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# Dkk1 and Msx2-Wnt7b Signaling Reciprocally Regulate The Endothelial-Mesenchymal Transition In Aortic Endothelial Cells

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## Abstract

**Objective—**Endothelial cells (ECs) can undergo an endothelial-mesenchymal transition (EndMT) with tissue fibrosis. Wnt- and Msx2-regulated signals participate in arteriosclerotic fibrosis and calcification. We studied the impact of Wnt7, Msx2, and Dkk1 – a Wnt7 antagonist -- on EndMT in primary aortic endothelial cells (AoECs).

**Approach and Results—**Transduction of AoECs with vectors expressing Dkk1 suppressed EC differentiation and induced a mineralizing myofibroblast phenotype. Dkk1 suppressed *claudin 5, PECAM*, *cadherin 5 (Cdh5)*, *Tie1* and *Tie2*. Dkk1 converted the cuboidal cell monolayer into a spindle-shaped multilayer and inhibited EC cord formation. Myofibroblast and osteogenic markers –*SM22, type I collagen*, *Osx*, *Runx2*, *alkaline phosphatase* -- were upregulated by Dkk1 via activin-like kinase / Smad pathways. Dkk1 increased fibrotic mineralization of AoECs cultured under osteogenic conditions – the opposite of mesenchymal cell responses. Msx2 and Wnt7b maintained morphology and upregulated markers of differentiated ECs. Deleting EC *Wnt7b* with the Cdh5-Cre transgene in *Wnt7b(fl/fl);LDLR−/−* mice upregulated aortic osteogenic genes (*Osx*, *Sox9*, *Runx2*, *Msx2*) and nuclear pSmad1/5, and increased collagen and calcium accumulation.

**Conclusions—**Dkk1 enhances EndMT in AoECs, while Wnt7b and Msx2 signals preserve EC phenotype. EC responses to Dkk1, Wnt7b, and Msx2 are the opposite of mesenchymal responses, coupling EC phenotypic stability with osteofibrogenic predilection during arteriosclerosis.

## INTRODUCTION

With advanced age, longstanding hypertension, and certain dysmetabolic states, the arterial vasculature becomes stiff, losing the elastic compliance necessary to ensure smooth distal tissue perfusion[1, 2]. This arteriosclerotic stiffening arises from atherosclerotic plaque burden, mural thickening and fibrosis, medial calcification, elastin fragmentation, non-enzymatic matrix glycation, and endothelial dysfunction[1]. Inflammatory cytokine and redox signals elicited by metabolic stressors (dyslipidemia, hyperglycemia and hyperphosphatemia), innate immune responses, neuroendocrine cues, and disturbed blood flow represent key pathophysiological stimuli[1, 2]. Multiple labs have now identified bone morphogenetic

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proteins (BMPs) and Wnts – polypeptides that play critical roles during bone morphogenesis – as rate-limiting components of the pro-fibrotic and pro-calcific injury responses that induce arteriosclerotic disease[3]. In our studies of the LDLR−/− mouse fed high fat diets (HFD), we identified that osteogenic Msx-Wnt signals in vascular myofibroblasts were activated in response to the diabetes, dyslipidemia, and obesity arising with HFD challenge[4]. Vascular myofibroblast expression of the osteoblast transcription factor *Msx2* and Wnt/β-catenin activation was shown to be upregulated by TNF-dependent signals that support aortic calcification responses in LDLR−/− mice[5]. Wnts capable of activating pro-calcific responses downstream of Msx2 in adventitial myofibroblasts, vascular smooth muscle (VSMC) and C3H10T1/2 cells were identified as Wnt3a, *Wnt7b*, and *Wnt7a*[4–7].

However, during development, Msx2[8] and Wnt7 family members[9, 10]are also expressed in endothelial cells (ECs). ECs participate in valve calcification in part by providing osteoprogenitors via the endothelial –mesenchymal transition (EndMT)[11]. During the EndMT process, ECs down-regulate homotypic cell-cell interactions that stabilize EC monolayer function and phenotype, with concomitant upregulation of proliferative and synthetic myofibroblast program -- including the deposition of type I collagen[3]. An EndMT process also contributes to the pathogenesis of myocardial fibrosis with ischemia[12]. Furthermore, in the ectopic muscle ossification of fibrodysplasia ossificans progressiva, constitutive activin-like kinase (ALK)-2 activity drives EndMT to generate osteoprogenitors[13]. Thus, EndMT has emerged as an important process in the pathobiology of fibrotic and calcific tissue injury responses[3, 14]. Of note, Msx genes[15] and Wnt/β-catenin signaling[16] support EndMT during cardiac development, modulated by mechanical strain[17].

We wished to better understand the role of endothelial Wnt/β-catenin signaling and arteriosclerosis. Therefore, we studied the actions of Wnt7, Dkk1, and Msx2 in primary cultures of bovine aortic ECs (AoECs). We find that Msx2 and Wnt7 family members stabilize the AoEC phenotype whereas Dkk1 promotes an EndMT in cultured bovine AoECs. Via an ALK/Smad-dependent mechanism, Dkk1 increased whilst Msx2 and Wnt7b decreased fibrotic mineralization of AoECs cultured under osteogenic conditions. Furthermore, targeted knockout of EC *Wnt7b* in vivo increased aortic fibrosis. Thus, AoEC arteriosclerotic responses to cell-autonomous Msx2, Wnt7b, and Dkk1 are the opposite of those elaborated by mesenchymal cells, providing a mechanism that may couple angiogenesis with osteofibrogenic predilection during tissue injury.

## METHODS

Materials and Methods are provided in the online-only Supplement.

## RESULTS

### Dkk1 inhibits epithelial cobblestone morphology and capillary-like network formation in primary bovine AoEC cultures

We previously identified that activation of osteogenic *Msx-Wnt* signaling in arterial myofibroblasts promotes medial VSMC β-catenin transactivation and calcium accumulation in diabetic arteriosclerosis[4, 18]. This cascade can be phenocopied by mesenchymal expression of Msx2, a transcriptional stimulus for myofibroblast *Wnt7b, Wnt7a*, and *Wnt3a* expression and inhibitor of *Dkk1* transcription[4, 7]. We wished to understand the role for *Msx-Wnt* signaling in endothelial cells, a vascular cell type that also elaborates *Msx2*[8], *Dkk1*[9], and *Wnt7b*[199]expression. Therefore, we studied the actions of retroviral SFG-Msx2 transduction on primary AoECs. When grown to high cell densities, AoECs transduced with the control virus SFG-LacZ adopt the cobblestone epithelial morphology characteristic of ECs (Figure 1A, **upper left**). This organized epithelial morphology appeared more

pronounced in cells transduced with SFG-Msx2 (Figure 1A, **upper right**) and preserved with SFG-Wnt7b (Figure 1A, **lower left**). By contrast, cells transduced with SFG-Dkk1, expressing the potent Wnt-LRP5/6 receptor antagonist Dkk1[20], lost epithelial features and adopted a markedly spindle-shaped morphology characteristic of the myofibroblast (Figure 1A, **lower right panel**). This change was associated with the up-regulation of cellular phospho-β-catenin and down-regulation of dephospho-β-catenin as expected with Dkk1-mediated inhibition of canonical Wnt signaling (Supplement Figure S-I). To demonstrate that the spindle-shaped cells were derived from epithelioid ECs, AoECs were tagged with fluorescent acetylated-LDL (DiI AcLDL), a ligand for the scavenger receptor expressed on ECs but not myofibroblasts[21]. As shown in Figure 1B, DiI AcLDL-labeled only the epithelioid cells in SFG-LacZ - and SFG-Dkk1 - transduced cultures when imaged following a 4 hour labeling "pulse" (left panels). The spindle-shaped cells that were already present in the SFG-Dkk1 transduced AoEC cultures were not labeled by DiI AcLDL fluorescent dye immediately after pulse (Figure 1B, Left lower panels). Of note, only 3.9% of AoEC primary cells stain for VSMC alpha-actin (online Supplement Figure S-II), in excellent agreement with the observation that 97% +/− 1.9% of cells in primary AoEC cultures were labeled with DiI AcLDL (N = 369 cells scored in 6 separate wells, data not shown). Thus, in primary AoEC culture, approximately 4% of cells were of non-endothelial phenotype. Following a 4 day "chase," achieved by incubation in the absence of DiI probe, the initial DiI AcLDL labeling of SFG-LacZ control AoEC cultures was retained only in cuboidal epithelial cells (Figure 1B, **right upper panel**); however, in SFG-Dkk1 cultures the DiI AcLDL label was mostly observed in spindle or oval-shaped cells (Figure 1B, **right lower panel**), confirming derivation of myofibroblastic cells from AoECs. The percentage of DiI-positive cells possessing a spindle or oval shaped morphology 4 days after labeling increased 10-fold with constitutive Dkk1 expression, ranging from 7% of cells in SFG-LacZ control AoEC cultures to just over 70% of the cell population in SFG-Dkk1 cultures (Figure 1C).

We wished to further confirm the inhibitory actions of Dkk1 on EC morphology and function. Therefore, we examined the effects of *Msx2* and *Wnt7b* vs. *Dkk1* expression on capillary-like network formation by bovine AoECs cultured in Matrigel, a permissive basement membrane-based extracellular matrix for vascular network assembly[22]. Expression of *Dkk1* significantly reduced endothelial cord morphogenesis (Figure 1D, left panels). In contrast, expression of *Msx2* and *Wnt7b* retained endothelial cord formation (Figure 1D, middle and right panels). Quantitative data obtained from digital images demonstrated a >50% reduction in capillary-like network density per high power field in Dkk1 cells (Figure 1E, left panel). By contrast, *Msx2* under these same conditions increased network density by 27% (Figure 1E, middle panel). Wnt7b expression induced a non-significant trend (p= 0.08) for increased network density as well (Figure 1E, right panel). Thus the Wnt receptor antagonist Dkk1 inhibits AoEC epithelial morphology and endothelial cord network assembly *in vitro*, exerting actions opposite to those of activated Msx-Wnt signaling.

## Dkk1 signaling inhibits the expression of EC differentiation markers and promotes the endothelial-mesenchymal transition in primary bovine AoEC cultures

Epithelial morphology of EC in culture is dependent upon cell adhesion molecules such as platelet endothelial cell adhesion molecule (*PECAM* a.k.a. *CD31*) that drive and demarcate EC differentiation[23]. Vascular endothelial cadherin (*VECAD* a.k.a. *Cdh5*)[24], PECAM[25], and claudin 5 (*Cldn5*)[26] are 3 EC phenotypic markers that maintain epithelioid morphology and endothelial functional integrity. Thus, we examined the impact of *Dkk1*, *Msx2* and *Wnt7b* on the expression of these EC markers. Consistent with the perturbation in epithelial morphology and capillary tube formation, SFG-Dkk1 significantly down-regulated *Cdh5*,

*PECAM*, and *Cldn5* in bovine AoEC cultures (Figure 2A; all $p < 0.05$ vs. SFG-LacZ control). The mRNA for *Tie1* (an EC- and early hematopoietic cell- restricted receptor tyrosine kinase[27]) was also down-regulated by 30% (Figure 2A; all $p < 0.05$). *Tie2* mRNA was also reduced by 45% ($p=0.02$; Figure 2A). By contrast, SFG-Wnt7b (Figure 2B) -- and to a lesser extent SFG-Msx2 (Figure 2C) -- significantly augmented expression of EC markers in cultured AoECs. Of note, SFG-Wnt7a elicited responses similar to those observed with SFG-Wnt7b in transduced AoECs (data not shown). Thus, Dkk1 inhibits and Msx2-Wnt7 signaling promotes EC differentiation programs and capillary-like network morphogenesis in AoECs.

The down-regulation of EC differentiation markers and epithelial morphology with concomitant adoption of spindle-shaped morphology suggested that SFG-Dkk1 was promoting an endothelial-mesenchymal transition (EndMT) -- a post-natal vascular injury response recently identified as contributing to tissue fibrosis, ectopic ossification and calcification in several disease contexts[12, 13]. To confirm this notion, we assessed the expression of *SM22* (*transgelin*) and *CNN1* (*calponin*) -- mesenchymal cell markers expressed by myofibroblasts[28] and vascular smooth muscle cells[29] – and the *Col1A1* transcript characteristic of wound myofibroblasts[30] and differentiating osteoblasts[31]. Consistent with an EndMT, SFG-Dkk1 upregulated *SM22, CNN1, and Col1A1* in AoECs (Figure 2D). Furthermore, SFG-Dkk1 concomitantly upregulated the expression of *Runx2*, *Osx*, and *ALP (alkaline phosphatase)* -- phenotypic markers characteristic of mineralizing osteogenic cells (Figure 2D). Conversely, SFG-Wnt7b suppressed myofibroblast and osteogenic gene expression in AoECs (Figures 2E). These genomic responses to Wnt7b and Dkk1 are the opposite of responses elicited in C3H10T1/2 mesenchymal cells[4, 7]. Thus, *Dkk1* promotes while *Wnt7b* signaling inhibits gene expression changes characteristic of the EndMT in bovine AoEC cultures.

