Application of Small Organic Molecules Reveals Cooperative TGFβ and BMP Regulation of Mesothelial Cell Behaviors

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Ⓢ Supporting Information

ABSTRACT: Epicardial development is a process during which epithelial sheet movement, single cell migration, and differentiation are coordinated to generate coronary arteries. Signaling cascades regulate the concurrent and complex nature of these three events. Through simple and highly reproducible assays, we identified small organic molecules that impact signaling pathways regulating these epicardial behaviors. Subsequent biochemical analyses confirmed the specificity of these reagents and revealed novel targets for the widely used dorsomorphin (DM) and LDN-193189 molecules. Using these newly characterized reagents, we show the broad regulation of epicardial cell differentiation, sheet movement, and single cell migration by Transforming Growth Factor β (TGFβ). With the DM analogue DMH1, a highly specific Bone Morphogenetic Protein (BMP) inhibitor, we demonstrate the cooperative yet exclusive role for BMP signaling in regulation of sheet migration. The action of DMH1 reveals that small organic molecules (SOM) can intervene on a single epicardial behavior while leaving other concurrent behaviors intact. All SOM data were confirmed by reciprocal experiments using growth factor addition and/or application of established non-SOM inhibitors. These compounds can be applied to cell lines or native proepicardial tissue. Taken together, these data establish the efficacy of chemical intervention for analysis of epicardial behaviors and provide novel reagents for analysis of epicardial development and repair.

Mesothelium is the simple squamous lining of the body cavities and organs.[1] Mesothelia are important in development, as they are essential for blood vessel formation and organogenesis in general.[2-5] Still, generation of mesothelium is complex, and information on regulation of its various cell behaviors is only now emerging.[6-8] The epicardium, covering the heart, is the best-studied model of mesothelial development.[9,10] During embryogenesis, the proepicardium arises independently from the heart on the sinus venosus.[3,4,11-13] Development and differentiation of the proepicardium is dependent on multiple simultaneous cell behaviors as it migrates to the naked myocardium, adheres, and spreads over the heart as an epithelial sheet.[9,14] During this lateral migration event, selected epicardial cells undergo epithelial/mesenchymal transition, migrate throughout the heart, and differentiate into many lineages including vascular smooth muscle, fibroblasts, endothelial cells, and cardiomyocytes.[5-7,11,15] Epicardial development is likely to require cooperative signaling mechanisms.

The approach to understanding complex molecular interplay controlling cell behaviors has historically used gene mutation and knockdown studies to deactivate gene products.[16] While these studies have been successful, they are not without caveats as they may cause secondary effects on the system and are expensive in terms of time, effort, and materials. Additionally, knockdown or knockout studies may simultaneously remove all functions

performed by the gene, including regulation by noncoding microRNAs.[17,18] An emerging alternative employs organic chemical "perturbagens" that bind to proteins, alter their function, and replace or complement gene mutation or knockdown studies.[17] Small organic molecules (SOM) have an added advantage: when well-characterized, selected compounds have highly specific activity on discrete residues of their target proteins and may affect only one of multiple functions performed by that molecule.[17]

An excellent example of this concept is dorsomorphin (DM) and its family of analogues.[19,20] These structurally related compounds differentially target Bone Morphogenetic Protein (BMP) signaling. BMPs are part of the Transforming Growth Factor β (TGFβ) superfamily of growth factors[21,22] and have been implicated in regulating epicardial behaviors.[23-26] TGFβ and BMP signaling occurs through stimulation of ligand-specific type-I and type II serine/threonine kinase receptors on the effector cell membrane, where they dimerize in response to ligand.[27] The receptor complex stimulates signal transduction in effector cells via phosphorylation of Smad family mediator

j Characteristics of Small Organic Molecules

Small Organic Molecule	Affected Pathway	Molecular Target (if known)	Concentration
SB-431542	TGFβ Inhibitor[a]	ALK 4, 5, 7[a]	5μm
HI913	Hedgehog Inhibitor[b]	Unpublished	0.5μm
KA4541	NF-κB/Notch Inhibitor[b]	Unpublished	1μm
BIO	Wnt Activator[c]	GSK3β[c]	0.5μm
Cyclopamine	Hedgehog Inhibitor[d]	Smoothened[d]	10μm
XAV-939	Wnt Inhibitor[e]	Tankyrase[e]	3μm
Dorsomorphin	BMP/AMPK/VEGFR2 Inhibitor[f]	ALK2, 3, 6, TGFβR2, AMPK, VEGFR2[f]	2μm
LDN-193189	BMP/TGFβ Inhibitor[g-h]	ALK 2, 3, 4, 5, 6, AMPK, VEGFR2[g-h]	2μm
DMH1	BMP Inhibitor[h]	ALK 2, 3[h]	2μm
DMH4	VEGFR2 Inhibitor[h]	VEGFR2[h]	2μm

