

# A Human Pluripotent Stem Cell-Based Screen for Smooth Muscle Cell Differentiation and Maturation Identifies Inhibitors of Intimal Hyperplasia

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## SUMMARY

Contractile to synthetic phenotypic switching of smooth muscle cells (SMCs) contributes to stenosis in vascular disease and vascular transplants. To generate more contractile SMCs, we performed a high-throughput differentiation screen using a *MYH11-NLuc-tdTomato* human embryonic stem cell reporter cell line. We identified RepSox as a factor that promotes differentiation of MYH11-positive cells by promoting NOTCH signaling. RepSox induces SMCs to exhibit a more contractile phenotype than SMCs generated using PDGF-BB and TGF- $\beta$ 1, two factors previously used for SMC differentiation but which also cause intimal hyperplasia. In addition, RepSox inhibited intimal hyperplasia caused by contractile to synthetic phenotypic switching of SMCs in a rat balloon injury model. Thus, in addition to providing more contractile SMCs that could prove useful for constructing artificial blood vessels, this study suggests a strategy for identifying drugs for inhibiting intimal hyperplasia that act by driving contractile differentiation rather than inhibiting proliferation non-specifically.

## INTRODUCTION

Balloon angioplasty, stents, and bypass surgery are commonly used for occlusive arterial disease, a leading cause of morbidity and mortality worldwide (de Vries et al., 2016). However, restenosis occurs in a significant number of treated patients because of intimal hyperplasia (Beamish et al., 2010; Dangas and Kuepper, 2002). During the development of intimal hyperplasia, contractile smooth muscle cells (SMCs) decrease contractile protein expression and increase proliferation, migration, and extracellular matrix (ECM) production; a process described as contractile to synthetic phenotypic switching (Beamish et al., 2010; Rensen et al., 2007). Small molecules that promote the differentiation of contractile SMCs at the expense of synthetic SMCs could minimize the development of intimal hyperplasia.

Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) and platelet-derived growth factor BB (PDGF-BB) are widely used for the differentiation of SMCs from human pluripotent stem cells (PSCs) (Bajpai et al., 2012; Cao et al., 2013; Cheung et al., 2012; Dash et al., 2015; Karamariti et al., 2013; Lin et al., 2019; Patsch et al., 2015; Wang et al., 2014; Wanjare et al., 2013; Yang et al., 2016; Zhang et al., 2011). However, upregulation of PDGF and TGF- $\beta$  signaling has been shown to promote the switching of SMC phenotypes from contractile to synthetic, contrib-

uting to intimal hyperplasia (Muto et al., 2007; Nabel et al., 1993; Newby and Zaltsman, 2000; Raines, 2004; Swanabol et al., 2011; Wolf et al., 1994). As a result, SMCs generated from PSCs using PDGF-BB and TGF- $\beta$ 1 carry a risk of intimal hyperplasia if used in tissue-engineered vascular constructs. One strategy to overcome this problem is to use high-throughput screening to identify small molecules that can promote contractile SMC differentiation. Since normal vascular differentiation and the dedifferentiation observed in vascular disease share common pathways (Beamish et al., 2010; Mack, 2011), this screening strategy using human PSC-based SMC differentiation might also identify drugs for preventing restenosis caused by intimal hyperplasia. Compared with primary cell lines used in previous screening (Goel et al., 2014), human PSCs provide an unlimited and more consistent cell source for screening. In addition, current Food and Drug Administration-approved anti-restenosis drugs (rapamycin and paclitaxel), as well as previous screening, focused on proliferation antagonists (Goel et al., 2014). However, restoring the contractile phenotype of SMCs in intimal hyperplasia not only inhibits cell proliferation but also improves contractile protein expression and the suppression of cell migration and ECM production (Beamish et al., 2010; Rensen et al., 2007). Thus, using the *MYH11-NLuc-tdTomato* reporter cell line may identify drugs other than proliferation antagonists.





MYH11 is a specific protein expressed by SMCs and is a marker for the mature contractile phenotype. Mutation or reduced expression of MYH11 is associated with vascular disease (Owens et al., 2004; Pannu et al., 2007). Using CRISPR/Cas9 technology (Cong et al., 2013; Hou et al., 2013; Mali et al., 2013), we generated a *MYH11-NLuc-tdTomato* human embryonic stem cell (ESC) reporter cell line and used it in a high-throughput screen of 4,804 small molecules. In this screen, RepSox was identified as a potent small molecule that promoted NOTCH signaling and improved contractile SMC differentiation from human PSCs. SMCs generated by RepSox (RepSox-SMCs) demonstrated a more contractile phenotype compared with SMCs induced by PDGF-BB (P-SMCs), SMCs induced by TGF- $\beta$ 1 (T-SMCs), and SMCs induced by both TGF- $\beta$ 1 and PDGF-BB (PT-SMCs). RepSox also promoted synthetic to contractile phenotypic switching of primary human aortic smooth muscle cells (AoSMCs) *in vitro* and inhibited intimal hyperplasia *in vivo*.

## RESULTS

### High-Throughput Screening for Contractile SMC Differentiation

The *MYH11-NLuc-tdTomato* human ESC reporter line was generated by CRISPR/Cas9 technology (Figure S1). The reporter cell line was differentiated into mesoderm by E8BAC medium for 2 days (Zhang et al., 2017) and then treated with fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 4 (BMP4) to further mature mesoderm for another 2 days. The cells were then passaged into 96-well plates and exposed to small molecules for 10 days using a customized robotic workstation (Figure 1A). The media were changed every other day and small molecules were added during each feeding. Among the 4,804 small molecules tested, 42 increased contractile SMC differentiation, as evidenced by the increased MYH11 promoter-driven luciferase activity (Figures 1B and 1C; Table S1). We then validated these hits and optimized their concentration. Among them, RepSox was the most effective at promoting MYH11 expression (Figure 1C) and was used for further optimizing contractile SMC differentiation.