## Dkk1 promotes and Wnt7b inhibits osteogenic calcium deposition directed by AoECs cultured under mineralizing conditions

The changes observed strongly suggested that Dkk1 promotes, whilst Msx2 and Wnt7b inhibit, the EndMT in AoECs. To provide additional evidence supporting this notion, we examined the impact of SFG-Dkk1, SFG-Msx2, and SFGWnt7b on osteogenic calcium accumulation in AoECs cultured under conditions that support osteogenic mineralization. As shown in Figure 3A, SFG-Dkk1 upregulated ALP enzyme activity in bovine AoECs -- an enzyme absolutely required for osteogenic mineralization[32]. Moreover, matrix calcium deposition as assessed by Alizarin Red S staining[33] was significantly enhanced by SFG-Dkk1 (Figure 3B, 3C). By contrast, SFG-Wnt7b and SFG-Msx2 suppressed AoEC mineralization (Figure 3B, 3C). Following 11 days of culture, collagen protein accumulation was consistently greater in SFG-Dkk1 vs. SFG-LacZ cultures ($29.3 +/- 1.0$ ug collagen/cm$^2$ culture vs. $20.5 +/- 0.5$ ug/cm$^2$; $n = 3$, $p < 0.01$, seeding density 10,000 cells/cm$^2$). Of note, the pro-calcific actions of Dkk1 and anti-calcific actions of *Msx2-Wnt7* signaling in AoECs are the opposite of the responses elicited in adventitial myofibroblasts[4, 18], 3T3-L1 cells[34], and C3H10T1/2 cells[7] – i.e., cells already committed to the mesenchymal lineage. Thus, endothelial Dkk1 promotes and Wnt7b inhibits osteofibrogenic differentiation of cultured bovine AoECs.

## Dkk1 promotes Smad activation, Smad-dependent transcription, and ALK-mediated signaling in bovine AoECs

Smad transcription factor pathway activation is a consistent molecular mediator of both EndMT[35] and osteogenic mineralization[36, 37], signaling downstream of ALK receptor activation. We posited that the responses induced by SFG-Dkk1 transduction of AoECs might be dependent upon these same Smad-dependent signaling pathways. To test this

notion, we examined the expression and nuclear accumulation of phospho-Smad1/5 (pSmad1/5) as an index of osteogenic pathway activation[36]. As shown in Figures 4A, bovine AoECs transduced with SFG-Dkk1 exhibited between 1.5- to 3.1 -fold higher levels of pSmad1/5 as compared to SFG-LacZ controls. Conversely, bovine AoECs transduced with SFG-Wnt7b accumulated levels of pSmad1/5 that were reduced by 40% (Figure 4A). Moreover, cells transduced with SFG-Dkk1 consistently yielded greater extent and relative intensity of nuclear pSmad1/5 staining (Supplement Figures S-IIIA and S-IIIB). Similar responses were noted for pSmad3 in both nuclear and cytoplasmic compartments (Supplement Figure S-IIIC, and data not shown). Furthermore, co-transfection of a pcDNA3-Dkk1 expression vector upregulated the activity of SBE-LUC (Smad binding element promoter –luciferase reporter) in AoECs, as well as the Smad-dependent COL1A1[6] and SM22[38] promoter-LUC reporters (Figure 4B). The SM22 promoter encodes a GTCTG Smad binding element in exon I that is required for VSMC expression in vivo[39]. Deletion of this exonic Smad response element -- as occurs by 3' promoter truncation of SM22[−215/+44] to SM22[−215/+5] -- abrogated induction by pcDNA3-Dkk1 co-transfection (Figure 4B), providing additional evidence for the importance of Smad signaling in Dkk1 control of AoEC gene expression.

To further confirm the functional contributions of Smad signaling, we examined the impact of dorsomorphin, an inhibitor of ALK receptors mediating Smad1/5 phosphorylation and activation[40]. As shown in Figure 4C, treatment of AoECs with dorsomorphin abrogated Dkk1 mediated induction of *Col1A1*, *SM22*, and *Runx2* – and prevented Dkk1 suppression of *claudin 5*. Dorsomorphin also inhibited basal and Dkk1-enhanced mineralization of AoEC cultures (Figure 4D). Thus, Dkk1 exhibits "cross-talk" with ALK/Smad-dependent pathways previously identified to support EndMT and osteogenic mineralization. ALK/Smad-signaling is necessary for osteofibrogenic signaling responses elicited in Dkk1 in AoECs.

## Conditional deletion of Wnt7b in aortic endothelial cells increases aortic collagen accumulation in LDLR−/− mice fed high fat diabetogenic diets

By immunohistochemistry, Wnt7b is detected in the endothelium of adult aortic and aortic valve endothelial cells[10](Supplement Figure S-IVA and S-IVB), consistent with prior reports[919]. SFG-Wnt7b "super-transduction" of bovine AoECs previously transduced with SFG-Dkk1 effectively reverses *Col1A1* induction by Dkk1 and restores *claudin 5* expression (Supplement Figure S-V). This confirmed that Dkk1 and Wnt7b reciprocally regulate EC physiology, with Wnt7b maintaining EC function and mitigating sclerotic responses *in vitro*. We wished to test this notion *in vivo*. Therefore, using existing murine reagents we generated Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice in which the Cdh5 promoter is used to direct EC Cre transgene expression[41] and thus delete the floxed Wnt7b gene[42] in ECs. As compared to Cdh5-Cre;LDLR−/− controls, Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice exhibited greater levels of aortic osteogenic transcription programs (Figure 5A). Moreover, after 3 months of high fat diet challenge, Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice exhibited histological evidence of larger, more fibrotic atherosclerotic lesions (Figure 5B). Biochemical analysis of aortic collagen content by Sircol assay confirmed the histological data; Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− possessed 52% greater levels of aortic collagen content (Figure 5C; $p = 0.003$). Moreover, in this same cohort Alizarin Red S staining for calcium deposition revealed increased intensity in the aortas of Cdh5Cre;Wnt7b(fl/fl);LDLR −/− mice (Supplement Figure S-VI). Of note, in an independent experimental cohort, biochemical analysis confirmed increased aortic calcium accumulation in Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice vs. controls (0.24 +/− 0.02 ug calcium / mg aortic dry weight vs. 0.19 +/− 0.01 ug/mg; $p = 0.03$; $n = 10$ and 12 per group, respectively) following 6 weeks of HFD. Immunohistochemistry demonstrated increased intensity and extent of

nuclear pSmad1/5 in aortas of Cdh5Cre;Wnt7b(fl/fl);LDLR−/− mice vs. controls (Supplement Figure S-VIIA–C; 1.5 fold, $p<0.0001$, Mann-Whitney U test). No significant differences existed between the two groups in fasting serum glucose, cholesterol or triglyceride levels (Supplement Figure S-VIII). Immunohistochemistry verified reduction in endothelial Wnt7b in Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice vs. Cdh-Cre;LDLR−/− controls. Because the Wnt7 antibody used detects both Wnt7a and Wnt7b, the lack of significant Wnt7 staining after Wnt7b deletion suggested that Wnt7b was the major Wnt7 protein expressed by ECs at this stage (Supplement Figure S-IX). Thus, as observed in vitro with primary bovine AoECs, endothelial Wnt7b serves to limit expression of vascular osteogenic gene program and mitigate the arteriosclerotic injury response *in vivo*.

## DISCUSSION

The spectrum of bioactivities recognized as being conveyed by Wnt signaling in vascular development and disease is rapidly expanding[43, 44]. Early data demonstrated placental capillary defects in Wnt2 knockout mice that cause embryonic lethality[43, 44]. Subsequent reports emphasized the expression, signaling, and biological consequences of Wnt/β-catenin and Wnt/Ca++ signaling in the developing vasculature[43, 44]. Both ECs and VSMCs express several Wnt ligands including Wnt7b[9]. Wnt7b, Wnt7a, and β-catenin signaling maintain the blood-brain barrier and vascular integrity of the developing central nervous system[19, 44]. With respect to postnatal vascular disease, Rajamannan's group[45] and our own[4] identified canonical Wnt signaling as being an important component of aortic valve and vascular calcification. Recently, LRP5 signaling and downstream claudin 5 actions have been shown to control retinal EC proliferation in response to Wnt3a and Wnt7a[46], relevant to the retinopathies of prematurity and diabetes.

The roles for the Dickkopf (Dkk) family of Wnt/LRP5/6 antagonists in vascular biology have only begun to be studied. Mice lacking both *Dkk1* and *Dkk2* die in utero with ventricular septal defects and hypertrophic failure arising from abnormal epicardial cell fating and myocardial proliferation[47]. Isolated deficiency in either *Dkk1* or *Dkk2* does not cause an overt myocardial phenotype, indicating some functional redundancy47. However, Dkk2 can differ markedly from Dkk1 in other contexts. Dkk2 enhances angiogenesis whilst Dkk1 suppresses angiogenesis via differential modulation of EC LRP6/Cdc42 signals[48]. The precise structural features conveying differential vascular responses of Dkk2 vs. Dkk1 have yet to be elucidated. Of note, Mercola identified that the N-terminal domain of Dkk1 promotes cardiogenesis independent of canonical Wnt signaling[49]. Structurally, Dkk1 and Dkk2 diverge most significantly in the N-terminal domain, and it is the conserved C-terminal CYS2 domain that binds LRP5 and LRP6[50]. Thus, the unique activities elaborated by Dkk1 and Dkk2 likely relate in part to the N-terminal structural divergence. Nevertheless, the CYS2 domain of Dkk2 can activate rather than inhibit LRP6 signaling in certain cellular contexts,[50] indicating that C-terminal domains also encode unique paralog functions. Circulating Dkk1 levels are elevated in patients with clinically significant atherosclerosis, and Dkk1 elicits an inflammatory endothelial response conveyed in part via inhibition of Wnt/β-catenin signaling[51].

Our studies of Msx2-Wnt signaling and Dkk1 in cardiovascular calcification initially emphasized responses in adventitial myofibroblasts – the site of most robust *Msx2* gene expression in diseased vessels[4]. In this mesenchymal cell background, others and we have shown that Msx2-Wnt signaling promotes early VSMC[38, 52] and osteofibrogenic[4, 7, 34, 53]differentiation in part via canonical Wnt pathways inhibited by Dkk1. We now identify that EC responses are the opposite of those elicited in mesenchymal cells[4, 7, 18]. Dkk1 upregulates myogenic, myofibrogenic and osteochondrogenic programs in ECs – programs that are suppressed by Wnt7b in ECs. *In vivo*, the arteriosclerotic

consequences of reduced endothelial Wnt agonism / Dkk1 antagonism were apparent; Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice exhibited more significant aortic fibrosis, collagen accumulation, and calcium deposition following challenge with atherogenic high fat diets. Based upon these data and the recent literature, a working model begins to emerge (Figure 6). Wnt7b helps to stabilizes the aortic EC phenotype and maintains endothelial integrity, consistent with activity first identified in CNS vasculature[19]. By inhibiting EC Wnt7b signals, Dkk1 provokes EC phenotypic modulation and increases the pool of mesenchymal progenitors in the VSMC lineage available for tissue repair. In this way, platelet-derived Dkk1[51] may synergize with other growth factors during the early phases of arterial injury. However, within the mesenchymal lineage, Msx-Wnt signaling supports the early myogenic and osteogenic differentiation programs – actions also opposed by Dkk1. Under such a model, the balance of Wnt7/Dkk1 “tone” modulates vascular cell access to EC, VSMC, and osteogenic fates. While providing a mechanistic advantage for responses to traumatic arterial wound healing, long-term endothelial Dkk1 exposure may worsen arterial fibrosis, calcification, and vascular stiffening in diabetic vascular disease. This latter notion has yet to be tested, but Dkk1 has been strongly implicated in the pathobiology of renal fibrosis in a preclinical model of diabetic nephropathy[54].