k Structures of Small Organic Molecules

SB431542 · BIO · Cyclopamine · XAV-939 · Dorsomorphin · LDN-193189 · DMH1 · DMH4

Figure 1. Dorsomorphin and SB-431542 inhibit epicardial α-smooth muscle actin (SMA) expression. (a, b) Epicardial/mesothelial cells (hereafter termed "epicardial cells") treated with serum-free medium (hereafter termed "medium") plus or minus growth factors serve as negative and positive controls for SMA expression. (c) Epicardial cells stimulated with growth factors and treated with SB- 431542 ablated SMA expression. (d−h) Growth factor-stimulated cells treated with KA4541, BIO, Cyclopamine, or XAV had no effect on SMA expression. (i) Growth factor-stimulated cells treated with dorsomorphin (DM) also strongly inhibited SMA expression. (j) This information panel provides details about the SOMs tested in these studies. (k) The structures of the small molecules tested in this study are presented here. [(Scale bar = 50 μm. Footnotes: [a]Reference 26, [b]unpublished data, [c]Reference 41, [d]Reference 42, [e]Reference 43, [f]Reference 44, [g]Reference 30, [h]Reference 22. SFM = Medium; GF = Growth Factor Medium].

molecules, which translocate to the nucleus and stimulate transcription of target genes.[27] Identification of SOMs with broad or specific inhibitory effects on these pathways would produce versatile tools to study intricate developmental processes regulated by concurrent signaling cascade mechanisms.

Chemical biology is particularly amenable for unraveling the complexity of epicardial development, where many signaling cascades coordinately impact cell behaviors. In the present study, we conducted SOM screens of epicardial differentiation, sheet movement, and single cell migration to elucidate signaling pathways regulating these independent yet concurrent events. With compounds that broadly or specifically intervene on signaling pathways, we show a dependence on TGFβ signaling in the regulation of all three activities. Conversely, using a newly characterized and highly specific BMP inhibitor, the DM analogue DMH1, we show that both TGFβ and BMP signal cascades interdependently regulate epicardial sheet migration. Intervention with these perturbagens demonstrates that a single epicardial behavior can be inhibited while simultaneous cell activities are left intact in both clonal cell lines and native tissue. Taken together, these data demonstrate the efficacy of chemical intervention to identify cooperative signaling in the regulation of epicardial behaviors. In addition, we provide the field with well-characterized reagents for intervention in both embryogenesis and wound healing.

■ RESULTS

Specific Small Molecules Intervene in Epicardial Smooth Muscle Differentiation. In an effort to establish a broadly applicable approach to analyze regulation of specific epicardial cell behaviors, a panel of SOMs was tested for effects on early smooth muscle differentiation. Previous work from our laboratory and others determined that expression of smooth muscle α-actin (SMA) in epicardial cells is a hallmark of epithelial/mesenchymal transition, an initial step in smooth muscle differentiation, and can be stimulated by multiple growth factors.[10,28,29] Treatment of epicardial cell lines with a combination of known SMA-stimulating growth factors for 48 h in serum-free medium (hereafter termed medium) evoked a robust and highly replicable SMA response (Figure 1, panel a *versus* b). Representative results of selected SOMs are presented in Figure 1, panels a−i. The characteristics and structures of representative molecules are listed in Figure 1, panels j and k. Most compounds had no or minimal effect on the number of cultured cells expressing SMA. One exception was the TGFβ inhibitor SB-431542,[30] which completely ablated SMA expression (Figure 1, panel c). This is a valuable internal control, as TGFβ is known to stimulate SMA expression in epicardium. Interestingly, dorsomorphin (DM), an inhibitor of BMP signaling cascade receptors along with other signaling pathways including vascular endothelial growth factor (VEGF),[31−33] also ablated SMA expression (Figure 1, panel i). This is an unexpected result, as BMP signaling is thought to regulate only myocardial differentiation in the heart and is not a well-described activator of smooth muscle differentiation.[27,28,34−36] Thus, these surprising results raised the possibility that BMP signaling may be involved in epicardial cell smooth muscle differentiation.