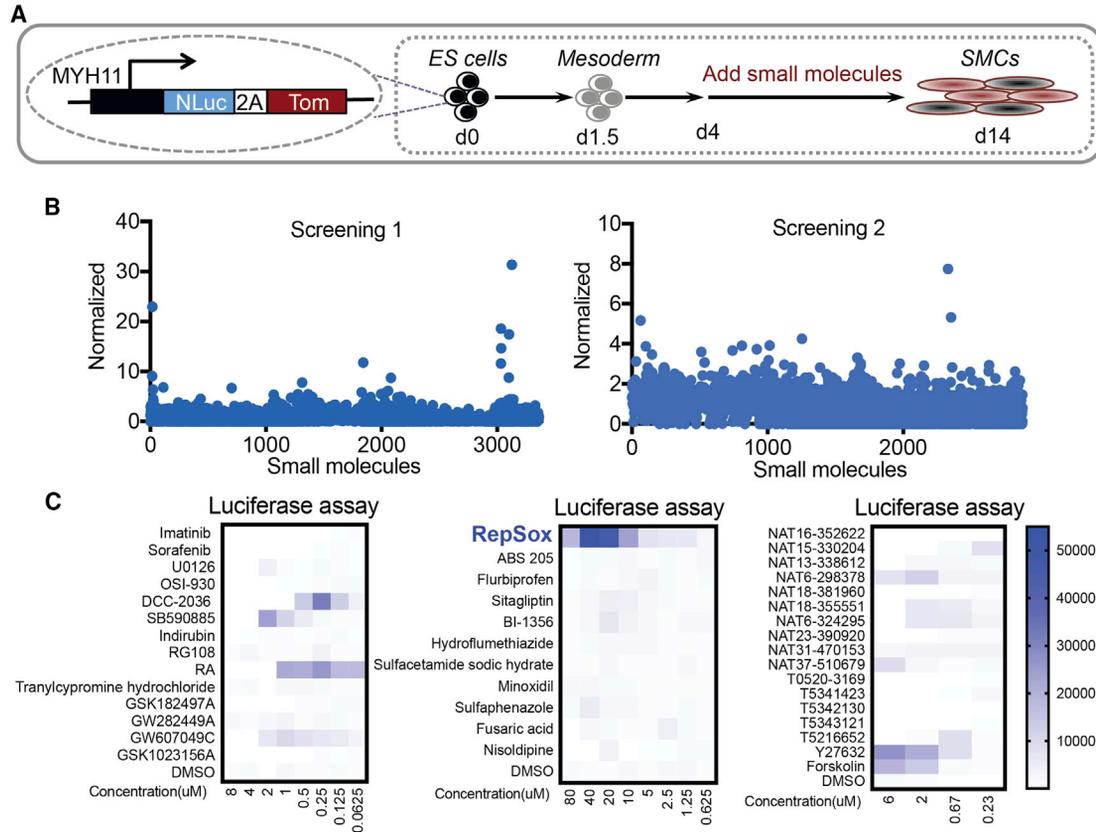
### RepSox Modulates NOTCH Signaling to Promote MYH11<sup>+</sup> SMC Differentiation

Previously, RepSox was shown to be a TGF- $\beta$  signaling inhibitor (Ichida et al., 2009). However, inhibition or knockdown of TGF- $\beta$  receptors failed to increase MYH11 expression (Figures 2A–2C), even though RepSox inhibited pSMAD2 during SMC differentiation (Figure 2D). The re-

sults suggested that RepSox was acting through another pathway to regulate SMC differentiation. Further investigation revealed that RepSox increased full-length NOTCH1 and its intracellular domain (NICD, activated form) expression (Figure 2D) as early as 1 h after RepSox treatment (Figure 2E). The NOTCH inhibitors DAPT, DBZ, and RO4929097 all eliminated the effect of RepSox on the increase in MYH11-Tom<sup>+</sup> SMC differentiation (Figure 2F). Similarly, knockdown of NOTCH1 mRNA suppressed the increase in MYH11 expression (Figure 2G). In a gain-of-function experiment, the doxycycline-induced overexpression of NICD1 increased MYH11-Tom<sup>+</sup> differentiation to levels similar to those obtained by RepSox (Figures 2H and 2I). Inhibition of TGF- $\beta$  did not further enhance MYH11-Tom<sup>+</sup> SMC differentiation when combined with overexpression of NOTCH signaling (Figure S2). Taken together, these data demonstrate RepSox acts through the NOTCH signaling pathway in promoting MYH11-positive SMC differentiation.

### Optimization of RepSox-Induced SMC Differentiation in Fully Defined, Xeno-Free Medium

Previous SMC differentiation protocols used serum or serum replacement products that contain animal components (Bajpai et al., 2012; Cao et al., 2013; Cheung et al., 2012; Dash et al., 2015; Karamariti et al., 2013; Lin et al., 2019; Patsch et al., 2015; Wang et al., 2014; Wanjare et al., 2013; Yang et al., 2016; Zhang et al., 2011). Here, we performed a stepwise optimization to further improve SMC differentiation efficiency in completely defined, xeno-free medium (Figures S3 and 3A). After optimization (Figure 3A), human PSCs were differentiated into mesoderm in E8BAC medium (Zhang et al., 2017) for 36 h and then treated with TGF- $\beta$ 1 for another 18 h. FGF2 was used for another 5 days. Next, the cells were treated with FGF2, VEGFA, and RESV (a NOTCH agonist) to induce SMC progenitors from day 8 to day 12. RESV and RepSox were then used to further mature SMCs from day 12 to day 24. The optimized RepSox protocol generated >95% MYH11<sup>+</sup> SMCs, while PDGF-BB and TGF- $\beta$ 1 treatment generated 30%–60% of MYH11<sup>+</sup> SMCs (Figures 3B–3D). Compared with pericytes, these RepSox-SMCs expressed lower levels of mesenchymal markers, PDGF-RB and NG2, further confirming the SMC fate (Figures S4A and S4B). We then performed a comparison with a previous protocol that generated 80% MYH11<sup>+</sup> SMCs (Cheung et al., 2012). Flow-cytometric analysis showed that RepSox-SMCs expressed much higher levels of MYH11 compared with Cheung's LM-SMCs (Figure S4C). qRT-PCR further confirmed that RepSox induced a 23-fold increase of MYH11 expression over that protocol (Figure S4D). qRT-PCR also revealed that RepSox-SMCs expressed higher levels of other structural genes, including *CNN1*, *MYH11*,



### Figure 1. High-Throughput Screening

(A) Schematic of high-throughput screening for generating contractile smooth muscle cells and restenosis drug discovery. The *MYH11-NLuc-Tom* reporter cell line was differentiated into mesoderm by E8BAC medium (E8 medium [Chen et al., 2011] supplemented with 5 ng/mL BMP4, 25 ng/mL activin A, and 1  $\mu$ M CHIR99021) for 2 days and then treated with 50 ng/mL FGF2 and 20 ng/mL BMP4 in E6 medium (E8 medium minus FGF2 and TGF- $\beta$ 1) for another 2 days. The cells were passaged at day 4 and seeded on the 96-well plate for screening ( $2 \times 10^6$  cells/plate). The small molecules were added to the medium from day 4 to day 14. d, day; ES, embryonic stem; SMCs, smooth muscle cells; Tom, tdTomato.