There are, of course, limitations to our study. We identified that Dkk1 augments and Wnt7b inhibits ALK/Smad signaling to modulate EC phenotype; however, we have not yet identified how these ligands for multiple heterodimeric receptor complexes – e.g., LRP4/5/6, Frizzled and Kremen families -- control ALK/Smad signaling[55]. LRPs differentially heterodimerize with co-receptors dependent upon the ligand[56], and we speculate that Dkk1 may alter the production or localization of LRP-ALK receptor signaling complexes. Of note, the genomic programs elaborated by EC are determined by epigenomic regulators[57]. It will be interesting to assess whether differential EC vs. VSMC chromatin modifications determine responses to Wnt7b and Dkk1[58]. Moreover, we have not yet examined how EC-mediated juxtacrine control[59] of previously committed VSMCs is altered as ECs undergo phenotypic change with Wnt7b deficiency. Changes in EC-mesenchymal cell “cross-talk” in the absence of EC Wnt7b may also contribute to the total myofibroblast numbers and phenotype in vivo (Figure 6). Furthermore, vascular cell types other than ECs and VSMCs[9] express Wnt7b[60]. We have noted non-endothelial Wnt7b – expressing cells in aortas and valves of Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice (supplement Figure S-IVA), and are currently determining the lineages of these cells. Nevertheless, our results extend emerging data that indicate roles for Dkk1 and Wnt in vascular pathobiology. Sophisticated strategies that selectively target EC Dkk1 actions may help limit aortic fibrosis and reduce calcium accrual – and thus improve arterial compliance and lower extremity perfusion in diabetic vascular disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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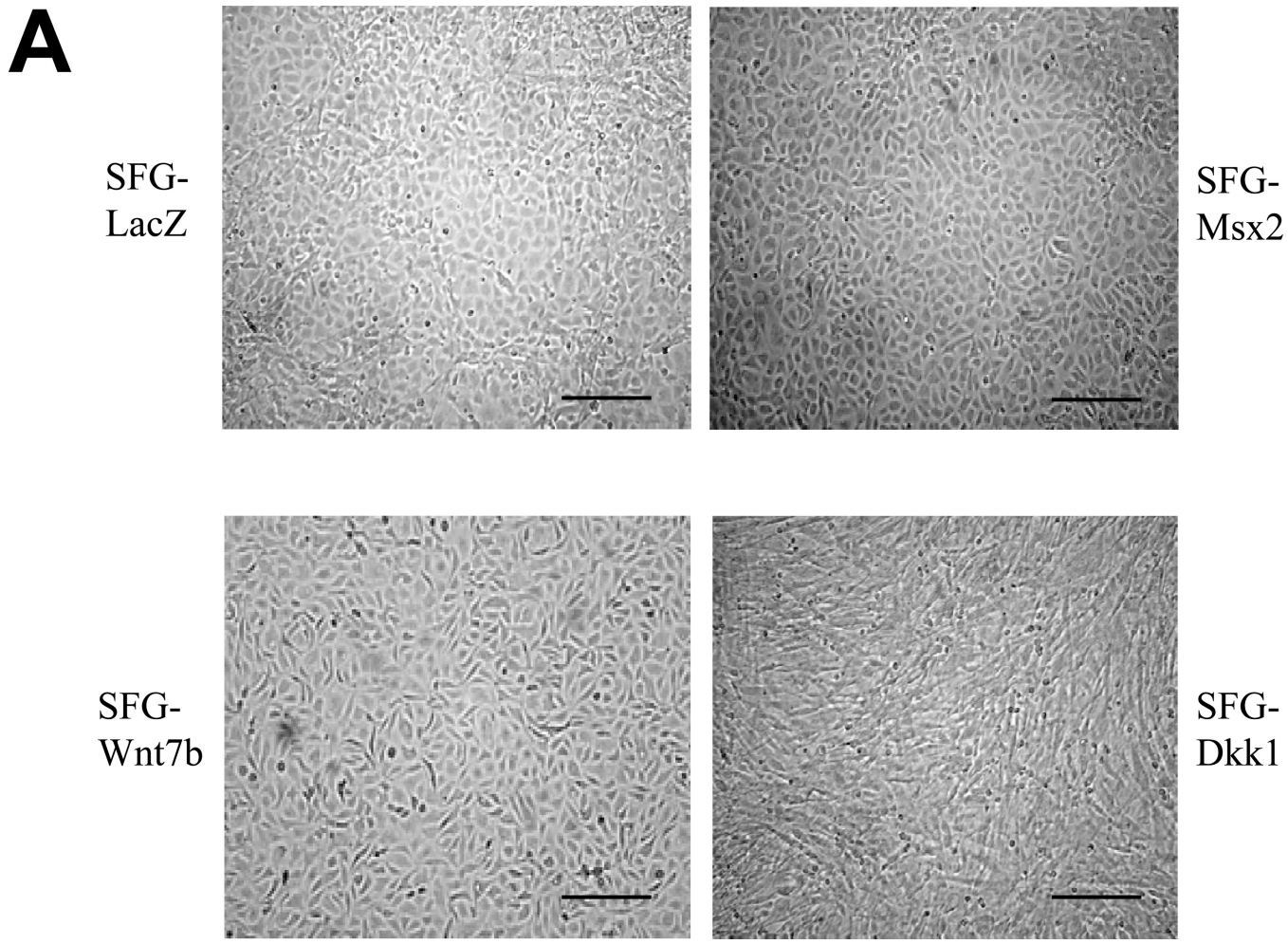
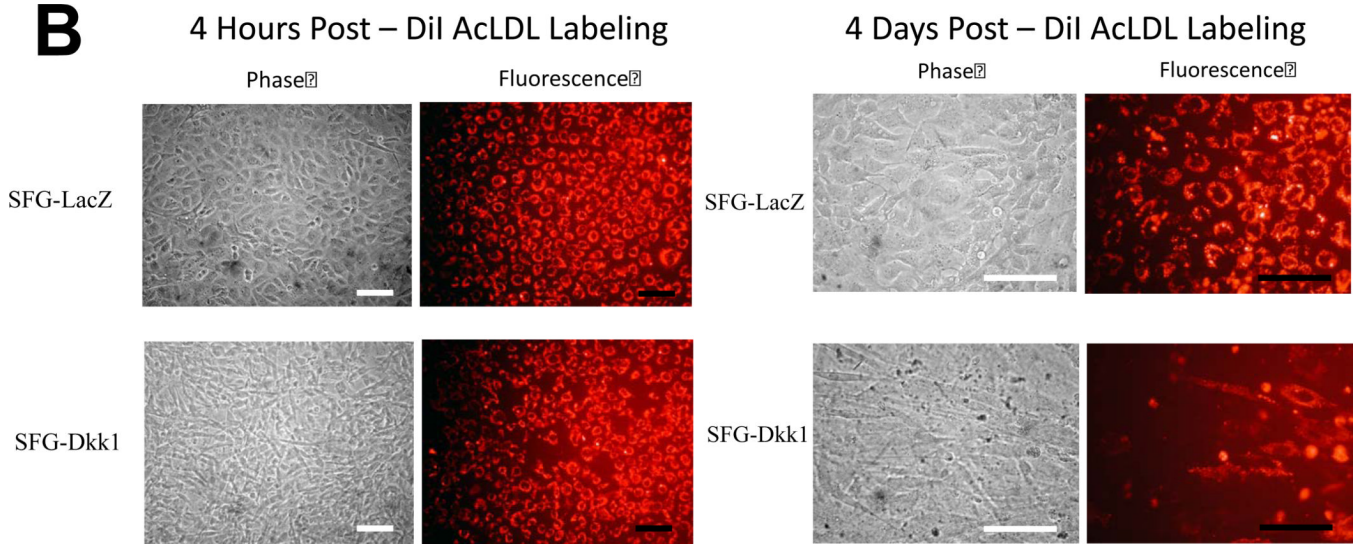
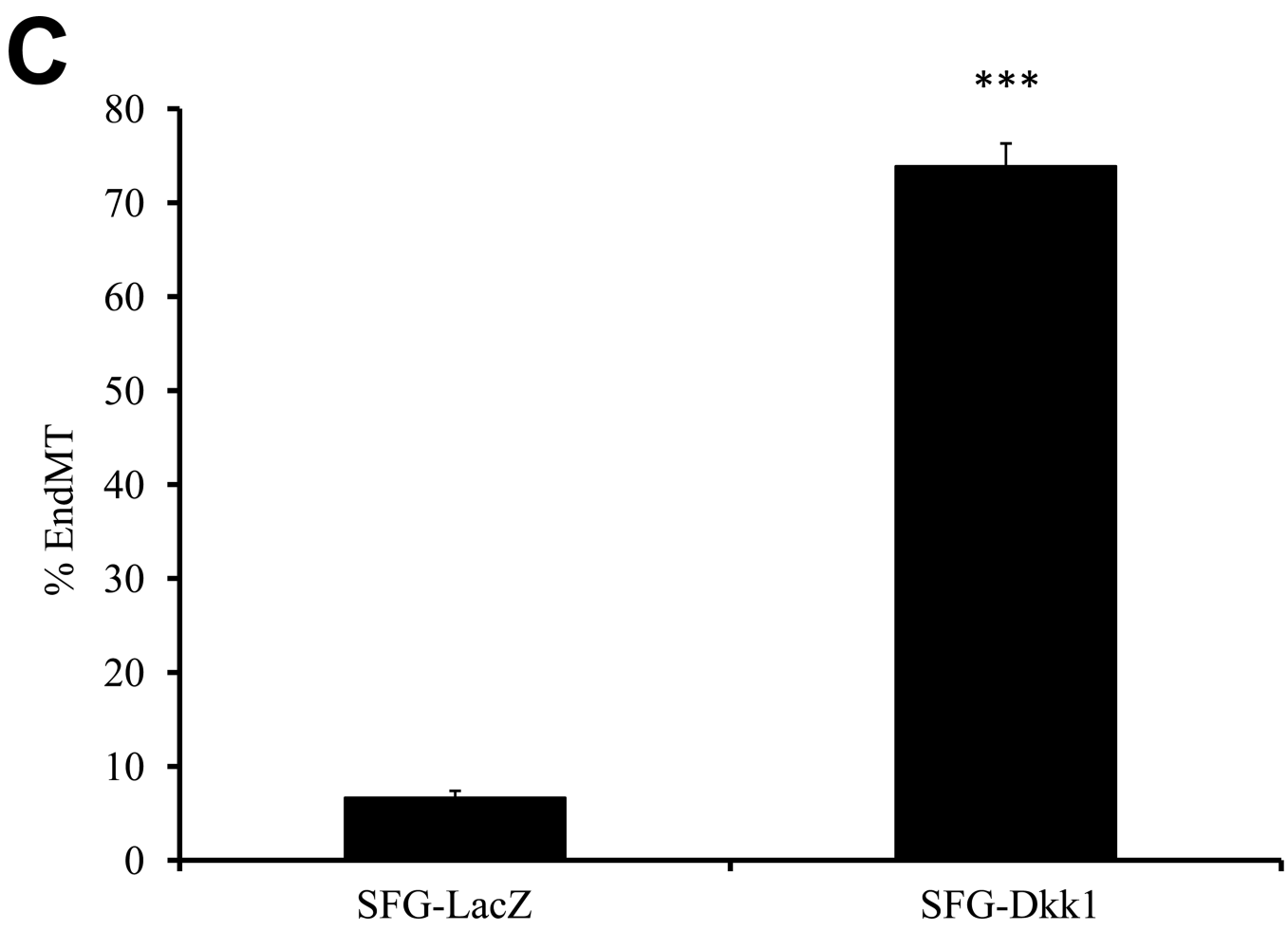
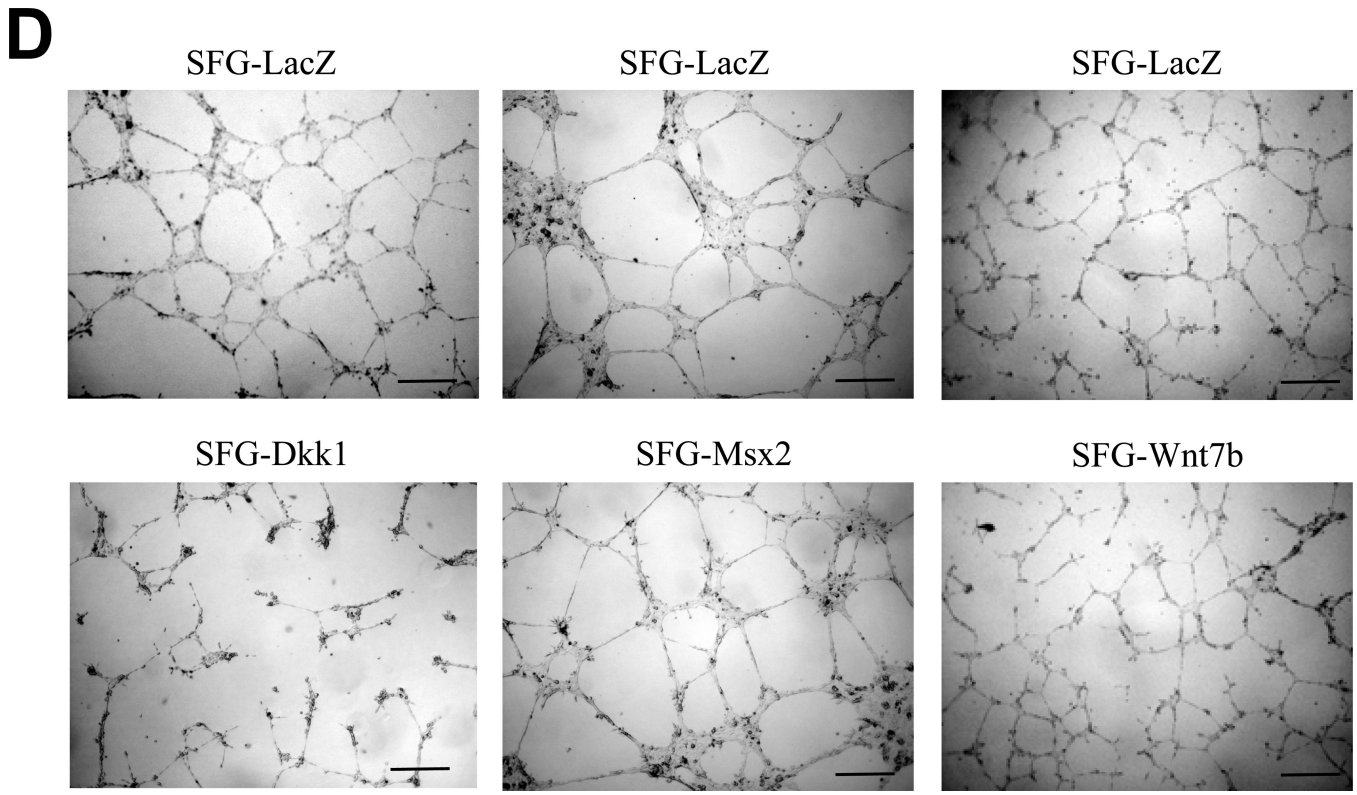
## Significance