Figure 2. Analysis of growth factor regulation of SMA expression in epicardial cells. (a, d, g, j) Epicardial cells cultured in low-serum medium did not express SMA. (b, e, h, k) Growth factors added to low-serum medium induced high levels of epicardial SMA expression. (c, f, m) Epicardial cells cultured with growth factors and treated with DM/LDN-193189 significantly inhibited SMA expression compared to untreated cells. (i, m) Epicardial cells cultured with growth factors and treated with the BMP-specific inhibitor DMH1 marginally inhibited SMA expression. (l, m) Epicardial cells cultured with growth factors and treated with DMH4 were unaffected (from growth factor $p = 0.237$). (n−p) Epicardial cells cultured in BMP2 or 4 did not stimulate SMA expression, at low or high concentrations. (q, r; t, u) Conversely, TGFβ_1 treatment stimulated expression of the early differentiation markers, SMA and SM22. (s, v) TGFβ_1-induced SMA and SM22 expression was ablated by addition of DM. [$n = \geq 20$ images; scale bar = 50 μm; (*$p <$ 0.01; $\sim p < 0.05$) TGFβ = Transforming Growth Factor β; BMP = Bone Morphogenetic Protein].

Application of DM Analogues Elucidates Differential Requirements for TGFβ and BMP Signaling for Epicardial Differentiation. We next determined whether the previous results revealed a novel role for BMP signaling in epicardial differentiation, an effect of DM on an alternate pathway, or both. To examine these possibilities, SMA expression was assayed following treatment with 63 structural analogues of DM that have selectivity for individual pathways greater than that of the parent compound.[33,37] Treatment of cells with LDN-193189, an inhibitor of BMP and VEGF pathways,[33,37] eliminated SMA expression (Figure 2, panels d−f and m). Conversely DMH1, an extremely selective inhibitor of the BMP type-I receptors Activin receptor-like kinases 2 and 3 (ALK2 and ALK3), had minimal effect (Figure 2, panels g−i, m). Additionally, DMH4, a selective inhibitor of VEGF receptor-2 (VEGFR2),[33] had no impact on epicardial SMA expression (Figure 2, panels j−m). Thus, use of these more selective inhibitors suggests that BMP signaling is not responsible for epicardial differentiation and that DM and LDN-193189 act on a previously unidentified target regulating this behavior.

To explore these unanticipated results, we conducted the inverse experiment adding specific growth factors to epicardial cultures and determining their effects on SMA expression. Addition of BMP2 and 4, both canonical stimulators of the BMP receptors ALK2/3[38,39] and highly expressed in epicardial

progenitors,[23,34] had no effect on SMA or SM22 expression at multiple concentrations (Figure 2, panels n−p, Supplemental Figure S1). TGFβ_1 was the only growth factor to induce expression of these early smooth muscle differentiation markers in epicardial cells (Figure 2, panels q, r and t, u), reflecting results obtained from other groups.[8,27,28] Interestingly and consistent with our previous data, this stimulation was completely ablated by addition of DM, identical to the inhibitory effects of SB-431542 (Figure 2, panels s and v). Corroborating our previous results in which the BMP inhibitor DMH1 had little or no effect on TGFβ stimulation of epicardial differentiation (Figure 2, panel i), these results support the data presented above, that BMP has no effect on this epicardial differentiation and that DM and LDN-193189 may affect this particular behavior in epicardial cells *via* inhibition of TGFβ signaling.

Identification of Novel DM Analogue Targets Reveals Differential Regulation of Epicardial Receptors. To precisely determine the signal cascades inhibited by DM and its analogues, *in vitro* kinase inhibitory activities (as assessed by IC$_{50}$, concentration causing 50% inhibition) against potential BMP and TGFβ receptors were performed. As expected, all DM analogues tested strongly inhibited ALK1 and ALK2, both type-I receptors selective for BMP ligands (Table 1; refs 38 and 39). Our previous work has shown that DM analogues also potently inhibited ALK3, another BMP type-I receptor.[33] Importantly, DMH1 had minimal

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Table 1

	confirmatory in vitro kinase assay: IC$_{50}$ (nM)				
	DM	LDN	DMHI	DMH2	DMH3
ALK1/ACVRL1	484	13.3	27	12	5
Alk2/ACVRI	**148**	**41**	**108**	**43**	**27**
ALK4/ACVR1B	*a*	1825	9,622	1,407	643
ALK5/TGFβR1	*a*	565	*a*	2,418	2,062
TGFβR2	178	140	*a*	86	246
AMPK	235	1,122	*a*	3,527	1,940
VEGFR2	22	215	*a*	2,418	2,062
PDGFRβ			*a*		

a None (>30 K).

activity on any of the TGFβ receptors tested (Table 1: ALK4, ALK5 and TGFβR2; ref 33). Conversely and unexpectedly, our data revealed for the first time that the widely used DM and LDN-193189 inhibited TGFβR2, a type-II receptor for TGFβ.[40]

