Leiden, The Netherlands) from the original mSlugGFP vector kindly donated from Prof. Savagner. The expression vector for Slug was also obtained from Prof. Savagner. The BAT-Luc and FOP-Flash-Luc reporter plasmids, TCF4wt and TCF4ΔN vectors were previously described. For transient knock-down experiments the control siRNA (ON-TARGETplus control pool, non targeting pool, D-001810-10) and the murine β-catenin siRNA (ON-TARGETplus SMARTpool L-040628-00) were purchased from Dharmacon (Fisher Scientific, Landsmeer, The Netherlands).

Immunostaining

For immuno-fluorescent labeling of ECs in vitro, cells grown on coverslips were fixed with 3.7% formaldehyde in PBS, washed, blocked in 10% normal goat serum, then incubated with antibodies for β-CATENIN, acetylated α-TUBULIN or SLUG (see above). Next, the cells were washed and incubated with Alexa Fluor FITC goat anti-mouse IgG, Alexa-Fluor 555 anti-rabbit IgG (Invitrogen, Breda, the Netherlands), or Cy3 goat anti-mouse IgG (Jackson ImmunoResearch, Suffolk, England). Subsequently, the preparations were mounted in Prolong Gold (Invitrogen, Breda, the Netherlands). Immunofluorescent labeling of tissues was performed after deparaffination and rehydration of sequential sections. Antigen retrieval was performed by boiling in 0.01 M sodium citrate, pH 6.0, for 10 minutes followed by overnight incubation at 4°C with acetylated α-tubulin (1:2000) or PECAM-1 antibodies. Incubation with secondary antibodies and mounting of the preparations was performed as described above. Overview images were acquired using a Panoramic Confocal Slide Scanner from 3DHistech Ltd (Budapest, Hungary). Magnified images acquisition was performed with a Leica SP8 confocal scanning laser microscope. A representative picture from the atherosclerotic human (n=4) and murine (n = 3) aorta is shown.

Immuno-histochemical staining was performed as described before. In short, sections were deparaffinized, rehydrated and endogenous peroxidase activity was quenched. Antigen retrieval was performed by boiling in 0.01 M sodium citrate, pH 6.0, for 10 minutes followed by overnight incubation at room temperature with anti-SLUG at 1:500 or 1:2000 dilution range for murine and human sections, respectively. Anti-rabbit polyclonal phosphorylated SMAD1/5/8 antibody (1:50) was used to stained human samples. Sections were incubated with biotinylated anti-rat antibodies, streptavidin–biotin complex (Dako, Glostrup, Denmark) and staining was visualized using diaminobenzidine and H2O2. Representative photomicrographs were taken with a Leitz Diaplan system Nikon DXM1200 digital camera (Nikon instruments, Melville, NY, USA). A representative picture from the murine aorta is shown (n = 3). Representative pictures were obtained using a Leica DMIL LED microscope with 10 times magnification. Semi-quantitative scoring of SLUG staining in the human aorta was performed by counting the number of positive ECs within a capillary in relation to the total number of ECs within the same capillary. At least 130 capillaries from 4 independent donors were scored per condition. All immunostainings were evaluated by two independent observers.

Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies.

Lentivirus production and transduction

Lentiviral vectors were produced in HEK293T cells with the helper plasmids pCMV-VSVG, pMDLg-RRE (gag/pol) and pRSV-REV as described before. Cell supernatants were harvested 48 hours post transfection. For stable infection, cells were treated for
24 hours with the lentivirus-containing supernatants in the presence of 5 µg/ml of polybrene. For western blot or quantitative real-time PCR analysis, cells were re-seeded under puromycin selection (2.5 µg/ml) in 6-well plates. Lentiviral vectors expressing specific shRNAs were obtained from Sigma (MISSION® shRNA). Five shRNAs were tested, and two effective shRNAs were chosen for experiments. For murine ift88, TRCN0000176589 (sh ift88 #1) and TRCN0000178064 (sh ift88 #2) were used in this study. For murine Slug, TRCN0000096225 (sh Slug #1) and TRC0000096227 (sh Slug #2) were used.

Statistical analysis

Student’s t-test was used for statistical analysis and P<0.05 was considered to be statistically significant. All experiments were performed at least in triplicate, unless indicated.

REFERENCES


ONLINE SUPPLEMENTAL MATERIAL

SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMENTARY FIGURE I.- Proposed model for EndoMT mediated vascular calcification. Hereby we propose a model by which the primary cilium in ECs arrests them into a quiescent stage by controlling β-CATENIN expression (A). Stimulation of the BMP receptors by extracellular ligands (B), activates the BMP signaling pathway, which is not sufficient to trigger cell mineralization. Disruption of the primary cilium leads to an increase in β-CATENIN expression, which is now able to translocate into the nucleus and activate the expression of target genes, including Slug (C). In the presence of BMP stimulation, SLUG expression is further increased, thereby contributing to the osteogenic differentiation of ECs (D). This results in the generation of a calcified matrix, thus contributing to vascular calcification and atherosclerosis.