With advancing age, longstanding hypertension, and diabetes, the arterial vasculature becomes stiff, losing elastic compliance necessary for smooth distal tissue perfusion. This arteriosclerotic stiffening arises from atherosclerotic plaque burden, mural thickening and fibrosis, medial calcification, elastin fragmentation, non-enzymatic matrix glycation, and endothelial dysfunction. Msx-Wnt signaling induces fibrosis and calcification in myofibroblasts (mesenchymal cells) and is inhibited by Dkk1. However, endothelial cells (ECs) can contribute to fibrosis via the endothelial-mesenchymal transition (EndMT), and roles for canonical Wnt signaling, Msx2, and Dkk1 in EndMT remain unclear. We show that Dkk1 enhances EndMT in aortic ECs, while Wnt7b and Msx2 preserve EC phenotype. Msx2-, Wnt7b- and Dkk1- regulated arteriosclerotic responses in ECs are opposite of those elicited in mesenchymal cells. These cell type-specific responses provide a mechanism that couples angiogenesis with osteofibrogenic predilection during tissue injury. Sophisticated strategies that selectively target vascular cell type - specific Wnt signaling may help limit arteriosclerotic disease.







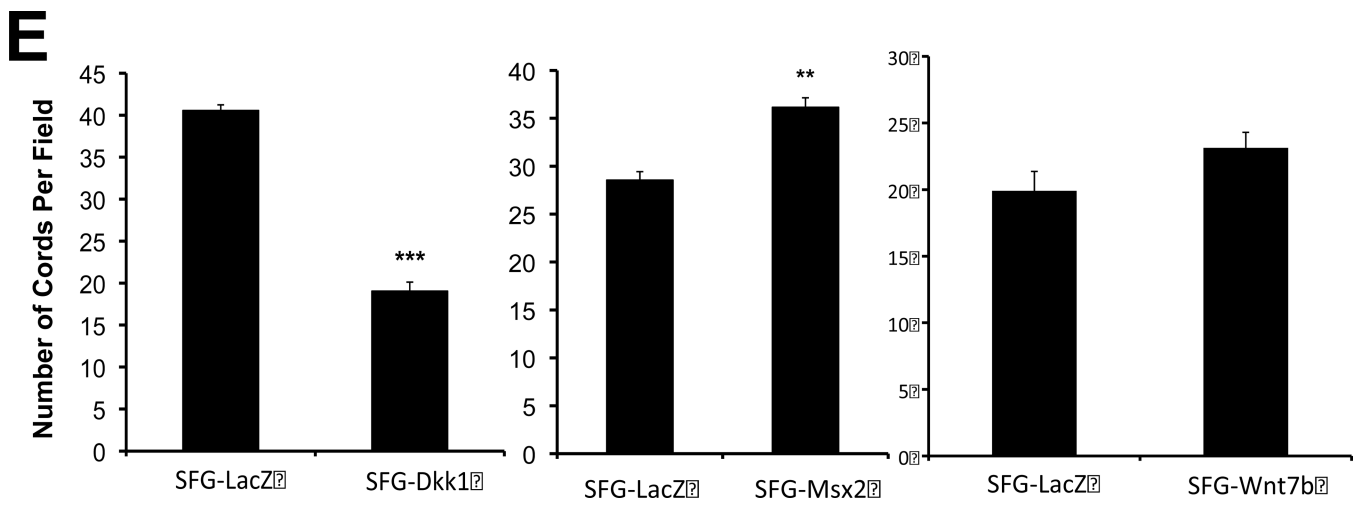
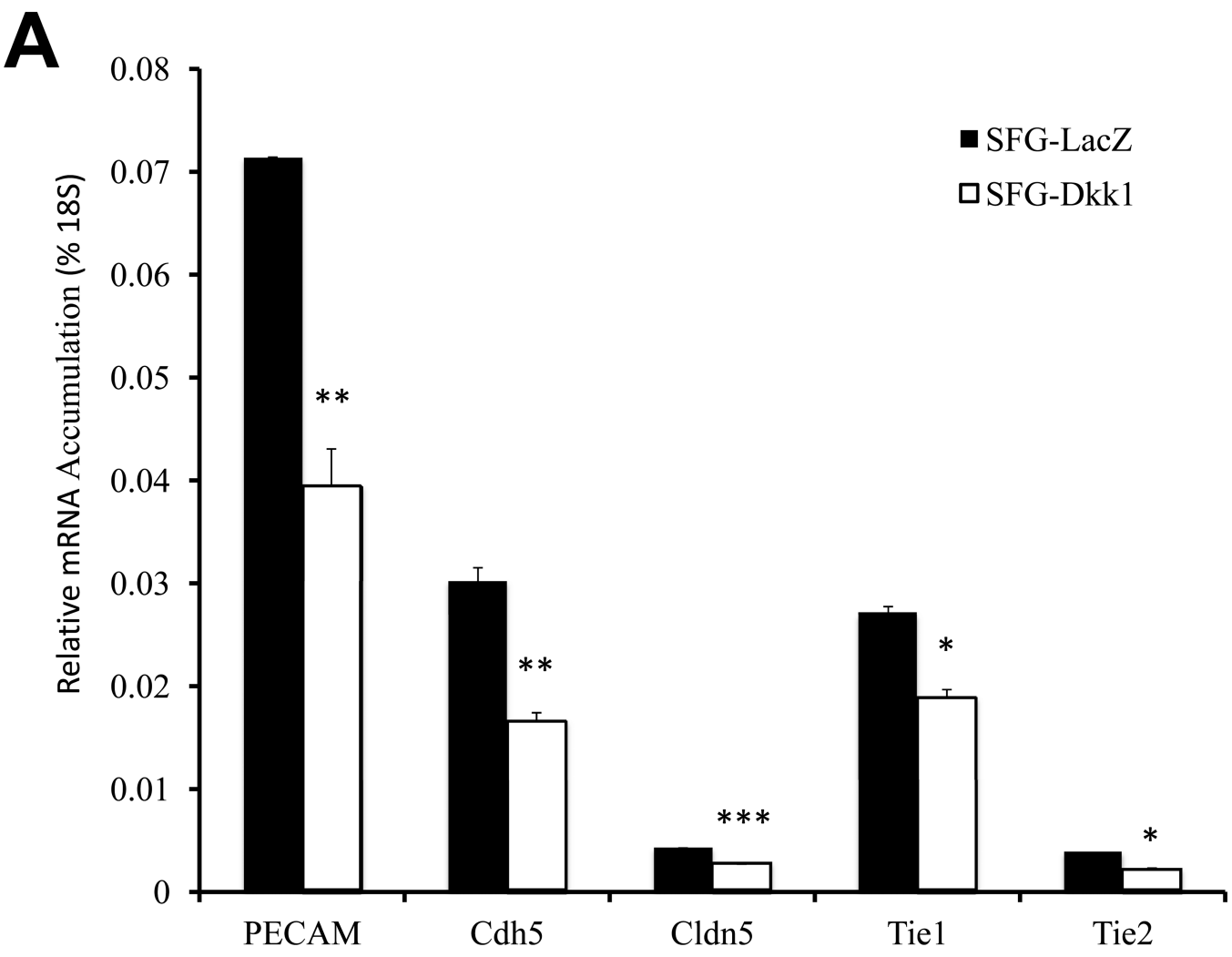
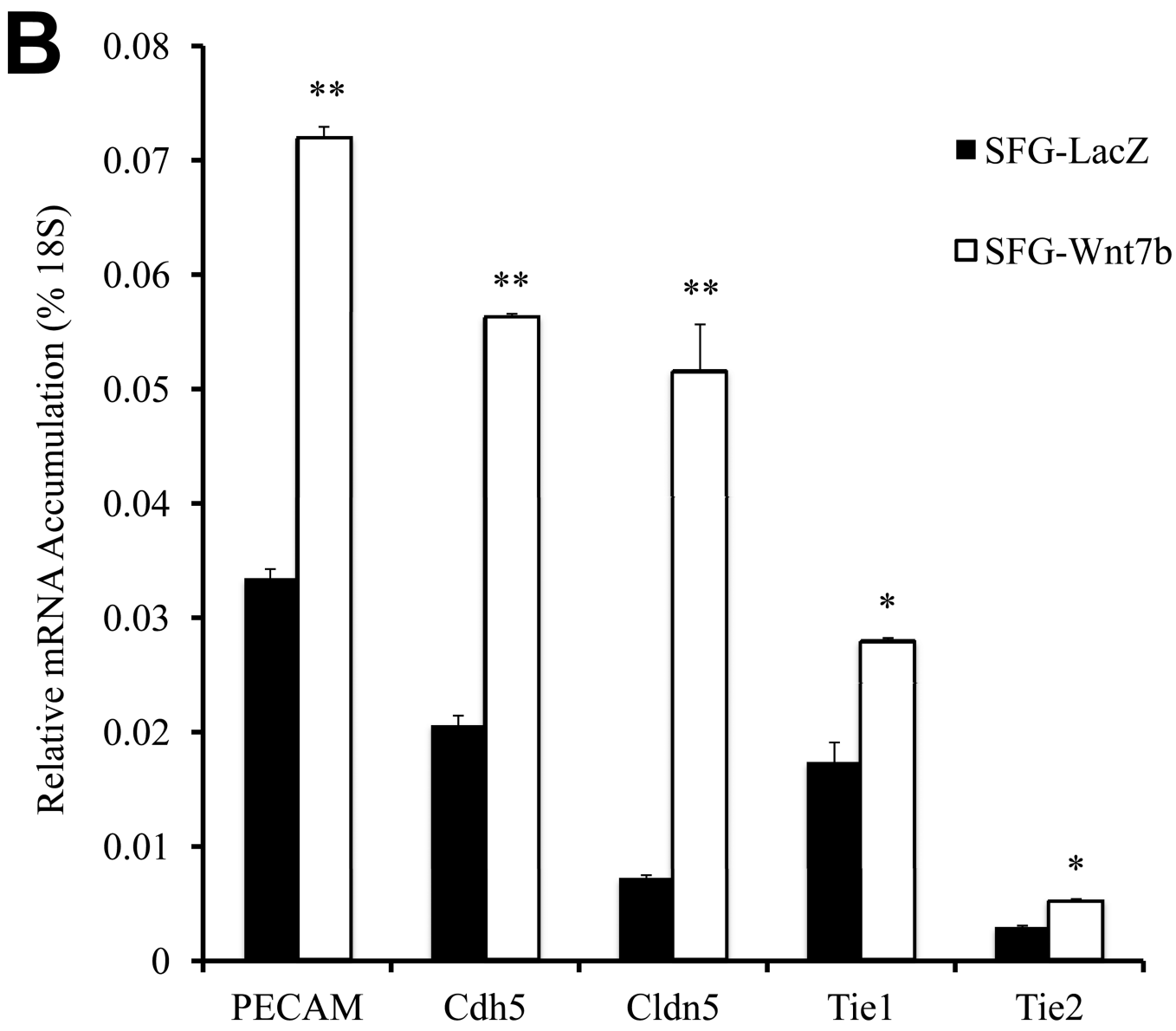