We next examined whether the apparent differential effects of DM and DMH1 on BMP and TGFβ receptors in biochemical analyses extended to epicardial cells. Since BMP and TGFβ signaling occur *via* distinct receptors to trigger Smad1/5/8 and Smad2/3 phosphorylation, respectively,[21,22] we utilized pathway-specific Smad phosphorylation to determine the inhibitory precision of DM and DMH1 in epicardial cells. TGFβ_1 administration induced Smad3 phosphorylation, and consistent with our *in vitro* kinase assay data, DM but not DMH1 inhibited TGFβ_1-dependent Smad3 phosphorylation (Figure 3, panel a). Conversely, both DM and DMH1 ablated Smad1/5 phosphorylation below baseline (Figure 3, panel a). Total Smad3, Smad1/5, and housekeeping gene (cyclophilin) protein levels were unchanged in all conditions. Additionally, the Wnt signaling cascade was unaffected under all conditions, further conferring specificity to the DM analogues (Figure 3, panel a). Immunofluorescence analysis demonstrated that BMP2 stimulated high levels of phospho-Smad1/5 nuclear localization, which was completely inhibited by both DM and DMH1 (Figure 3 f, g and data not shown). TGFβ had minor stimulatory effect on nuclear localization, which was also inhibited by DM and DMH1 (Figure 3, panels c–e). Collectively, our data confirm that TGFβ, but not BMP, signaling is critical for epicardial cell differentiation and identify new targets of the widely used DM and LDN-193189 molecules. With a precise understanding of the activities of the DM analogues in epicardial cells it was now possible to examine the concurrent regulation of additional behaviors by the two signaling pathways.

Elimination of BMP Signaling Inhibits Epicardial Sheet Movement, but Not Differentiation. A more precise understanding of DM and DMH1 activities at the level of BMP and TGFβ receptors enabled us to use these reagents to explore whether TGFβ and/or BMP signaling regulate epicardial cell behaviors other than SMA expression. Epithelial sheet migration is a function basic to epicardial cells and can be analyzed after scratch injury.[3,4,6] In the presence of TGFβ_1, epicardial wounds incubated for 6 h exhibited significantly increased regrowth rate (Figure 4, panels a–c). Application of DM and LDN-193189 inhibited sheet migration to control rate, indicating that elimination of TGFβ and/or BMP signaling disrupts this function (Figure 4, panels d and e). Interestingly, DMH1, the exquisitely selective BMP inhibitor, intervened in TGFβ-induced sheet migration, having the strongest inhibitory effect of all DM analogues (Figure 4, panel f compared

Figure 3. DMH1, but not dorsomorphin, specifically inhibits Smad1/5 activity. (a) The TGFβ responsive Smad3 molecule was activated in the presence of TGFβ_1 and inhibited with addition of dorsomorphin, but not DMH1 (row: P-Smad3). Alternatively the phosphorylation of the BMP responsive Smad1/5 molecules was lost when cells were treated with DM or DMH1 (row: PSmad1/5). Lysates from epicardial cells in all treatments expressed equal amounts of cyclophilin (loading control), Smad1/5, and βcatenin total proteins (rows: cyclophilin, Smad3, Smad1/5, and βcatenin, respectively). βcatenin phosphorylation was unchanged, conferring specific activity to DM and DMH1 on Smad transduction molecules (row: P-βcatenin). (b, c) Phospho-Smad1/5 nuclear localization was minimal in serum free medium and was marginally upregulated by TGFβ_1. (f) Alternatively, BMP2 treatment greatly increased activated Smad1/5 nuclear localization. (e, g) TGFβ-stimulated cells wounded, treated with DM and DMH1 did not localize Smad1/5 to the nucleus. [n = 3, P-Smad3 = phosphorylated Smad3, P-Smad1/5 = phosphorylated Smad1/5 protein, P-βcatenin = phosphorylated β-catenin.].

to a). In fact, DMH1 significantly decreased migration rate below the negative control, similar to the strong TGFβ inhibitor SB-431542 (Supplementary Figure S2, panels a–c). Because of this surprising result, we determined whether activated BMP receptors might overcome the requirement for TGFβ_1 ligand. Thus, we overexpressed constitutively active ALK2/3 and assayed sheet movement in the absence of TGFβ_1. Overexpression of these receptors strongly enhanced epicardial sheet migration, which was again inhibited upon application of DMH1 (Figure 4, panels l–o). Further, in subsequent experiments we demonstrated that exogenous application of BMP ligands can also stimulate sheet movement in the absence of TGFβ (Figure 7, panels f and g). Thus, activated BMP signaling in the absence of TGFβ is sufficient to elicit this response, complementing the strong loss-of-migration phenotype observed with DMH1. Because addition of TGFβ is the only exogenously applied growth factor in this context, it is possible that an endogenous source of BMPs exists or that the BMP targets ALK1–3 may be directly activated by TGFβ_1 ligand. We performed experiments outlined below that resolve this question (Figure 7). Taken together, these data illustrate the concurrent requirement for TGFβ and BMP signaling in the regulation of epicardial sheet movement.