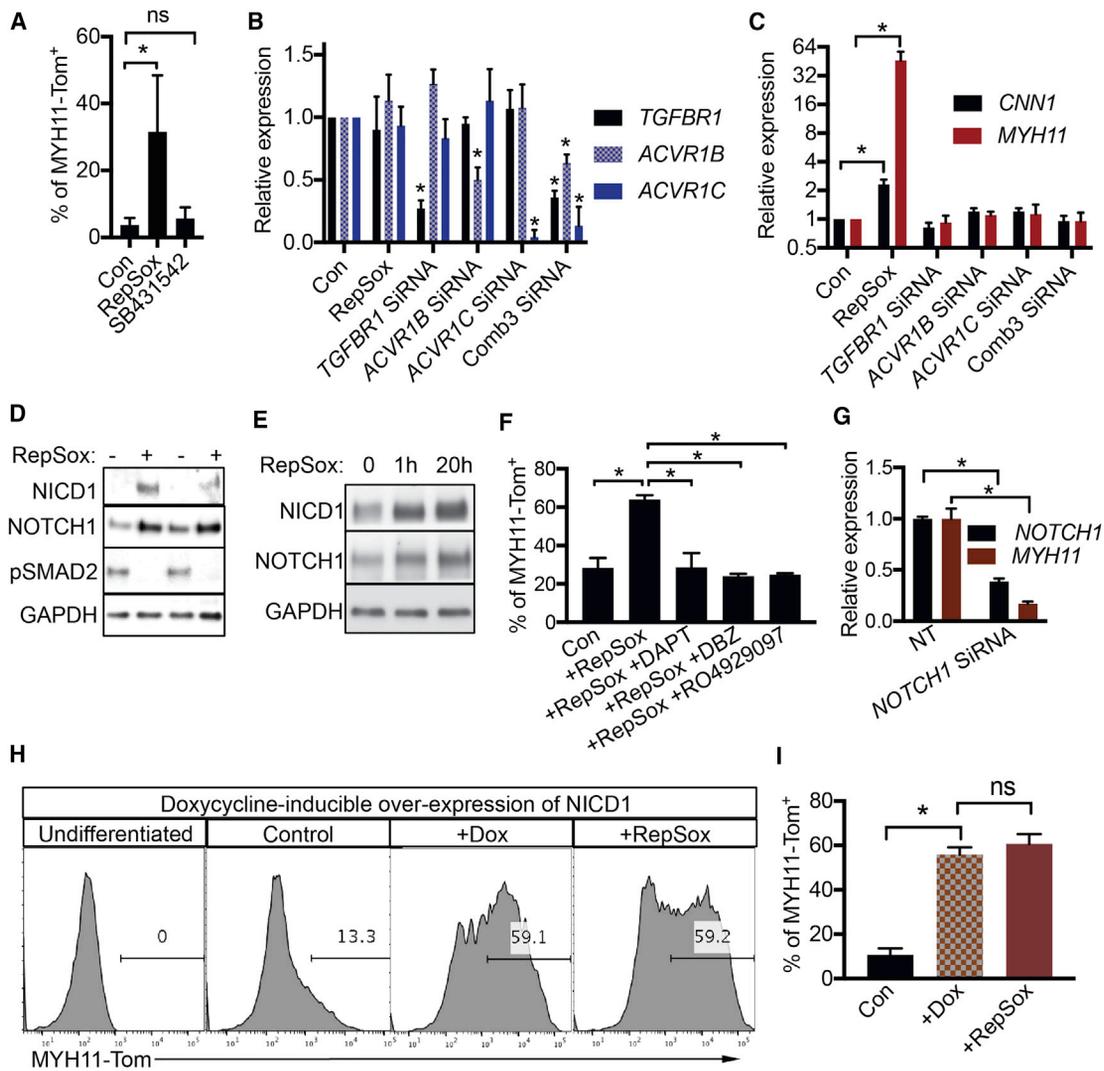
(B) Screening results. Two screens were performed with a total of 4,804 small molecules. The luciferase assay results of individual small molecules were normalized to the average reads of all samples for each batch. The small molecules were selected for further analysis when the normalized reads were greater than "average +  $3 \times$  SD."

(C) Optimizing the concentration of the selected small molecules.

*SMTN*, and *ELN*, compared with PDGF-BB- and/or TGF- $\beta$ 1-treated cells (Figure 3E). In contrast, the expression of collagen (*COL1A1*), a major ECM gene that is increased in the synthetic state (Wanjare et al., 2013; Yang et al., 2016), was lower in RepSox-SMCs compared with P-SMCs, T-SMCs, and PT-SMCs (Figure 3E). Immunostaining showed that RepSox-SMCs strongly expressed structural proteins SMA and SM22 $\alpha$  (Figure 3F). We further measured elastin protein expression. Fastin Elastin Assay revealed that RepSox-SMCs produced higher elastin levels than PT-SMCs (Figure S4E). Finally, we tested whether these cells maintained SMC phenotype over long-term culture. We found that the expression of MYH11 could be maintained in RepSox-SMCs for at least 8 weeks after differenti-

ation, while its expression was almost undetectable in PT-SMCs (Figures S4F and S4G). These RepSox-SMCs were less proliferative compared with PT-SMCs (Figure S4H), indicating a contractile phenotype.

The RepSox protocol also worked robustly for human induced PSC (iPSC) lines 005B23.1 and PBMC-3-1, which are derived from skin punch fibroblasts and peripheral blood mononuclear cells, respectively. Eighty-seven percent to 98% of MYH11<sup>+</sup>, SMA<sup>+</sup>, or SM22 $\alpha$ <sup>+</sup> SMCs were generated from these two iPSC lines and non-targeted H1 human ESCs (Figures S5A and S5B). In addition, qRT-PCR revealed that the expression of the contractile genes and ECM genes were similar among iPSC- and ESC-derived SMCs (Figure S5C).



**Figure 2. RepSox Promotes NOTCH Signaling**

(A) Flow-cytometric analysis of MYH11-Tom<sup>+</sup> cells after treatment with RepSox (25  $\mu$ M) or SB431542 (10  $\mu$ M) from day 10 to day 14. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. ns, not significant; \*p < 0.05, Student's t test.

(B) qPCR analysis of gene expression. Cells were treated with RepSox (25  $\mu$ M) or small interfering RNA (siRNA). Comb3: Knockdown of *TGFBR1*, *ACVR1B*, and *ACVR1C* at the same time. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test.

(C) qPCR analysis of *CNN1* and *MYH11* expression. Cells were treated with RepSox (25  $\mu$ M) or siRNA. Comb3: Knockdown of *TGFBR1*, *ACVR1B*, and *ACVR1C* at the same time. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test.

(D) Western blot. During smooth muscle cell differentiation, cells were treated with or without RepSox from day 10 to day 11.