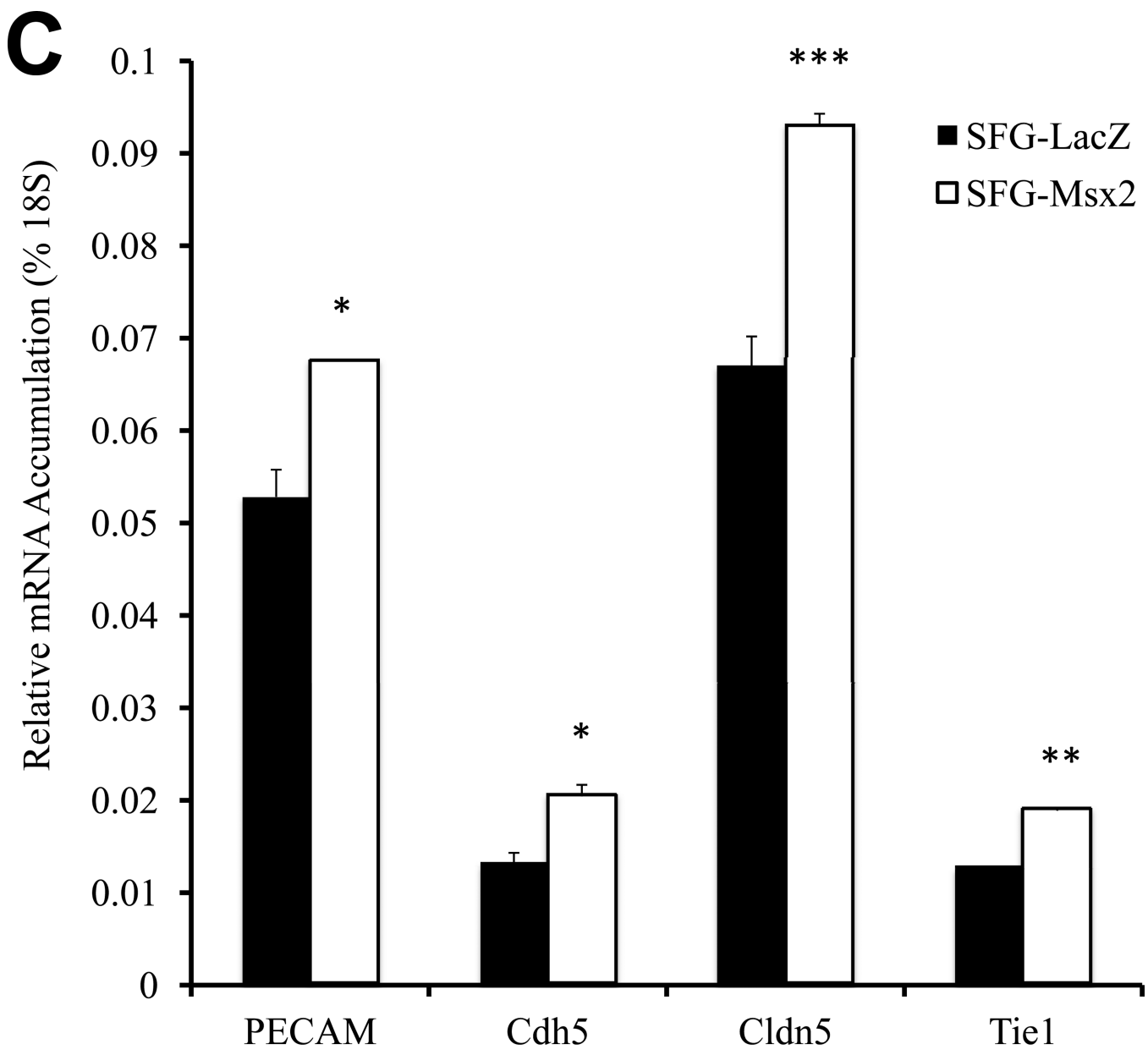

**Figure 1. Dkk1 inhibits EC epithelial cobblestone morphology and capillary–like network formation in primary bovine AoEC cultures**

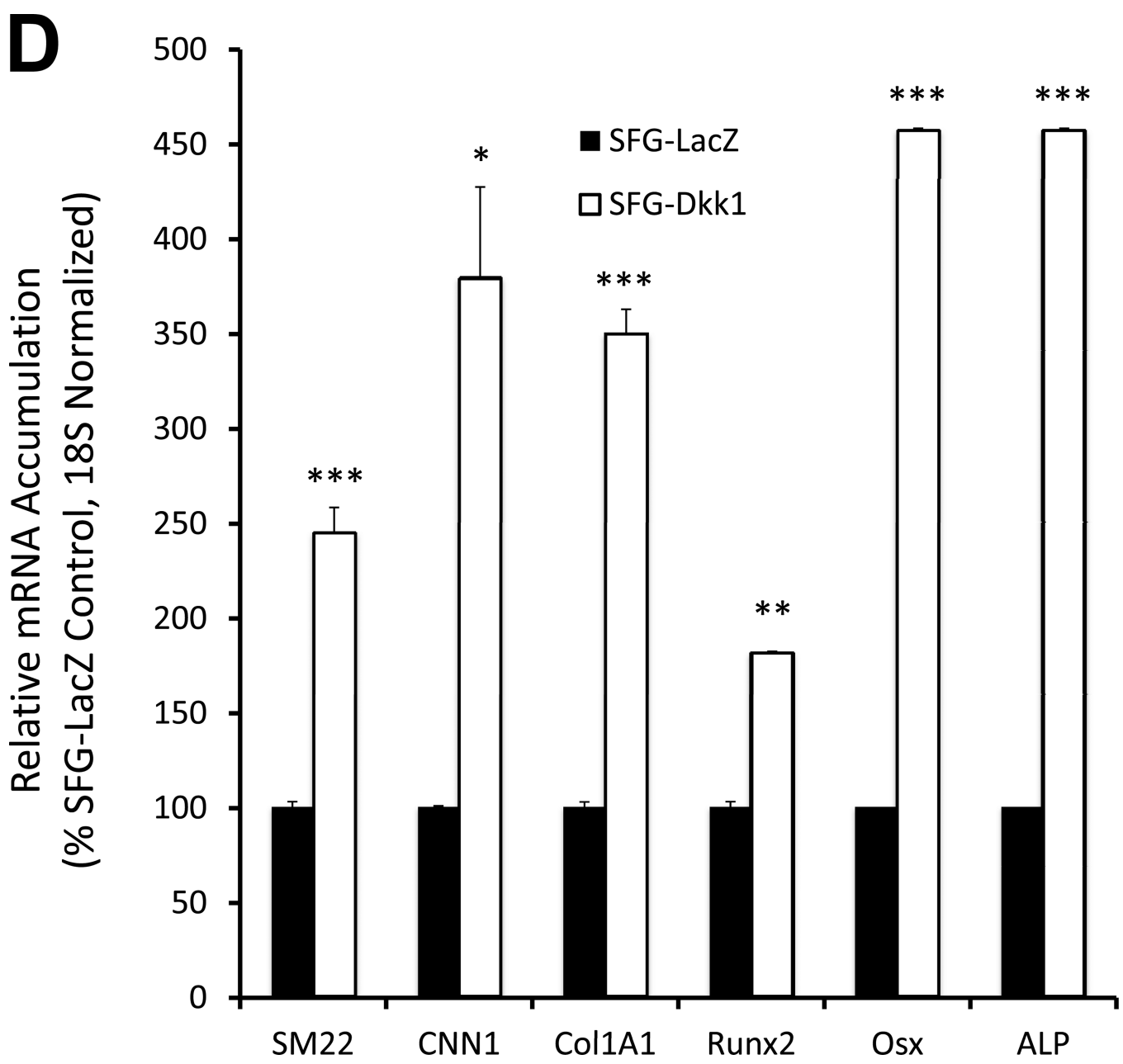
Panel A, as compared to SFG-LacZ controls (upper left), SFG-Dkk1 transduced AoEC cultures (lower right) exhibit spindle-shaped morphology and perturbed cobblestoning. SFG-Wnt7b and SFG-Msx2 transduced cultures more closely resemble SFG-LacZ controls (scale bars 100μm). Panel B, pulse-chase labeling of endothelial cell cultures indicate that AoECs labeled with DiI AcLDL for 4 h (left panel, 100×) adopt a spindle- or oval-shape in SFG-Dkk1 transduced cells after a 4 day chase (right panel, 400×). Note that the spindle-shaped cells, which were already present in the SFG-Dkk1 transduced EC culture at the beginning of pulse, were not labeled by the DiI AcLDL fluorescent dye during the 4 h pulse (lower left panels). (scale bars = 100μm).

Panel C, SFG-Dkk1 increases the percentage of spindle- and oval-shaped cells derived from labeled cuboidal AoEC. Cells were labeled with DiI AcLDL for 4 hours. Four days later, the number of labeled spindle- and oval-shaped cells and the total number of labeled cells were quantified. %EndMT is defined as (number of DiI AcLDL-labeled spindle- and oval-shaped cells/total number of labeled cells) X 100. SFG-LacZ: n=37 fields assessed; SFG-Dkk1: n=56 fields assessed. ***, $p<0.001$ vs. SFG-LacZ. Panel D, as compared to SFG-LacZ controls, AoECs transduced with SFG-Dkk1 exhibit reduced capillary-like network formation on Matrigel while SFG-Msx2 and SFG-Wnt7b maintain this activity. Shown are representative photomicrographs taken at 40× magnification (scale bars 200μm). Panel E, SFG-Dkk1 significantly decreases capillary–like network formation by AoECs cultured on Matrigel. Unlike SFG-Dkk1, SFG-Msx2 and SFG-Wnt7b network formation. ***, $p<0.001$ vs. SFG-LacZ; **, $p<0.01$ vs. SFG-LacZ. The cords formed were counted in at least 6 fields per well and there were 3 wells for each cell line.