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Figure 4. DMH1 strongly inhibits epicardial sheet movement, but not SMA expression. Epicardial sheet migration rate was assessed by wound healing assays with representative images of wounds at 6 h in panels b−f. (a, c) Wounding epicardial sheets in the presence of $TGF\beta_1$ significantly increased sheet migration rate in comparison cells wounded in medium. (a, d, e) Epicardial wounds cultured with $TGF\beta_1$ and concurrently treated with DM/LDN-193189 healed at medium-equivalent rates. (a, f) Interestingly, sheets wounded with $TGF\beta_1$ and the BMP inhibitor DMH1 healed at a rate significantly slower than that for unstimulated sheets. (g) Epicardial sheets wounded in medium spontaneously expressed SMA after an 18h incubation. (h) When wounds were cultured with $TGF\beta_1$, SMA expression was strongly increased. (i, j) Conversely, when sheets were cultured with $TGF\beta_1$ and treated with DM/LDN-193189, SMA expression was inhibited. e) DMH1 marginally inhibited $TGF\beta_1$-stimulated SMA expression. (l, n) Epicardial sheet migration was strongly stimulated by constitutively active ALK2/3. (l, o) Migration was modestly inhibited by treatment of constitutively active-ALK2/3 cells with DMH1, which differs from the significant inhibition that DMH1 exerts on sheet migration in wildtype cells. [$n \geq 8$ wounds; Scale bar = 50 μm; (∗ = $p < 0.001$ from $TGF\beta_1$-stimulated cells; ∼ = $p < 0.001$ from untreated cells; ∧ = $p < 0.001$ from untreated CA-ALK2/3); CAALK2/3 = Constitutively Active-ALK2/3].

Simultaneous with migration of a wounded epithelial sheet is the expression of SMA in cells on its leading edge.[41] Analysis of SMA expression during sheet migration afforded us the opportunity to

examine two distinct epicardial cell behaviors at the same time and to explore whether specific perturbagens reveal differential regulation of these events. Sheets incubated with $TGF\beta_1$ exhibited a

Figure 5. DMH1 does not ablate single cell migration. (a) Epicardial cells cultured in medium have minimal single cell migration in Boyden chamber assays. (b, d) Single cell migration is significantly enhanced by stimulation with TGFβ_1. (c, d) TGFβ_1 stimulation was inhibited by DM/LDN-193189 to a migration rate equivalent with medium ($p = 0.056$). (d) TGFβ_1 stimulation was only marginally inhibited by DMH1, unlike the TGFβ inhibitor SB-431542, which ablated this behavior. [$n = 30$ images; ($* = p < 0.001$ from TGF$\beta_1 \sim = p < 0.001$ from SFM)].

marked increase in SMA expression, along with robust sheet migration (Figure 4, panel h). Application of DM, LDN-193189, or SB-431542, all shown to inhibit TGFβ/Smad2/3 signaling in this system, strongly blocked both behaviors (Figure 4, panels i and j; Supplemental Figure S2, panel b). Interestingly, while DMH1 had the greatest inhibitory effect on sheet migration, SMA expression was untouched (Figure 4, panel k). These data demonstrate that inhibition of TGFβ has broad inhibitory effects on epicardial behaviors, while BMP disruption is highly selective for sheet migration. Application of DM and DMH1 to epicardial cells allowed the simultaneous analysis of multiple cell behaviors, and revealed specific intervention in only one behavior while leaving the other concurrent behaviors intact.

DMH1 Does Not Ablate Epicardial Single Cell Migration. Our current data suggest that BMP signaling regulates only sheet movement in epicardial cells. However, single cell migration is also a critical behavior for epicardially derived cells,[4] and as such, we analyzed the impact of BMP signaling on this activity. It has been previously noted that TGFβ stimulates single cell invasive migration,[42] and thus we used this factor as an activator of the behavior and then perturbed it with the DM analogues. When epicardial cells were stimulated with TGFβ_1 a significant increase in single cell migration rate occurred compared to controls (Figure 5, panels a *versus* b and d). Interestingly, application of DMH1 did not interfere with single cell migration (Figure 5, panel d). Conversely, DM, LDN-193189, and SB-431542 all strongly inhibited single cell migration, (Figure 5, panels c and d). BMP2 and 6 were tested for their influence on single cell migration in the same assay and, as expected, did not stimulate

this behavior, corroborating the DMH1 data (Supplemental Figure S3, panels b—d). Taken together, these data indicate that BMP signaling specifically regulates epicardial sheet migration, having little influence on other behaviors in epicardial cell lines.