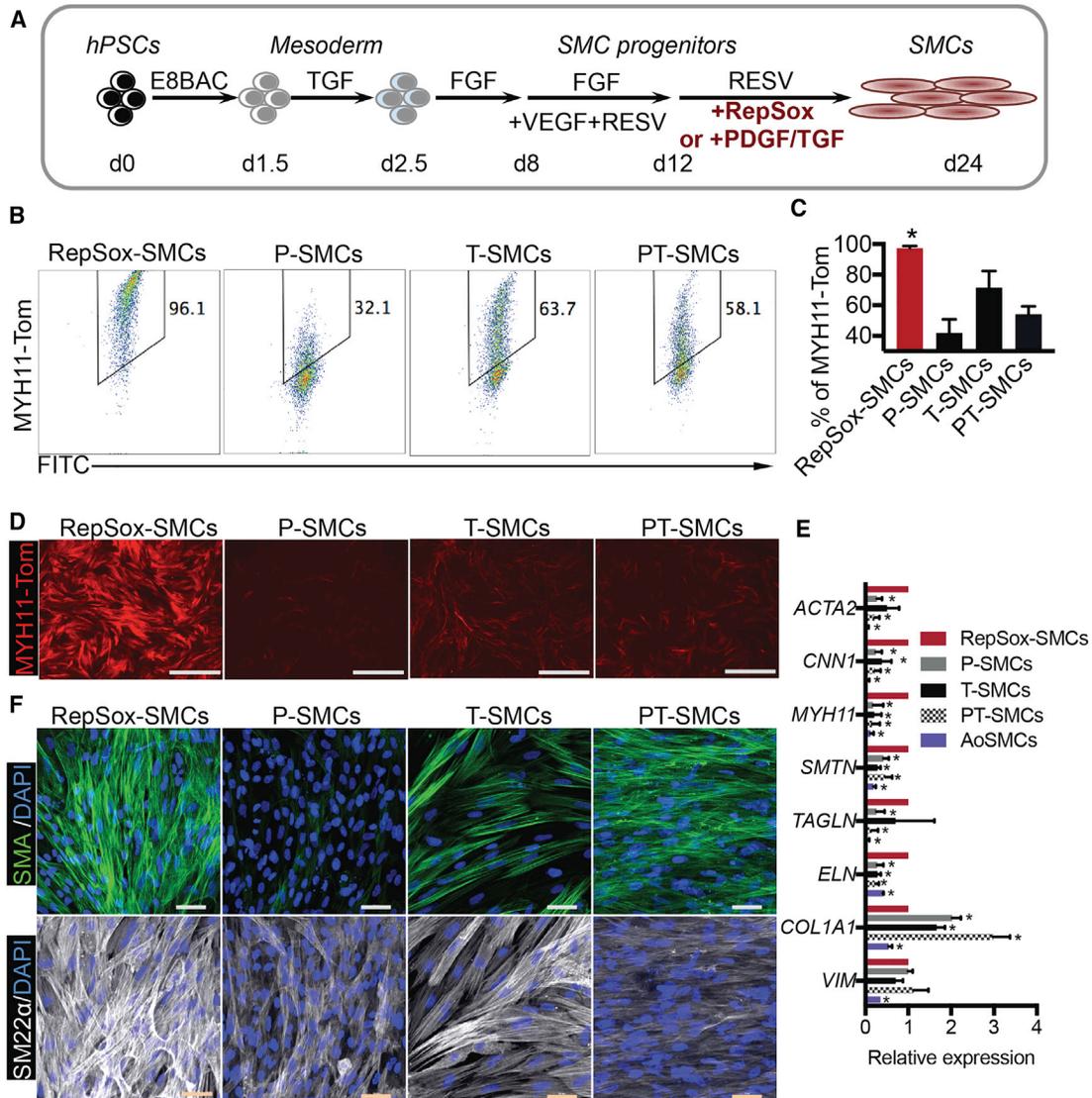
(E) Western blot. During smooth muscle cell differentiation, cells were treated with RepSox for 1 or 20 h at days 10–11.

(F) Flow-cytometric analysis of MYH11-Tom<sup>+</sup> cells after treatment with DMSO, RepSox (25  $\mu$ M), DAPT (20  $\mu$ M), DBZ (10  $\mu$ M), or R04929097 (10  $\mu$ M) from day 10 to day 16. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test.

(G) qPCR analysis of *NOTCH1* and *MYH11* expression. Cells were treated with RepSox and non-targeting control (NT)/siRNA at day 10. The RNA was isolated at day 14. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test.

(H) Flow-cytometric analysis of MYH11-Tom<sup>+</sup> cells. The cells were treated with doxycycline (1  $\mu$ g/mL) to induce the expression of NICD1, or RepSox (25  $\mu$ M) from days 10–16 or days 12–16.

(I) Statistical data for NICD1-induced MYH11-Tom<sup>+</sup> cells. Data are presented as mean  $\pm$  SD, n = 6 independent experiments. ns, not significant; \*p < 0.05, Student's t test.



### Figure 3. Molecular Characterization of SMCs

(A) Schematic of the optimized SMC differentiation protocol. Matrigel coating was used for all the following experiments. The *MYH11-NLuc-tdTomato* reporter human ESC line was differentiated into mesoderm by using E8BAC medium (E8 medium supplemented with 5 ng/mL BMP4, 25 ng/mL activin A, and 1  $\mu$ M CHIR99021) for 36 h and then treated with 1.7 ng/mL TGF- $\beta$ 1 in E6 medium (E6 is defined as E8 medium minus FGF2 and TGF- $\beta$ 1) for 18 h. FGF2 (100 ng/mL) in E5 medium (E5 is defined as E6 minus insulin) was used for another 5 days. Next, the cells were treated with FGF2 (100 ng/mL), VEGFA (50 ng/mL), and RESV (5  $\mu$ M, a NOTCH agonist) in E6 medium to induce SMC progenitors from day 8 to day 12. RESV in E6 medium supplemented with RepSox (25  $\mu$ M), PDGF-BB (10 ng/mL), or TGF- $\beta$ 1 (1.7 ng/mL) was then used to further mature SMCs.