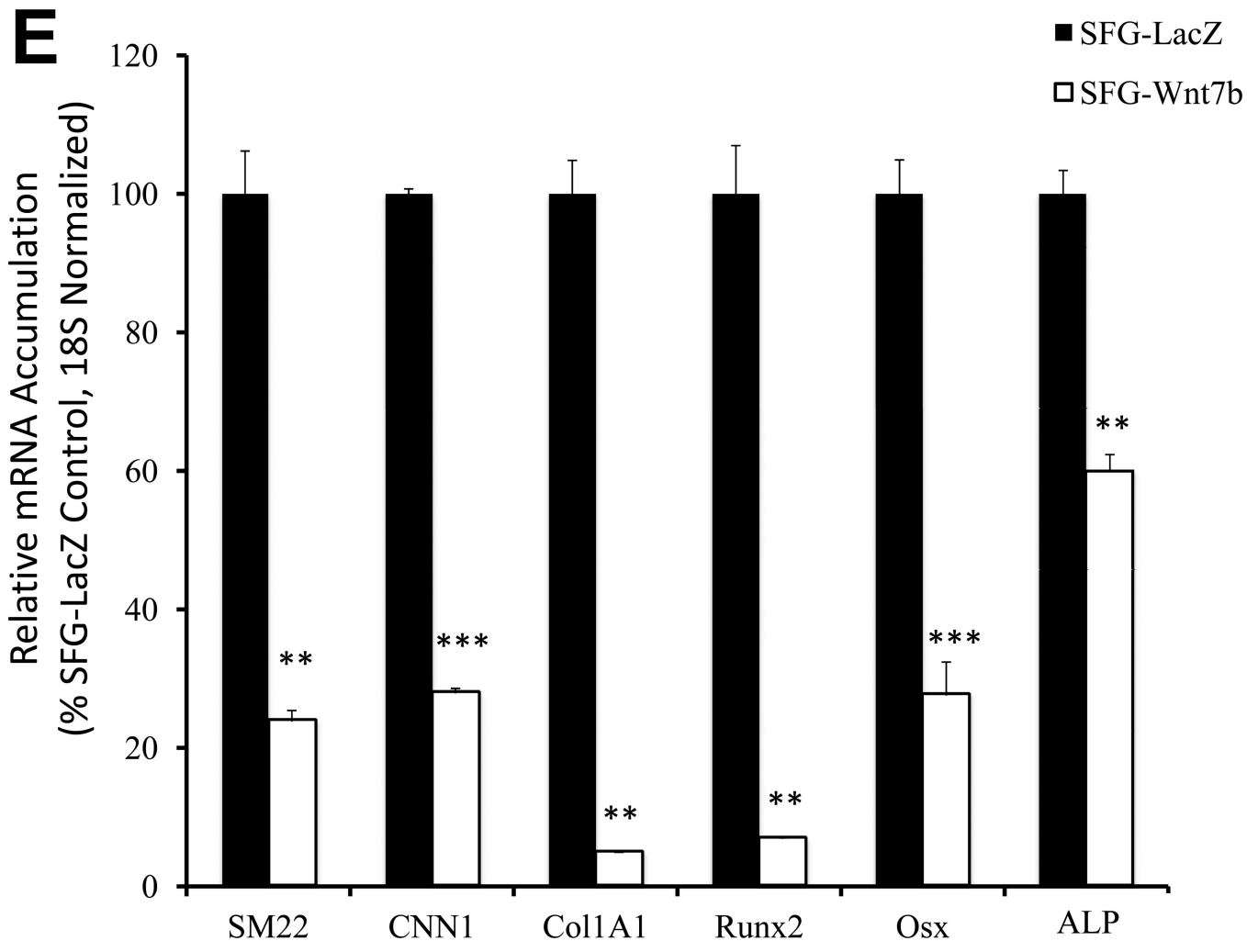
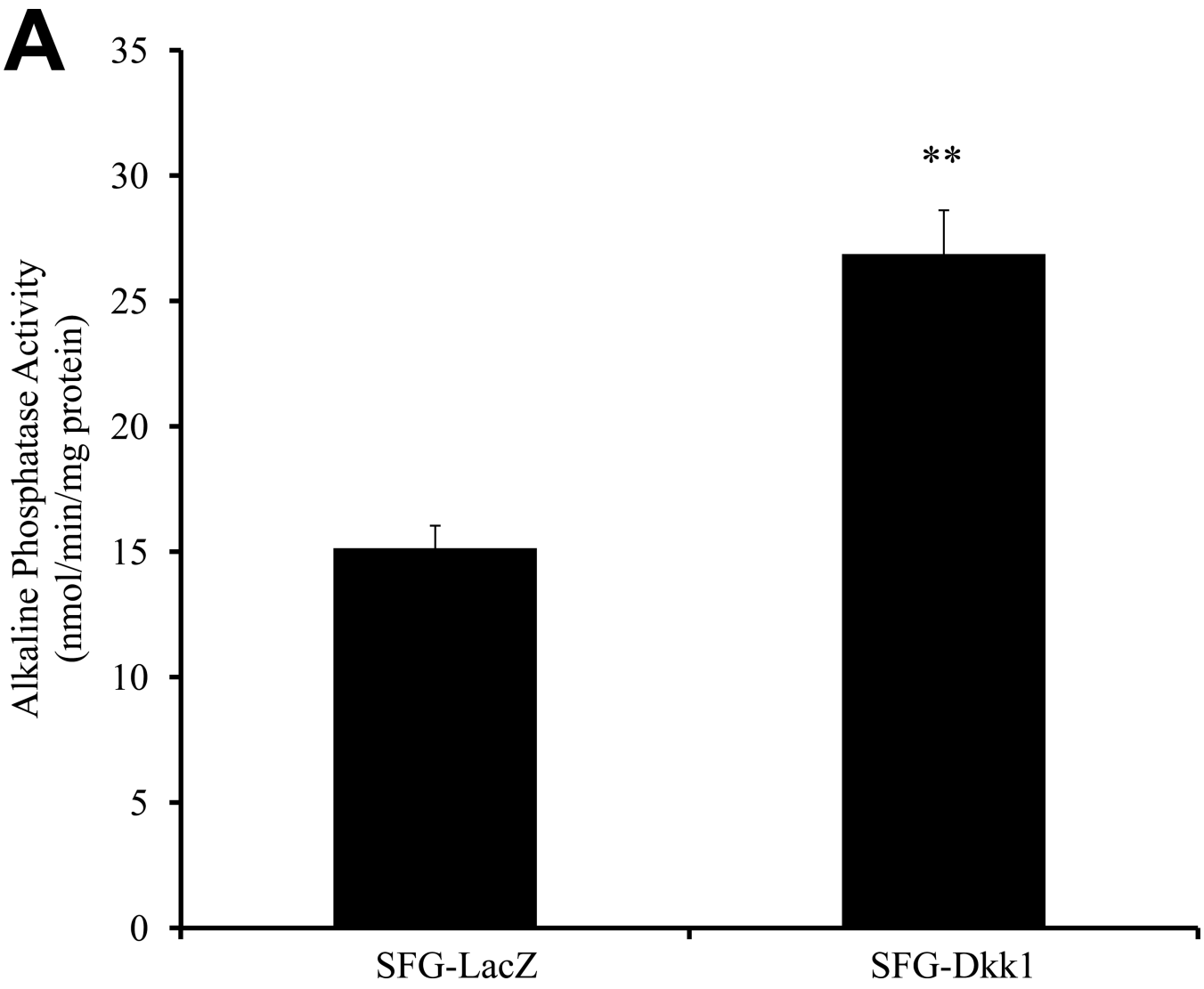



**Figure 2. Dkk1 signaling inhibits the expression of EC differentiation markers and promotes the endothelial-mesenchymal transition in primary bovine AoEC cultures**

Panel A, SFG-Dkk1 down-regulates expression of EC differentiation markers. RNA was harvested for analysis following 5 days in culture. Panel B, by contrast, SFG-Wnt7b promotes expression of the EC phenotype. Panel C, SFG-Msx2 also upregulates EC markers. Panel D, SFG-Dkk1 concomitantly upregulates VSMC and osteofibrogenic gene expression. Panel E, SFG-Wnt7b suppresses VSMC and osteofibrogenic gene expression in aortic endothelial cells. *, $p < 0.05$ vs. SFG-LacZ; **, $p < 0.01$ vs. SFG-LacZ; ***, $p < 0.001$ vs. SFG-LacZ. All experiments were performed in triplicate and repeated at least twice.



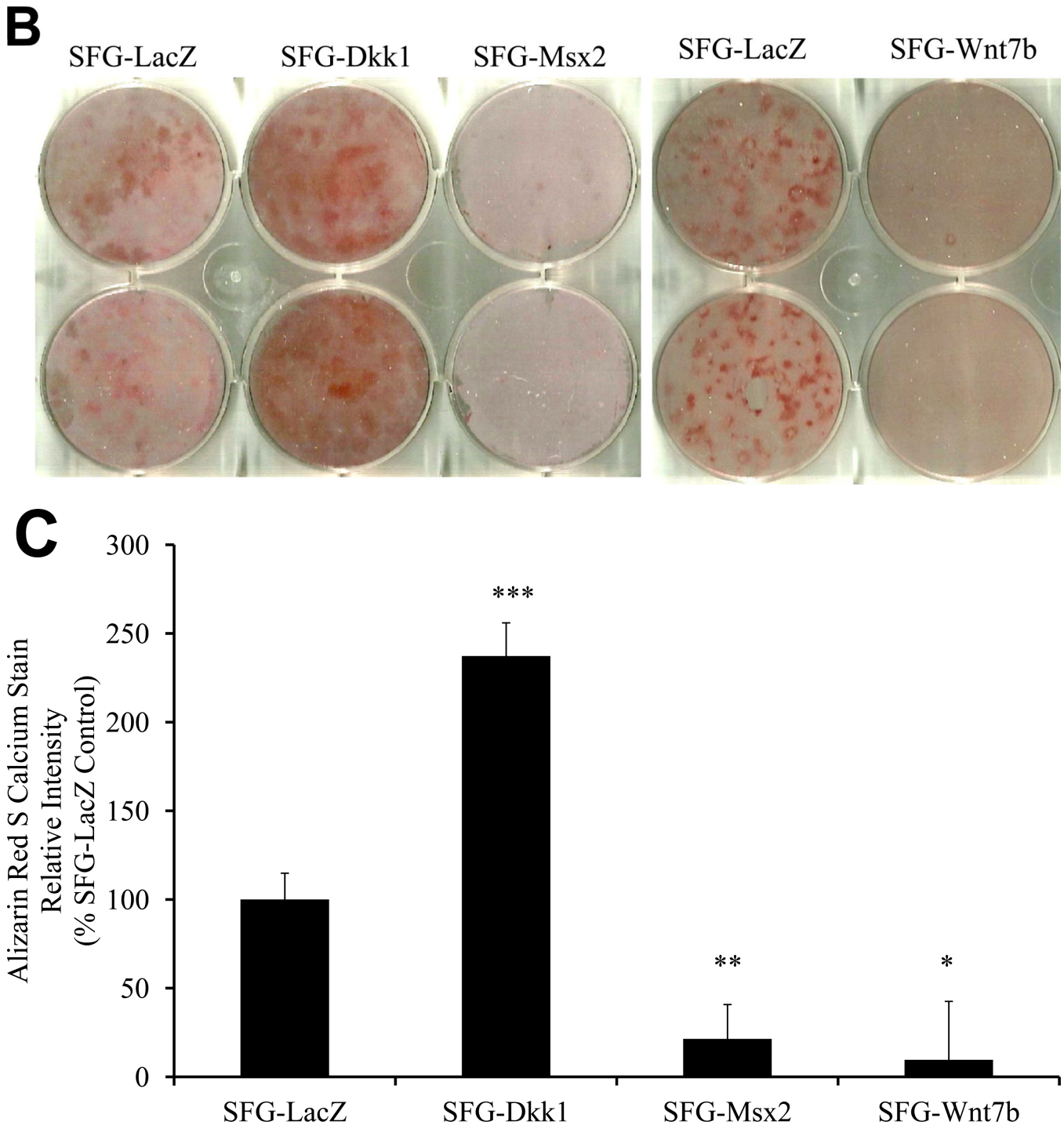
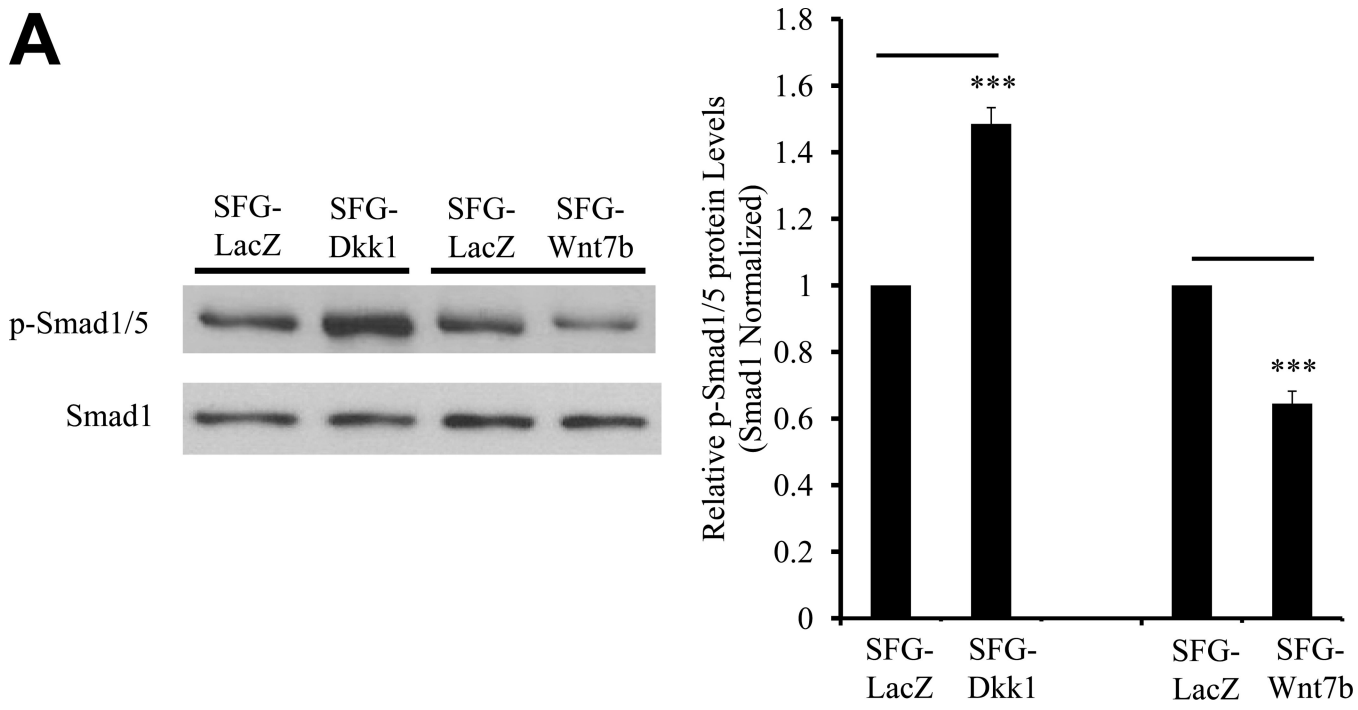
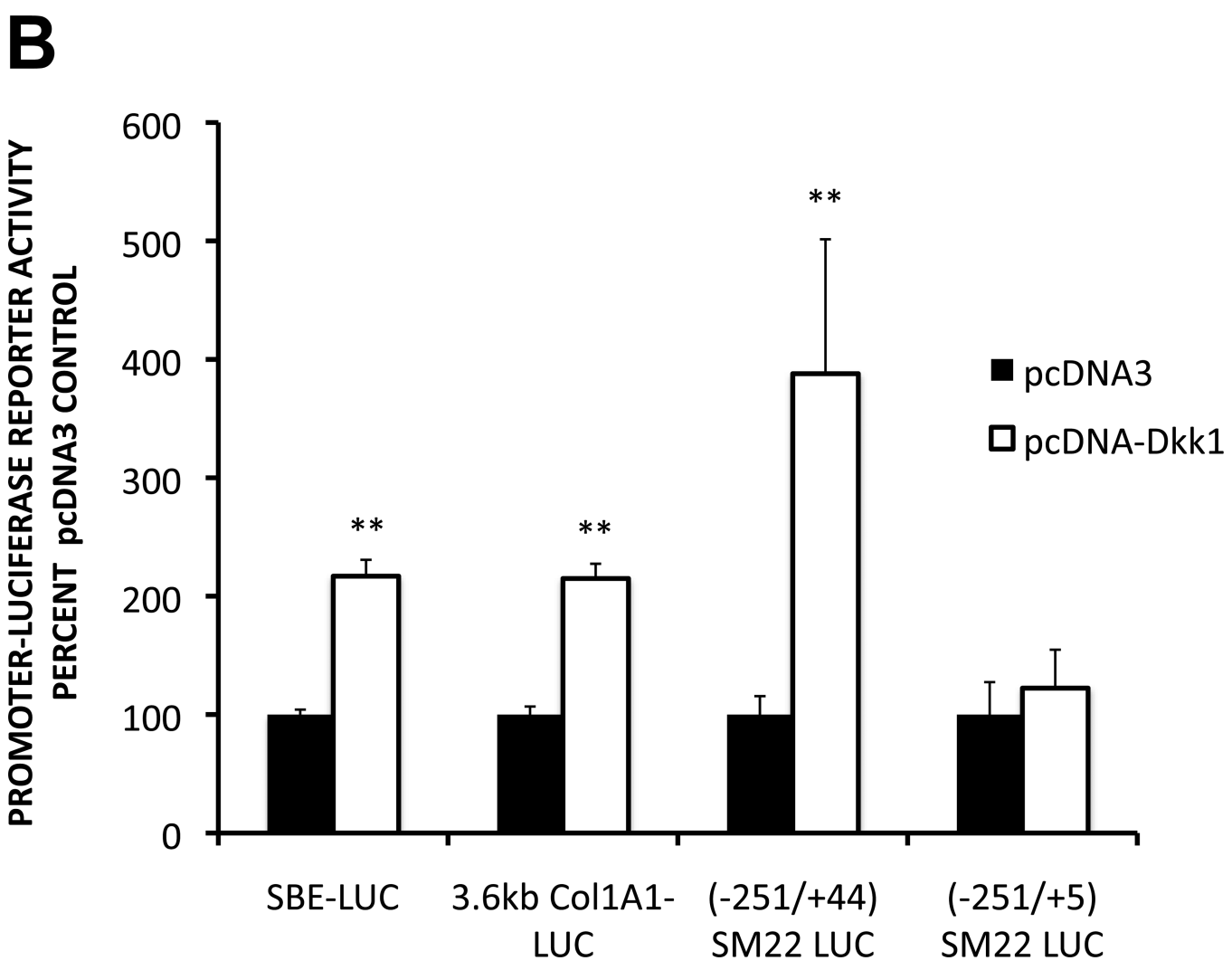