DMH1 Inhibits Sheet Migration, but Not Early Differentiation, in Proepicardial Explants. The proepicardium is the source of epicardial cells in the developing embryo.[3,4,12,13] Several groups have shown that proepicardial explants grown *in vitro* retain the developmental properties analyzed in this study.[4,6,28,43] Thus, it was essential to determine whether SOMs are effective tools to modulate specific cell behaviors in native embryonic epicardial tissues. Proepicardial explants grown in basal medium spread on fibronectin to form a radial outgrowth with an average radium of 904 μm (Figure 6, panels a, d, g). These outgrowths had membrane localized Zonula Occludins-1 (ZO-1; a commonly used epithelial junction marker[44]) and SMA expressing cells at their periphery. Proepicardial explants incubated in TGFβ_1 had outgrowths of approximately the same size as untreated explants (av radius = 1003 μm; Figure 6, panel g). This similarity was unsurprising given the native mobility of embryonic epicardial tissue. However, TGFβ_1 evoked increased peripheral SMA expression and stimulated expression within the epicardial sheet itself (Figure 6, panels b and e). In contrast, when proepicardial explants were grown in the presence of DM, there was a significant decrease in the spread of the epicardial sheet at 24 h (av radius = 489 μm; Figure 6, panels c, f, g). Similar results occurred with LDN-1923189 treatment (Figure 6, panel j). Additionally, in DM and LDN-193189 treated explants, SMA expression was restricted to the peripheral cells and was greatly diminished in intensity compared to TGFβ_1-treated

Figure 6. DMH1 inhibits sheet migration, but not SMA expression, in proepicardial explants. HH stage 16 proepicardia were excised from chick embryos and incubated for 24 h. (a, d) Proepicardia incubated in medium exhibited a uniformly spread epicardial cell sheet with SMA expression at the distal edge and ZO-1, marking epithelial cells, in the interior of the sheet. (b, e, g) Proepicardia cultured in TGFβ₁ had upregulated SMA expression centrally and peripherally in the outgrowths, but TGFβ₁ treatment did not increase outgrowth migration rate from medium controls. (c, f, g, j) Proepicaria stimulated with TGFβ₁ and treated by DM/LDN-193189 had reduced epicardial sheet migration and SMA expression. (k) Proepicardia stimulated by TGFβ₁ and treated with DMH1 inhibited outgrowth migration but had enhanced SMA expression and diminished ZO-1 protein expression throughout the epicardial sheet. [Measurements assessed per condition = 40; Scale bars = 200 μm for panels a−c and 50 μm for panels d−f; (∗ = $p < 0.001$; $n \geq 5$ proepicardia); ZO-1 = Zonula Occludins-1].

cultures. In contrast, treatment of proepicardial explants with DMH1 resulted in the greatest inhibition of epithelial sheet migration. At the same time, DMH1 treated outgrowths displayed the highest level of SMA expression, with abundant numbers of positive cells at the periphery and within the sheet itself (Figure 6, panel k). These data corroborate our previous studies using adult epicardial cell lines and suggest that BMP signaling is required for epicardial sheet migration while having minimal or even perhaps a permissive effect on SMA expression. The specific inhibition of sheet migration from both clonal epicardial cells and native embryonic tissue further demonstrates the efficacy of DMH1 in perturbation of a single behavior while leaving other functions unaffected.

BMPs Are Expressed by Epicardial Cells and Regulate Cellular Behaviors. The present study suggests that TGFβ and BMP signaling cascades have both interdependent and independent roles governing epicardial development. While TGFβ and BMP signaling could display some cross-talk among transduction molecules after receptor activation, the most parsimonious explanation of this result is that BMPs are made by epicardial cells and work together with exogenously applied TGFβ to regulate sheet migration. Indeed, previous work has shown that both epicardium and compact myocardium produce TGFβ ligands and BMPs.[6,36,45] To determine whether epicardial cells express BMPs, cultures were incubated in medium for 24 h and assessed by RT-PCR. As seen in Figure 7, panel a, a myriad of BMPs were detected under basal conditions. In subsequent studies to quantify BMP expression using QRT-PCR, TGFβ₁ treatment enhanced expression of BMP6 only (Figure 7, panel b).