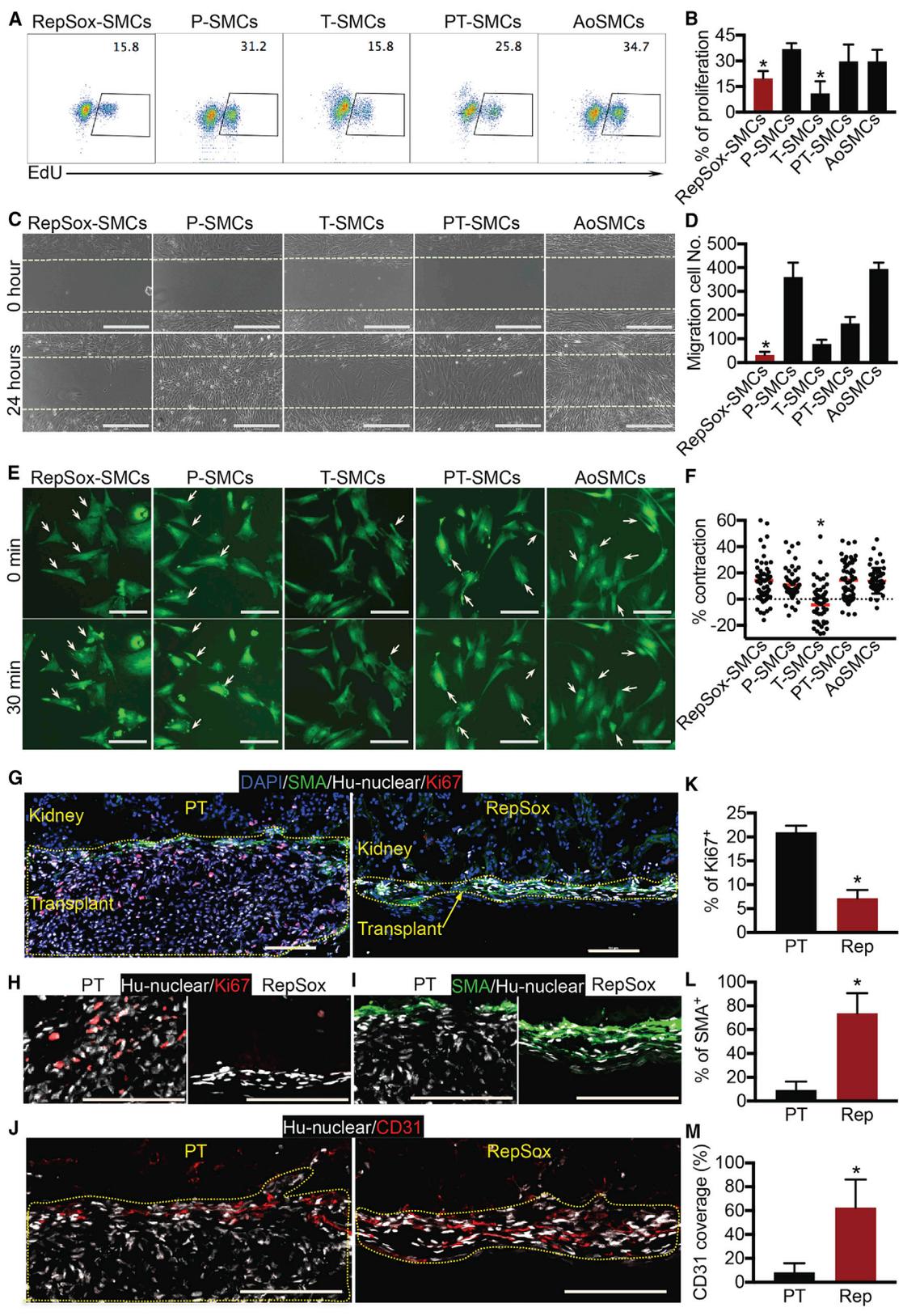
(B) Flow-cytometric analysis of MYH11-Tom<sup>+</sup> cells. RepSox-SMCs, P-SMCs, T-SMCs, and PT-SMCs induced from day 12 to day 24.

(C) Statistical data for MYH11-Tom<sup>+</sup> cells in each treatment group from (B). Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test.

(D) Live imaging of MYH11-Tom expression. Scale bars, 1 mm.

(E) qRT-PCR of SMCs. Data are presented as mean  $\pm$  SD, n = 3 independent experiments. \*p < 0.05, Student's t test. hPSCs, human pluripotent stem cells; Tom, tdTomato.

(F) Immunostaining of SMA and SM22 $\alpha$ . Scale bars, 50  $\mu$ m.



(legend on next page)



### RepSox-SMCs Have a Contractile Phenotype

Since low proliferation and migration rates are characteristic of contractile SMCs, we characterized these properties in PSC-derived SMCs and AoSMCs. RepSox-SMCs and T-SMCs had lower proliferation rates than the other SMCs (Figures 4A and 4B). In addition, RepSox-SMCs showed the lowest migration rate compared with all the other SMC groups (Figures 4C and 4D). SMC contraction controls vascular tone and blood pressure (Brozovich et al., 2016). Thus, we measured carbachol-evoked cell contraction. Time-lapse imaging revealed that P-SMCs, PT-SMCs, RepSox-SMCs, and AoSMCs exhibited a 10%–20% change of cell-surface area after carbachol treatment (Figures 4E and 4F). However, the average T-SMC surface area actually expanded during treatment (Figures 4E and 4F), suggesting that T-SMCs do not have physiologically normal contractile properties. In summary, RepSox treatment produced a more contractile SMC phenotype including lower proliferation and migration rates.

To test whether RepSox-SMCs can maintain a contractile phenotype *in vivo*, we transplanted RepSox-SMCs and PT-SMCs under the kidney capsules of immune-deficient mice. Immunostaining of transplanted grafts showed that RepSox-SMCs had a lower proliferative rate compared with PT-SMCs (Figures 4G, 4H, and 4K). In addition, most PT-SMCs lost smooth muscle actin expression (Figures 4G, 4I, and 4L) and recruited few endothelial cells (Figures 4J and 4M; highlighted by yellow dashed line). In contrast, most RepSox-SMCs (75%) maintained smooth muscle  $\alpha$ -actin expression (Figures 4G, 4I, and 4L) and were able to recruit more endothelial cells (Figures 4J and 4M). Thus, RepSox-SMCs maintained a more contractile phenotype than PT-SMCs after transplantation.

### RepSox Inhibits Intimal Hyperplasia

Multiple pathways are shared between vascular SMC differentiation and the dedifferentiation observed in vascular disease (Beamish et al., 2010; Mack, 2011). Therefore, we hypothesized that screening for molecules that drive ESC differentiation to a mature, contractile SMC phenotype might also block the dedifferentiation and restore the contractile phenotype of primary SMCs, and that such molecules might prove useful for inhibiting intimal hyperplasia *in vivo*. Indeed, among the molecules identified in our screen (Figure 1C) UO126, Y27632, and retinoic acid have all been shown to inhibit neointimal hyperplasia previously (DeRose et al., 1999; Gulkarov et al., 2009; Sawada et al., 2000).

Cultured primary AoSMCs undergo a contractile to synthetic switch upon culture (Beamish et al., 2010; Owens et al., 2004), so we first tested whether RepSox would reverse this switch. RepSox treatment reduced cell migration and proliferation rates of AoSMCs (Figures 5A–5D) without affecting contraction ability (Figures 5E and 5F). In addition, RepSox treatment increased contractile gene expression (Figure 5G). Thus, RepSox improved the contractile phenotype of primary AoSMCs *in vitro*.

The clinically used anti-restenotic drugs rapamycin (or analogs) and paclitaxel also suppress endothelial proliferation and repair (Prasad et al., 2005; Zheng et al., 2014). To examine potential side effects of RepSox on endothelial cells *in vitro*, we performed a proliferation assay. The result demonstrated RepSox had significantly less effect on arterial endothelial cell proliferation compared with rapamycin (Figures S6A and S6B). In addition, RepSox did not increase apoptosis (Figures S6C and S6D) and did not compromise nitric oxide production (Figure S6E), an arterial specific function (Zhang et al., 2017).

#### Figure 4. Functional Characterization of SMCs (H1 Cell-Derived)

(A) Click-iT 5-ethynyl-2'-deoxyuridine (EdU) analysis of cell proliferation.

(B) Statistical data of EdU-incorporated cells are presented as mean  $\pm$  SD,  $n = 4$  independent experiments. \* $p < 0.05$  compared with P-SMCs and AoSMCs, Student's  $t$  test.

(C) Cell migration. Cells were imaged at 0 and 24 h. Scale bars, 400  $\mu\text{m}$ .

(D) Statistical data of migration cells are presented as mean  $\pm$  SD,  $n = 4$  independent experiments. \* $p < 0.05$  versus all the other SMCs, Student's  $t$  test.