**Figure 3. Dkk1 promotes and Wnt7b inhibits osteogenic calcium deposition directed by bovine AoECs**

Panel A, SFG-Dkk1 upregulates alkaline phosphatase enzyme activity in AoECs, a key component of osteogenic mineralization. **, $p<0.01$ vs. SFG-LacZ. Panel B, Alizarin red S staining reveals SFG-Dkk1 upregulates while SFG-Msx2 and SFG-Wnt7b suppress calcification of bovine AoECs cultured under mineralization conditions. This is the opposite of the response elicited in mesenchymal cells (ref. 7). Panel C, digital quantification of

Alizarin Red S staining demonstrates significant and reciprocal regulation of calcification in bovine AoECs transduced with Dkk1, Msx2, and Wnt7b expression vectors. ANOVA $p < 0.0001$; *, $p < 0.05$ vs. SFG-LacZ; **, $p < 0.01$ vs. SFG-LacZ; ***, $p < 0.001$ vs. SFG-LacZ control. All experiments were performed in triplicate and repeated at least twice.





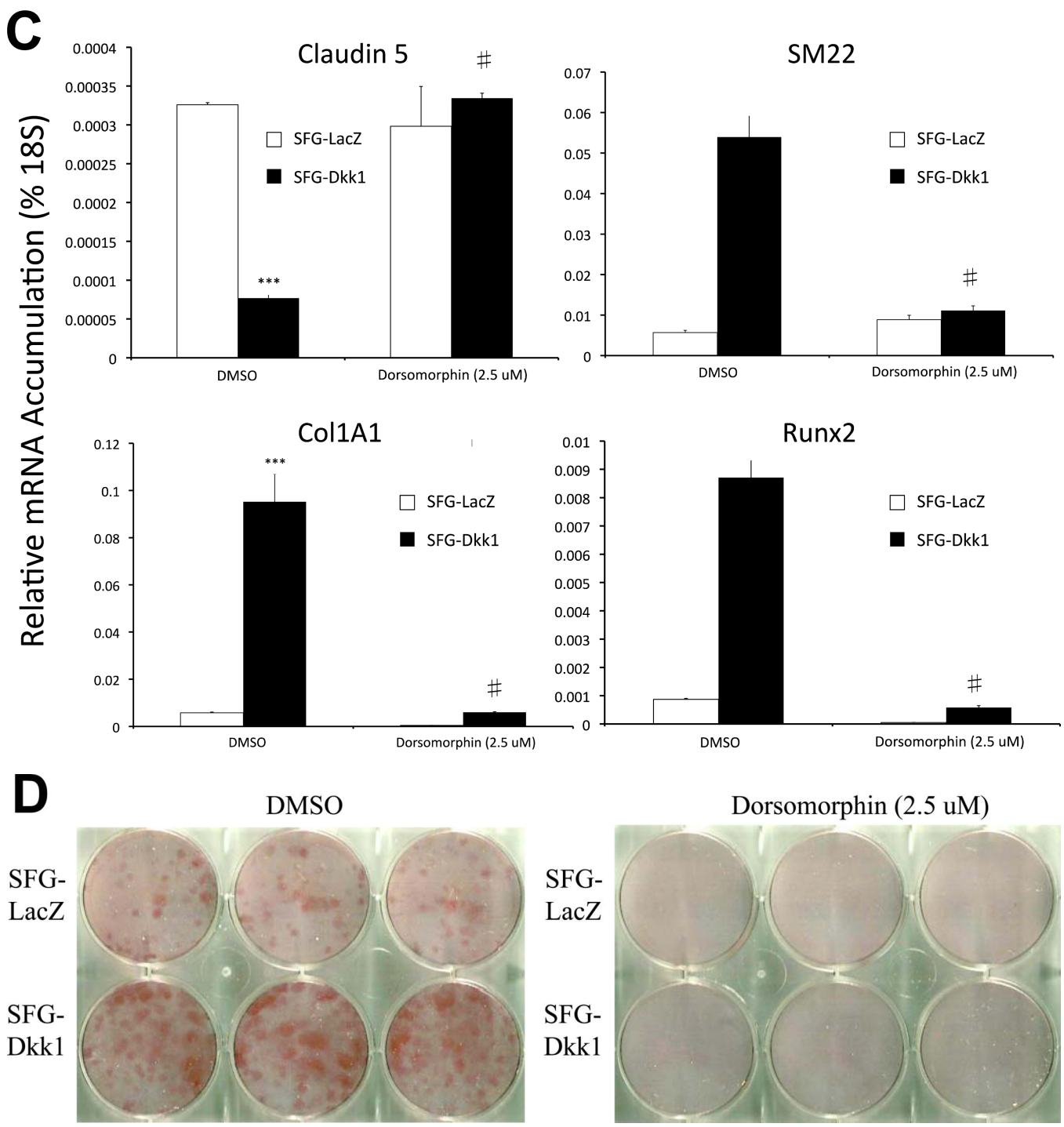
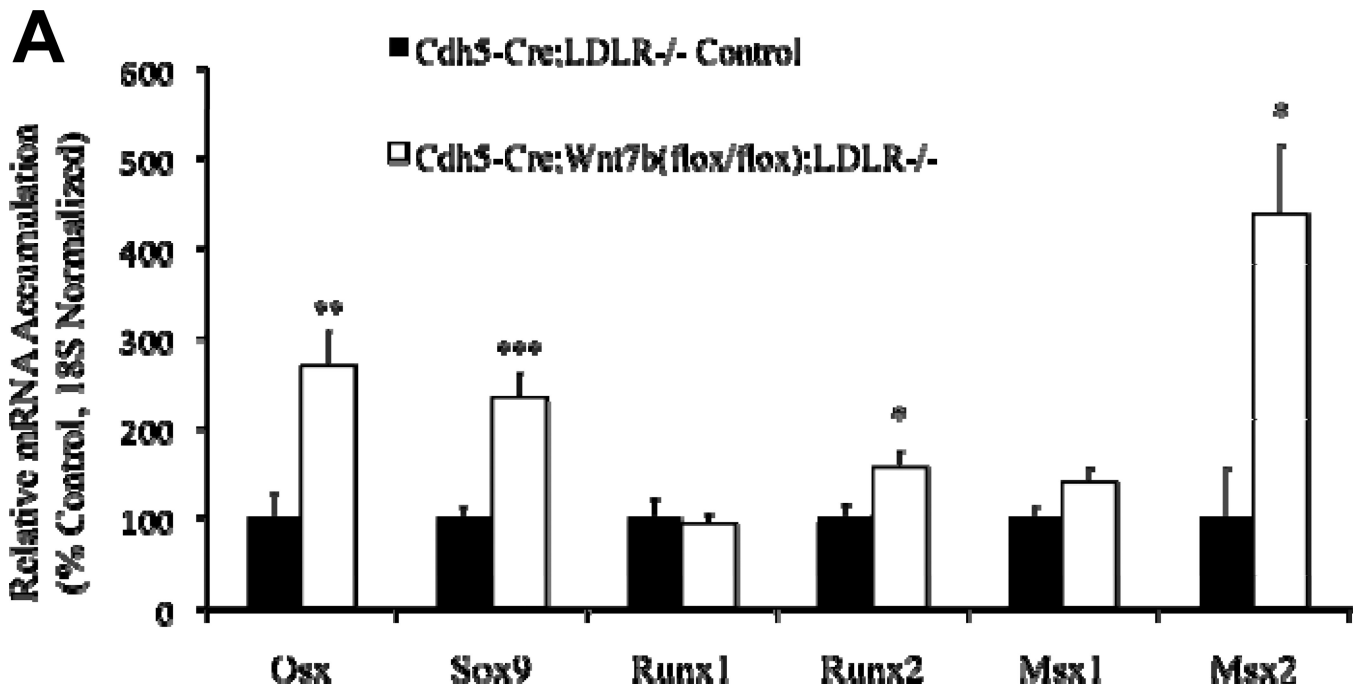
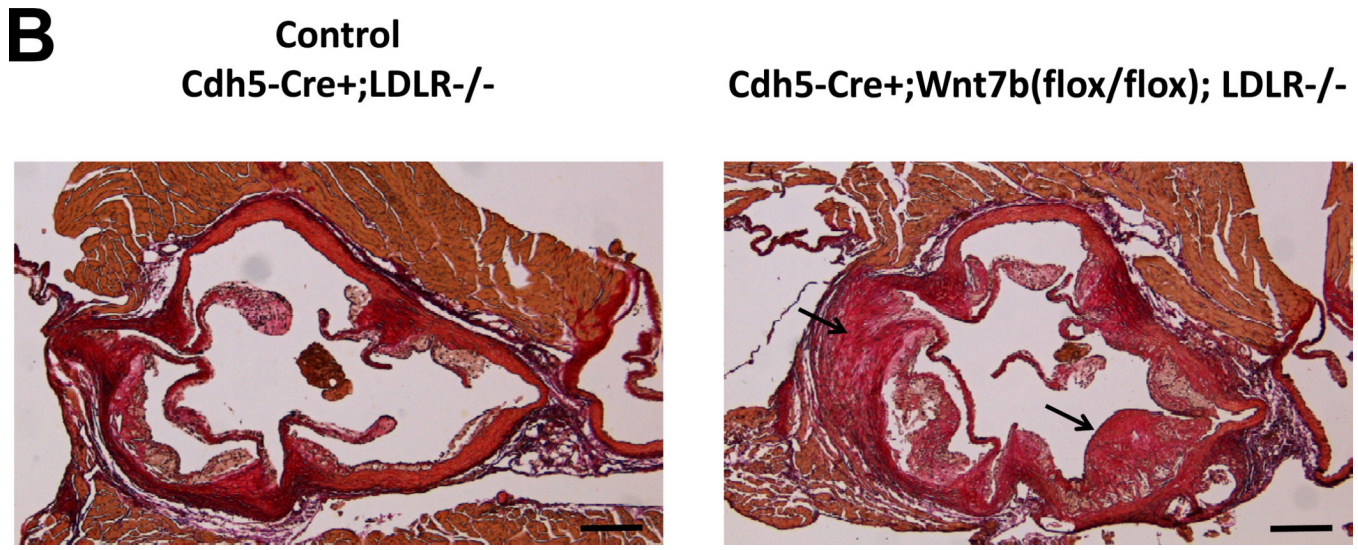