Finally, although epicardial cells make BMP growth factors, it is possible, as a result of effector cross-talk between TGFβ ligand and BMP signal receptors, that BMP ligands are not required to stimulate sheet migration. Previous studies indicate that while TGFβ- and BMP-induced signaling cascades are often independent,

TGFβ may activate BMP receptors.[22,28] To test whether BMP itself is a stimulating ligand, the classic BMP extracellular sequestering molecule Noggin[46] was used to remove any endogenously produced BMP ligands. Wounded epicardial sheets stimulated by TGFβ₁ and treated with Noggin had a significant reduction in sheet migration when compared to TGFβ₁ treatment alone (Figure 7, panels d and g). This result was similar to but not as pronounced as the reduction observed with DMH1 treatment (compare Figure 7, panel g with Figure 4, panel a). Thus, Noggin and DMH1 both inhibit extracellular stimulation of BMP receptors, but DMH1 has greater efficacy and specificity for sheet migration. In a complementary experiment, application of the canonical BMP pathway-activating protein, BMP2, enhanced epicardial sheet migration rate beyond that observed with endogenous levels of BMP (Figure 7, panels e and g). This differs from the effect of BMP2 on SMA expression, where addition of the growth factor had no stimulatory effect (Figure 2, panel n). Finally, as TGFβ₁ treatment enhanced BMP6 expression, this molecule was tested for effects on SMA expression and sheet migration. As observed with BMP2 treatment, BMP6 had no effect on smooth muscle differentiation (Figure 7, panels h−j) but strongly enhanced sheet migration (Figure 7, panel k), suggesting that BMP2 and 6 have similar stimulatory functions in this system. Interestingly, as shown above, BMP2 and −6 have no effect on single cell migration (Supplemental Figure S3, panels b−d). Taken together, these data indicate that extracellular BMP growth factors are required to stimulate epicardial sheet migration in conjunction with TGFβ.

Conclusion. Here we present simple, highly reproducible screens to identify SOM perturbagens of epicardial behaviors. Further, we have characterized selected compounds to precisely detail their molecular specificity and have identified important new targets of the widely used DM and LDN-193189 molecules. With these reagents, it was possible to determine their potential

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Figure 7. Epicardial cells express BMP factors that regulate epicardial behaviors. (a) Epicardial cells express a multitude of BMP mRNAs when assayed by RT-PCR. (b) Only BMP6 expression level was significantly increased after $TGF\beta_1$ treatment when assayed by QRT-PCR. (c, d, f, g) Epicardial cells treated with $TGF\beta_1$ and Noggin (200 ng/mL) inhibited epicardial sheet migration significantly when compared to untreated, $TGF\beta_1$-stimulated epicardial sheets. (e) Epicardial cells treated with BMP2 only stimulated sheet migration with a rate equivalent to $TGF\beta_1$-stimulated sheets. (f–h) Similar to BMP2, epicardial cells stimulated with BMP6 in medium did not express SMA when compared to $TGF\beta_1$-stimulated cells. However, BMP6 treatment enhanced sheet migration at a rate equivalent to that of $TGF\beta_1$-stimulated sheets. [($p = 0.92$). $n = 40$ images; scale bar = $50\,\mu$m; $* = p \leq 0.001$; $\wedge = p \leq 0.001$ from $TGF\beta_1$-treated].

intervention in three concurrent epicardial behaviors. Specifically, we demonstrate the broad requirement for $TGF\beta$ in epicardial differentiation, sheet movement, and single cell migration, while determining that BMP signaling is essential for only sheet movement, with little to no impact on the two other functions. Together, these data illustrate that well-characterized analogue families of SOMs are valuable tools to intervene on specific behaviors in multifaceted systems. Additionally, we are the first to show cooperative regulation of epicardial sheet migration by the $TGF\beta$ and BMP signaling cascades. Using the DM analogue family permitted a coanalysis of multiple signaling factor contributions to this epicardial behavior. During development, cells respond concurrently to a myriad of signals. It is highly probable that multiple signal cascades can and do contribute to the regulation of a single behavior. Developing tools like the DM analogue family will permit the identification of cooperative regulation of epicardial development, which might have been overlooked using less sensitive perturbation techniques.

■ METHODS

Quantification. For the assays described below statistics and percent closure were performed in Microsoft Excel. Error bars represent the standard error of the mean; Student's t tests were used to determine significance. All images being compared were adjusted equally.

Cell Culture, Cell and Tissue Processing, Antibodies, and Immunofluorescence Analysis. Epicardial cell culture conditions and processing were standard,[10] as was slide preparation.[47] Commercially available antibodies and their working dilutions are given in Supporting Information.