(E) Cell-contraction assay. Cells were imaged before and after treatment with 100  $\mu\text{M}$  carbachol. Arrows indicate contracting cells. Scale bars, 100  $\mu\text{m}$ .

(F) Statistical data of cell-surface changes are presented as mean  $\pm$  SD,  $n = 65, 61, 56, 59,$  and 53 cells (from 3 independent experiments) for RepSox-SMCs, P-SMCs, T-SMCs, PT-SMCs, and AoSMCs, respectively. \* $p < 0.05$  versus all the other SMCs, Student's  $t$  test.

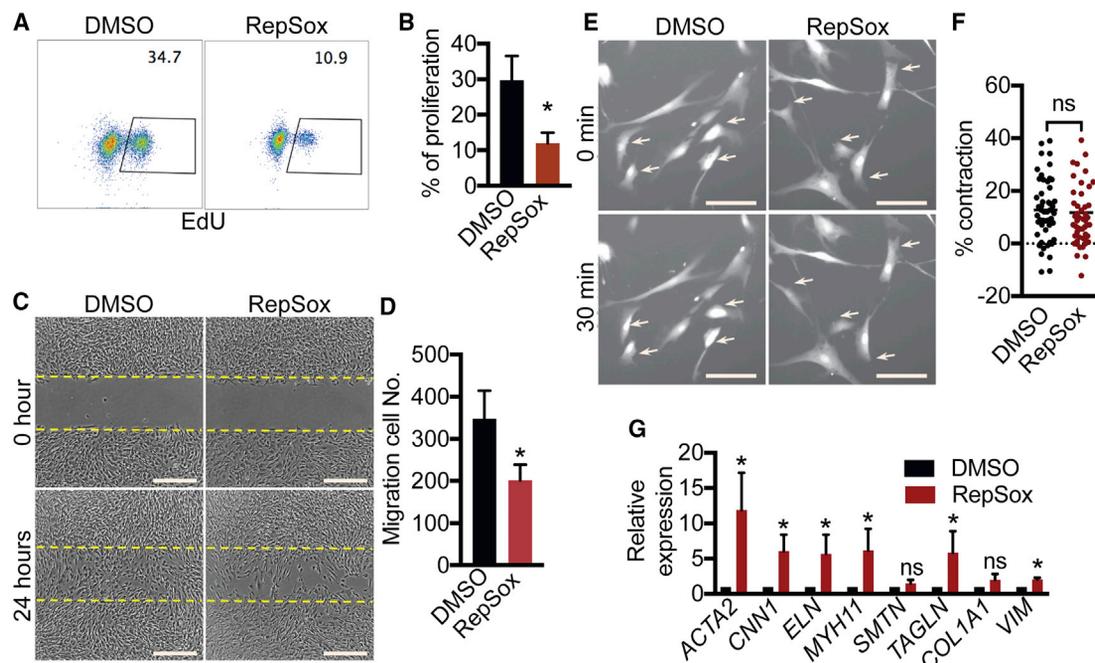
(G) Kidney capsule experiment. PT-SMCs and RepSox-SMCs were transplanted under the kidney capsule of NBSGW mice. Triple immunostaining of anti-smooth muscle actin (SMA; labeling smooth muscle cells), anti-hu-nuclear (labeling human cells), and anti-Ki67 (labeling proliferating cells). Scale bars, 100  $\mu\text{m}$ .

(H) Higher magnification of anti-hu-nuclear and anti-Ki67 staining. Scale bars, 100  $\mu\text{m}$ .

(I) Higher magnification of anti-SMA and anti-hu-nuclear staining. Scale bars, 100  $\mu\text{m}$ .

(J) Immunostaining of anti-hu-nuclear and anti-CD31 (labeling endothelial cells). Scale bars, 100  $\mu\text{m}$ .

(K–M) Statistical data for percentage of Ki67<sup>+</sup> cells (K), percentage of SMA<sup>+</sup> cells (L), and CD31 coverage (M) of human cells under kidney capsule. Data are presented as mean  $\pm$  SD,  $n = 3$  mice for PT-SMCs and  $n = 5$  mice for RepSox-SMCs. \* $p < 0.05$ , Student's  $t$  test.



**Figure 5. RepSox Promotes a Contractile Phenotype of Cultured AoSMCs**

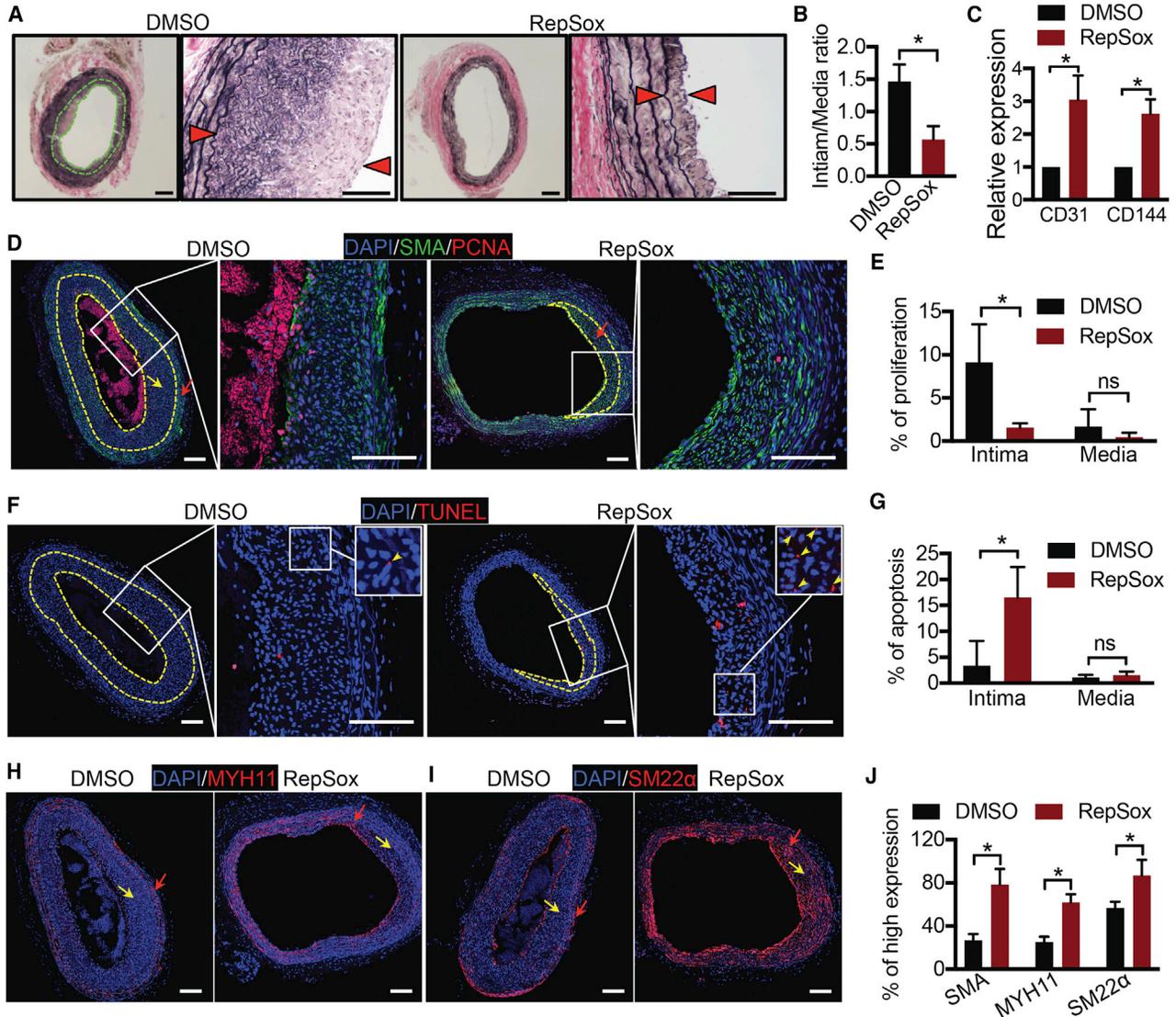
(A) Click-iT EdU analysis of cell proliferation.  
 (B) Statistical data of EdU-incorporated cells are presented as mean  $\pm$  SD,  $n = 4$  independent experiments. \* $p < 0.05$ , Student's  $t$  test. AoSMCs (primary human aortic smooth muscle cells) were treated with DMSO or 100  $\mu$ M RepSox for 2 days.  
 (C) Cell migration. AoSMCs were treated with DMSO or 100  $\mu$ M RepSox for 2 days before the migration assay and one more day during the assay. Cells were imaged at 0 and 24 h during migration assay. Scale bars, 500  $\mu$ m.  
 (D) Statistical data of cell migration are presented as mean  $\pm$  SD,  $n = 6$  samples from 3 independent experiments. \* $p < 0.05$ , Student's  $t$  test.  
 (E) Cell-contraction assay. AoSMCs were treated with DMSO or 100  $\mu$ M RepSox for 2 days before adding carbachol. Cells were imaged before and after the treatment with 100  $\mu$ M carbachol. Scale bars, 100  $\mu$ m.  
 (F) Statistical data of cell-surface changes are presented as mean  $\pm$  SD,  $n = 50$  cells/group. ns, not significant by Student's  $t$  test. Arrows indicate contracting cells.  
 (G) qRT-PCR of SMCs. AoSMCs were treated with DMSO or 100  $\mu$ M RepSox for 3 days. Data are presented as mean  $\pm$  SD,  $n = 3$  independent experiments. ns, not significant; \* $p < 0.05$ , Student's  $t$  test.