SUPPLEMENTARY FIGURE II.- BMP induce osteogenic differentiation of non-ciliated MECs. A) Wild type and Tg737orpk/orpk MECs were cultured for 1 week in the presence of TGF-β3 and BMP ligands at the indicated concentrations and stained for ALP activity. A representative picture is shown. B) Alizarin Red Staining of wild type and Tg737orpk/orpk MECs cultured for 2 weeks under osteogenic conditions (OM; Osteogenic medium) in the presence of BMP ligands. The BMPs -2, -6 and -7 exclusively promote the mineralization of non-ciliated MECs. A representative picture is shown. C) Tg737orpk/orpk MECs were incubated and stimulated with the indicated ligands and inhibitors under osteogenic conditions for 14 days and ARS assay was performed. Average of at least three independent assays ± SD is shown. Absorbance was measured at 570 nm after cetylpyridinium chloride solubilization (*** P<0.0001; ** P=0.0062).

SUPPLEMENTARY FIGURE III.- β-CATENIN and SLUG are necessary for BMP-dependent mineralization of non-ciliated MECs. A) ARS assay of stable empty vector and sh-ift88 wild type MECs stimulated with BMP-6 was quantified by ARS solubilization. Average of at least three independent assays ± SD is shown (sh ift88 #1 P=0.0024, sh ift88 #2 P=0.0004). B) Western blot assay of whole cell lysates obtained from wild type, two ift88-knock-down and Tg737orpk/orpk MECs. IFT88, β-catenin and SLUG expression was analyzed with specific antibodies. C) Analysis of SLUG expression by western blot in non-ciliated Tg737 orpk/orpk MECs stably transduced with shRNAs against murine Slug. D) ARS assay of stable empty vector, scrambled and sh Slug transduced Tg737 orpk/orpk MECs, stimulated with BMP-6 under osteogenic conditions. Calcium deposits were dissolved and absorbance was measured at 570 nm. Average of at least three independent assays ± SD is shown (P values were calculated according to the empty vector ^P=0.0315, ^^P=0.0036; as well as the scramble shRNA *P=0.0189 **P=0.0004). E) Co-transfection of 293t cells with an empty vector or a vector encoding for SLUG, and a reporter gene for Collagen-1-α (Col-1-α-LUC). Average of at least three independent assays ± SD is shown. (*** P < 0.0001). F) Co-transfection of 293t cells with an empty vector or a vector encoding for SLUG, and a reporter gene for Osteopontin (OPN-LUC) Average of at least three independent assays ± SD is shown. (* P <0.0118).

SUPPLEMENTARY FIGURE IV.- ROS scavenging reduces BMP-induced mineralization in non-ciliated MECs. A) Western blot assay of whole cell lysates obtained from non-ciliated Tg737 orpk/orpk MECs stimulated with BMP-6 for 16 hours, with and without the ROS scavengers N-acetyl-cysteine (NAC, 1 mM), Tempol (1 mM) and Tiron (1 mM). β-CATENIN, SLUG and phosphorylated-SMAD1/5/8 expression was
analyzed with specific antibodies. B) ARS assay of Tg737<sup>orpk/orpk</sup> MECs stimulated with BMP-6, with and without the ROS scavengers N-acetyl-cysteine (NAC, 1 mM), Tempol (1 mM) and Tiron (1 mM) under osteogenic conditions for 14 days. Experiment was independently performed three times and one representative picture is shown. C) ARS assay of Tg737<sup>orpk/orpk</sup> MECs stimulated with BMP-6, with and without the ROS scavengers NAC (1 mM), Tempol (1 mM) and Tiron (1 mM) under osteogenic conditions for 14 days. Average of at least three independent assays ± SD is shown. Absorbance was measured at 570 nm after cetylpyridinium chloride solubilization (*P=0.019; **P=0.0027). D) Western blot assay of whole cell lysates obtained from non-ciliated Tg737<sup>orpk/orpk</sup> MECs stimulated with BMP-6 for 16 hours, in combination with either the NO donor S-Nitroso-N-acetylpenicillamine (NOd, 0.5 μM) or the eNOS inhibitor Nitro-L-arginine Methyl Hester Hydrochloride (eNOSi, 1 μM). β-CATENIN, SLUG and phosphorylated-SMAD1/5/8 expression was analyzed with specific antibodies. E) ARS assay of Tg737<sup>orpk/orpk</sup> MECs stimulated with BMP-6, in combination with the aforementioned NOd (0.5 μM) and eNOSi (1 μM) under osteogenic conditions for 14 days. Experiment was independently performed three times and one representative picture is shown. F) ARS assay quantification was performed and the average of at least three independent assays ± SD is shown. Absorbance was measured at 570 nm after cetylpyridinium chloride solubilization.
A) Primary Cilia
- BMP Receptors

β-catenin

Endothelial Phenotype

B) Primary Cilia
- BMP Receptors

β-catenin

Smad1/5/8

Endothelial Phenotype

C) BMP Receptors

β-catenin

SLUG

Endothelial/Mesenchymal Phenotype

D) BMP Receptors

β-catenin

Smad1/5/8

SLUG

Osteogenic Differentiation
Calcification

Supplementary Figure I