SMA Expression and Wound Healing Assays. Initially, 10^5 epicardial cells were seeded to 4-well glass chamber slides (NUNC Lab-Tek 154526) in standard epicardial cell medium.[10] After the 18 h of incubation, the medium was changed to 2% FBS; 2% FBS/DMEM, supplemented with $TGF\beta_1$, PDGF, and EGF (Millipore GF111 and GF149, BD Biosciences 354001, respectively) at 10 ng mL^{-1} each, to induce epicardial cell differentiation with simultaneous addition of SOMs at the concentrations given in Figure 1, panel j. For induction by $TGF\beta_1$ alone, medium was supplemented with 20 ng mL^{-1} $TGF\beta_1$ in 2 μM DMSO. The negative control consisted of medium with DMSO. For induction by BMP2, 4, and 6 (R&D Systems 355-BM, 314-BM-010, 6325-BM, respectively) growth factor was added to SFM. Noggin (R&D Systems 3344-NG) was added at 200 ng mL^{-1} to the $TGF\beta_1$-supplemented medium. In all situations, cells were incubated for 48 h, until being processed.

To assay early differentiation and sheet migration rate, 2.5×10^5 cells were seeded to glass 4-well chamber slides and incubated overnight in standard epicardial cell media. After 18 h of incubation, cells were equilibrated in mediums. After 6 h of incubation, confluent cell sheets were scratched with a 200 μL pipet tip, incubated for an additional 18 h, and then analyzed for SMA expression as above. Sheet movement was

quantified at 0 and 6 h after wounding. For ALK2/3 overexpression assays, 5 μg of CA-ALK2/3 plasmids were expressed with the Nucleofector II Device (Amaxa Biosystems) using Solution L, program A-020; 2.5×10^5 cells were added to each well of a 4-well slide, and after 48 h incubation sheets were wounded and analyzed. This work was conducted at the Vanderbilt CISR.

Smad Phosphorylation Assays. For analysis by Western blot, cells were grown to confluence, serum starved, and stimulated with TGFβ as above. Lysates were collected, measured for total protein, and immunoblotted as previously reported.[47] AP detection of immunoreactive bands was standard.[47] For analysis by IF, cells were seeded, treated, and injured as above for Western blot analysis. Cells were processed for Phospho-Smad1/5 antibody as per Cell Signaling (9516). Z-series were captured at VUMC CISR, and two 0.6 μm Z-planes were projected with LSM Image Browser.

IC$_{50}$ Determination. Kinase binding and confirmatory *in vitro* kinase assays were performed by KinomeScan at www.kinomescan.com.

RNA Collection, cDNA Synthesis, and Quantitative RT-PCR. Eighteen hours preceding experimental intervention, 2×10^5 cells were seeded in triplicate to a 6-well plastic culture dish (Falcon 353502). Next, cells were stimulated with the TGFβ_1-induction conditions described above. After 24 h of incubation, cells were lysed (Qiashredder, Qiagen 79654), and RNA was purified as described.[48] Specific primers with a melting temperature of 59 °C were designed from the *Rattus norvegicus* cDNA database using PrimerBlast (NCBI) and are listed in Supplementary Table S4. QRT-PCR was performed on the 7900HT Fast Real-Time PCR System (ABI) (using the VUMC DNA resources core). Gene expression was compared using TaqMan Gene Expression assays: BMP2-Rn01484736_M1, BMP4-Rn00432087_m1, BMP6-Rn00432095_m1, GAPDH-Rn99999916_s1, 18s-HS99999901_s1. Data was managed in RQ Manager 1.2 software. All statistics were performed on the ΔCT values.

Modified Boyden Chamber Migration Assay. Cells (3.0×10^5 per experiment) were pelleted for 10 min at 1500 rpm in a desktop microcentrifuge and resuspended in 400 μL of control and experimental media described above. The cells were seeded to the basket of an 8.0 μM cell culture insert (Millicell P18P01250) and placed in 600 μL of the same media in a 24-well plastic culture dish (Falcon). Cells were incubated for 4 h and processed with standard methods.[48] Fifteen phase images were captured on an Olympus BX60 microscope and Olympus Camera.

Chicken Proepicardium Explant Differentiation and Migration Assay. *Gallus gallus* HH16.5 proepicardia were dissected from embryos and placed on 4-well glass chamber slides coated with 5 μg $(cm^2)^{-1}$ Fibronectin (Sigma F4759). Proepicardia were incubated for 24 h in TGFβ_1-induced culture conditions (above) and processed for immunochemistry with standard methods.[43] Images were captured and processed at VUMC EBC. Proepicardial explant size was ascertained with phase-contrast imaging and by total-protein content measurement (Supplementary Figure S5).

■ ASSOCIATED CONTENT

ⓢ Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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E.E.C., D.M.B., R.T.T., and C.C.H. designed the research; E.E.C., M.M., R.T.T., and C.R.H. performed the research; E.E.C. and D.M.B. analyzed the data and wrote the paper.

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