To test RepSox *in vivo*, we used a rat intimal hyperplasia model. RepSox or DMSO control was dissolved in a slow-released hydrogel and then applied to the outside of the injured artery segment immediately after balloon injury of the rat carotid artery (Chen et al., 2017). Carotid arteries were collected and sectioned 14 days after surgery. H&E staining revealed pronounced intimal hyperplasia in the control group (DMSO), which was reduced from 1.5 to 0.5 through treatment with RepSox (intima/media ratio, Figures 6A and 6B). To examine potential negative effects of RepSox on endothelial cells, we performed qPCR analysis for endothelial markers on treated arteries. RepSox increased endothelial cell marker expression compared with DMSO control-treated arteries (Figure 6C), suggesting that RepSox may not inhibit endothelium repair. To further understand how RepSox reduced intimal hyperplasia, we

performed immunostaining. RepSox decreased proliferation and increased apoptosis in the intima but not in the media (Figures 6D–6G) and also increased the expression of contractile proteins SMA, MYH11, and SM22 $\alpha$  (Figures 6D and 6H–6J). Thus, RepSox inhibited intimal hyperplasia *in vivo*.

## DISCUSSION

Vascular stent placement can abrade the endothelial cell layer and cause intimal hyperplasia and restenosis. In an attempt to reduce restenosis, drug-eluting stents have been developed to target either inflammation or cell proliferation (Pendyala et al., 2008). However, proliferation inhibitors currently used in stents, such as rapamycin and



### Figure 6. RepSox Inhibits Intimal Hyperplasia in Rat Balloon Injury Model

(A) Rat balloon injury experiment. H&E staining is shown. Arrowheads indicate the boundaries of the intima. Scale bars, 200  $\mu$ m.

(B) Statistical data of intima/media ratio are presented as mean  $\pm$  SD, n = 5 mice from 2 independent experiments. \*p < 0.05, Student's t test.

(C) qRT-PCR of endothelial cell marker expression. Data are presented as mean  $\pm$  SD, n = 3 mice. \*p < 0.05, Student's t test.

(D) Double immunostaining of anti-SMA and anti-PCNA (labeling endothelial cells). Neointima is the area between the yellow dashed lines. Red arrows indicate the region with high SMA expression. Yellow arrows indicate the region with low SMA expression. Scale bars, 100  $\mu$ m.

(E) Statistical data of proliferation are presented as mean  $\pm$  SD, n = 3 mice. ns, not significant; \*p < 0.05, Student's t test.

(F) TUNEL assay (labeling apoptotic cells). Neointima is the area between the yellow dashed lines. Scale bars, 100  $\mu$ m.

(G) Statistical data of apoptosis are presented as mean  $\pm$  SD, n = 3 mice. ns, not significant; \*p < 0.05, Student's t test.

(H) Immunostaining of MYH11. Red arrows indicate the region with high MYH11 expression. Yellow arrows indicate the region with low MYH11 expression. Scale bars, 100  $\mu$ m.

(I) Immunostaining of SM22 $\alpha$ . Red arrows indicate the region with high SM22 $\alpha$  expression. Yellow arrows indicate the region with low SM22 $\alpha$  expression. Scale bars, 100  $\mu$ m.

(J) Statistical data of SMA, MYH11, or SM22 $\alpha$  expression. The "% of high expression" denotes the ratio (area of high expression)/(total area of intima and media). These are presented as mean  $\pm$  SD, n = 3 mice. \*p < 0.05, Student's t test.



paclitaxel, are not cell-type specific and thus may inhibit endothelial repair (Prasad et al., 2005; Zheng et al., 2014). In an attempt to identify more cell-type-specific agents with fewer negative endothelial effects, we screened for drugs that reduce intimal hyperplasia by forcing differentiation of proliferative synthetic SMCs to a more mature, less proliferative contractile state. Interestingly, three compounds identified in our screen, UO126, Y27632, and retinoic acid, have all been shown to inhibit intimal hyperplasia in previous studies (DeRose et al., 1999; Gulkarov et al., 2009; Sawada et al., 2000); validating this as a suitable method for identifying inhibitors of intimal hyperplasia. Importantly, RepSox was identified in our assay as the most potent inducer of the contractile state, having not been described previously as such. In an *in vivo* artery balloon injury model, RepSox reduced neointimal formation, increased intimal apoptosis, and increased contractile protein expression in the neointima that did form (Figure 6). These results indicate potential as a very promising screening strategy for identifying additional candidate anti-restenotic drugs in larger libraries.