**Figure 4. Dkk1 promotes Smad activation, Smad-dependent transcription, and ALK-mediated signaling in bovine AoECs**

Panel A, SFG-Dkk1 upregulates the accumulation of Smad 1/5 Ser phosphorylation, an index of activated ALK/Smad signaling. By contrast, SFG-Wnt7b suppresses Smad 1/5 phosphorylation in AoEC cultures. Left: representative Western blot analysis; Right: Results of digital image analysis of relative pSmad band intensity; n = 6 / group. ***, p<0.001 vs. respective SFG-LacZ. Panel B, co-transfection of the expression plasmid pDNA-Dkk1

upregulates Smad-dependent promoter-luciferase reporters. (−251/+5) SM22LUC, lacking an exonic Smad binding element, is not regulated.**, $p < 0.01$ vs. pcDNA3 control. All transfections were performed in quadruplicate. Panel C, dorsomorphin, an inhibitor of multiple ALK Smad kinases, inhibits Dkk1 actions on AoEC and osteofibrogenic markers. ***, $p < 0.001$ vs. SFG-LacZ control. #, $p < 0.001$ vs. DMSO-treated SFG-Dkk1 transduced cells. Panel D, Alizarin Red S staining reveals that dorsomorphin inhibits basal and Dkk1-stimulated osteogenic calcium deposition of AoECs cultured under mineralizing conditions. All experiments were performed in triplicate and repeated at least twice.



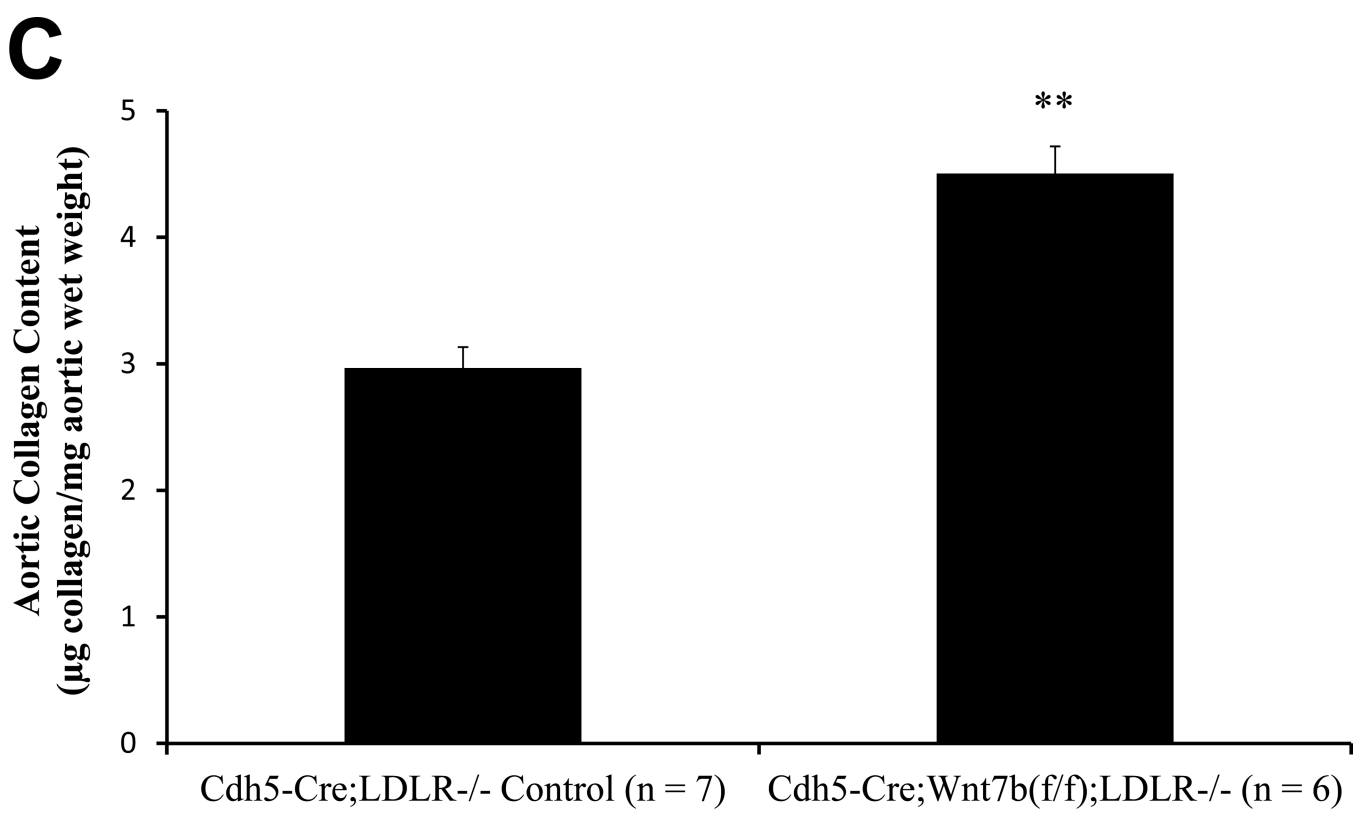



**Figure 5. Conditional deletion of Wnt7b in aortic endothelial cells increases aortic osteogenic gene expression, collagen accumulation, and calcification in LDLR−/− mice**

Panel A, Cdh5-Cre;Wnt7b(fl/fl);LDLR−/− mice express significantly greater levels of osteogenic transcription factor mRNAs in aortic tissue vs. controls. N = 6 – 7 per genotype. *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. Control; ***, $p < 0.001$ vs. Control. Panel B, picrosirius red histochemistry for collagen deposition indicates greater fibrosis in Cdh5-Cre:Wnt7b(fl/fl);LDLR−/− mice (arrows) following 12 weeks of high fat diet (HFD). (scale bars 200µm). Panel C, biochemical measurement of aortic collagen by Sircol assay demonstrates significantly increased collagen deposition in Cdh5-Cre:Wnt7b(fl/fl);LDLR−/− mice vs. Cdh5-Cre;LDLR−/− controls following 12 weeks of HFD. Calcium deposition is also increased in Cdh5-Cre:Wnt7b(fl/fl);LDLR−/− mice (see text). **, $p<0.01$ vs. Control.

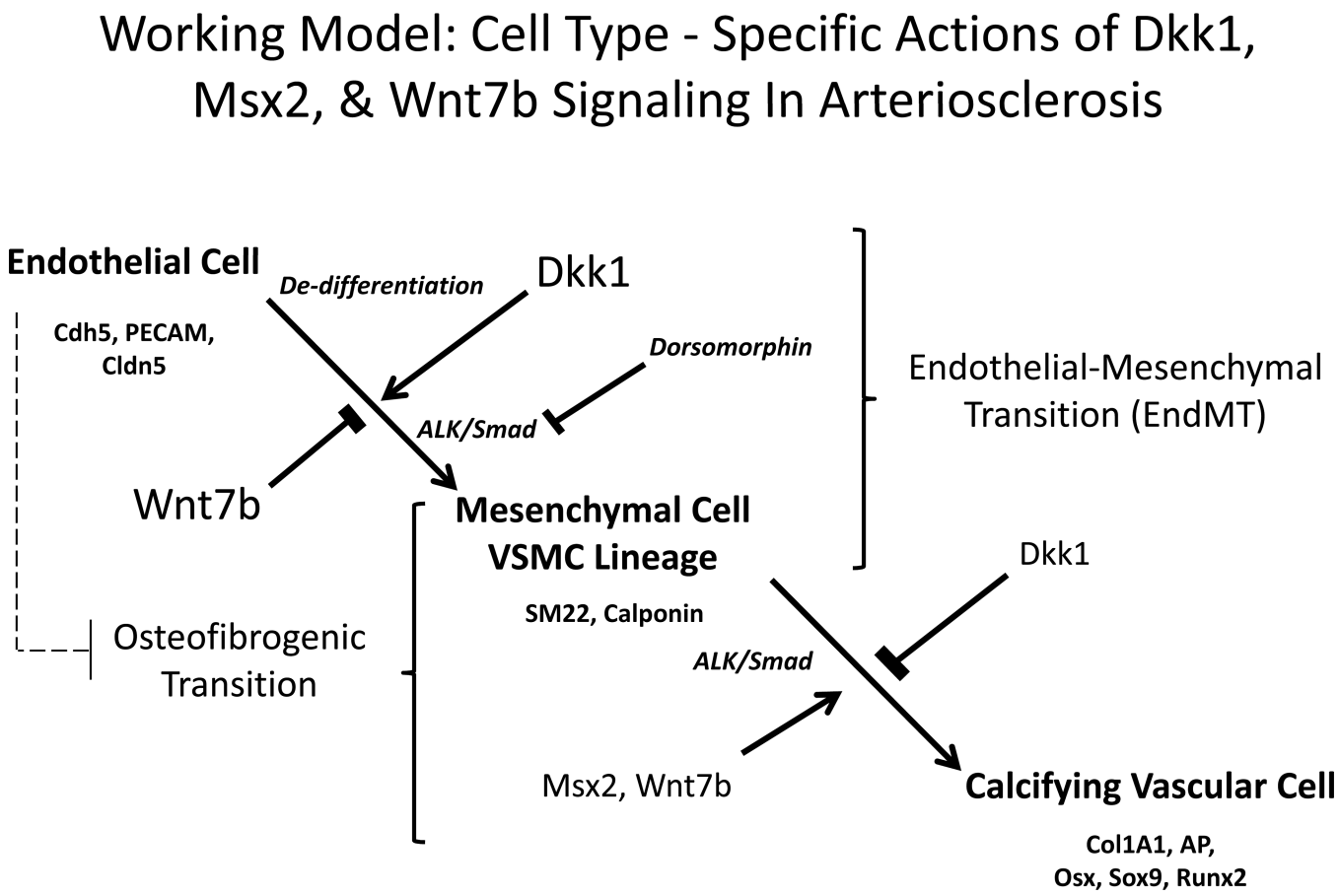



**Figure 6. Working model of the cell type – specific actions of Dkk1, Msx2, and Wnt7b signaling in arteriosclerosis**

Dkk1, arising from platelets or cytokine –stimulated vascular cells can impair the autocrine actions of EC Wnt7b that stabilize the EC phenotype. This promotes EndMT, thus increasing the mesenchymal cell pool that contributes to vascular fibrosis. Committed mesenchymal progenitors, by contrast, elaborate the calcifying vascular cell phenotype in response to Msx-Wnt signaling, e.g. an osteofibrogenic transition. Juxtacrine EC cues may also regulate this latter process (dashed line). ALK/Smad signals are required for both mesenchymal and calcifying vascular programs. See Discussion.