RepSox was reported previously to be a TGF- $\beta$  signaling inhibitor and was used to replace Sox2 in reprogramming (Ichida et al., 2009), but our results suggest that RepSox regulated SMC differentiation via a TGF- $\beta$  signaling-independent pathway. Our data show that RepSox promoted NOTCH signaling and inhibited TGF- $\beta$ -SMAD2 signaling (Figure 2B). However, inhibition of TGF- $\beta$  signaling alone failed to promote SMC differentiation to a contractile phenotype *in vitro* (Figures 2A–2C), nor did inhibition of TGF- $\beta$  further enhance the contractile phenotype when combined with NOTCH signaling (Figure S2). Instead, at least in these *in vitro* conditions, NOTCH signaling alone was sufficient to promote the contractile state. However, the function of NOTCH signaling in intimal hyperplasia *in vivo* is controversial (Fouillade et al., 2012). TGF- $\beta$  inhibition has been shown to inhibit intimal hyperplasia (Suwanabol et al., 2011), promote endothelial cell expansion (James et al., 2010), and promote arterial endothelial cell differentiation (Zhang et al., 2017); thus, our experiments cannot rule out that RepSox may be acting through distinct pathways in the more complex cellular environment *in vivo*. Finally, it is interesting to note that RepSox induced apoptosis in intima but not media layers (Figures 6F and 6G). NOTCH induces cell-cycle arrest followed by apoptosis (Chadwick et al., 2008). Given that RepSox reduced proliferation in intima (Figures 6D and 6E), it is possible that the apoptotic intima was caused by the cell-cycle arrest resulting from RepSox-activated NOTCH signaling.

The RepSox protocol described here produced SMCs in completely defined medium that were more mature than those reported previously. In a side-by-side comparison,

both flow-cytometric analysis and qRT-PCR results revealed that RepSox-SMCs were expressed on levels an order of magnitude higher than MYH11 (a marker for the mature contractile phenotype) compared with a previous protocol that generated 80% MYH11<sup>+</sup> SMCs (Cheung et al., 2012) (Figures S4C and S4D). In addition, previous protocols have used TGF- $\beta$ 1 and PDGF-BB, which have been reported to cause intimal hyperplasia *in vivo* (Muto et al., 2007; Nabel et al., 1993; Newby and Zaltsman, 2000; Raines, 2004; Suwanabol et al., 2011; Wolf et al., 1994). In contrast, RepSox promoted maturation *in vitro* and reduced intimal hyperplasia *in vivo*. Thus, RepSox-SMCs could reduce the risk of intimal hyperplasia if used in tissue-engineered vascular constructs.

Serum has been widely used in previous smooth muscle differentiation studies (Bajpai et al., 2012; Karamariti et al., 2013; Wang et al., 2014; Wanjare et al., 2013). However, serum suppresses the contractile phenotype of SMCs (Wanjare et al., 2013), suggesting that signals from serum may have compromised SMC maturation. Two potential signals could arise from PDGF-BB and insulin, both of which can be found in serum (Czarkowska-Paczek et al., 2006; Osei et al., 1993). PDGF-BB has been shown to promote the synthetic phenotype of SMCs as well as intimal hyperplasia (Muto et al., 2007; Newby and Zaltsman, 2000; Raines, 2004; Wanjare et al., 2013). Also, our data show that adding insulin from day 3 to day 8 reduced SMC differentiation (Figure S3F). In addition to being present in serum, insulin is one component of serum replacement used in previous protocols (Cao et al., 2013; Cheung et al., 2012; Patsch et al., 2015; Yang et al., 2016). Our differentiation medium is fully defined, and the differentiation could also be performed on a defined substrate (recombinant vitronectin) (Figure S3I). Thus, a fully defined xeno-free protocol will facilitate the generation of contractile SMCs and investigation of the molecular mechanisms underlying SMC differentiation. Finally, RepSox is a small molecule that is more stable and cheaper than growth factors previously employed; thus, our protocol is more suitable to large-scale applications and could be useful clinically.

Vascular SMCs have multiple origins *in vivo* (Sinha et al., 2014; Wang et al., 2015) including paraxial and lateral plate mesoderm, neural crest, second heart field, epicardium, pleural mesothelium, and nephrogenic stromal cells. In the mouse, a Cre-loxp lineage-tracing experiment demonstrated that AoSMCs are derived from a MEOX1-positive population (Wasteson et al., 2008). Because MEOX1 is a paraxial mesoderm marker, this suggests AoSMCs are derived from paraxial mesoderm. However, whether these MEOX1-derived SMC progenitors express other classical paraxial mesoderm markers *in vivo* is currently unknown, and they may represent a distinct subset of the paraxial



mesoderm. The SMC mesoderm population we produced expressed both paraxial (MEOX1 and MSGN1) and lateral plate (ISL1 and NKX2.5) mesodermal markers (Figure S3E). Thus it is likely that this mesoderm is a mixture of both lateral plate and paraxial mesoderm, although it is also possible that MEOX1 SMC progenitors display a unique molecular signature.

Human PSCs offer a scalable, consistent, genetically defined source of SMCs and arterial endothelial cells (Bajpai et al., 2012; Cao et al., 2013; Cheung et al., 2012; Dash et al., 2016; Karamariti et al., 2013; Patsch et al., 2015; Wang et al., 2014; Wanjare et al., 2013; Yang et al., 2016; Zhang et al., 2011, 2017) with applications in vascular disease modeling, drug discovery, and tissue engineering. Using the most active hit from our screen, a protocol was developed for differentiating contractile SMCs from human PSCs in fully defined, xeno-free medium. The protocol is efficient and works robustly for multiple iPSC and ESC lines. Such SMCs should also be useful in tissue-engineered vascular constructs, as their more mature state should reduce intimal hyperplasia, thereby reducing the likelihood of graft failure.

## EXPERIMENTAL PROCEDURES

### Smooth Muscle Cell Differentiation

Human PSCs (H1) were cultured in E8 medium on a Matrigel-coated plate. On the day of differentiation, ESCs were dissociated by Accutase and plated on a Matrigel-coated plate at 1:4 ratios ( $1 \times 10^5$  cells/cm<sup>2</sup>). The cells were cultured in E8BAC medium for 36 h. At 36 h, the cells were passaged and seeded on a new Matrigel-coated plate ( $1.6 \times 10^4$  cells/cm<sup>2</sup>). The cells were treated with E6T medium for 18 h to induce the transient and medium-level expression of MEOX1. E5F medium was used to suppress MEOX1 expression for another 5 days (days 3–8). Next, the cells were treated with FVR medium to induce SMC progenitors from day 8 to day 12. E6R medium supplemented with RepSox (25  $\mu$ M) was then used to further mature SMCs from day 12 to day 24. Cells were split ( $1 \times 10^5$  cells/cm<sup>2</sup>) at day 16 and further differentiated until day 24. For P-SMCs, T-SMCs, and PT-SMCs, RepSox was replaced by PDGF-BB, TGF- $\beta$ 1, or a combination of PDGF-BB and TGF- $\beta$ 1 from day 12 to day 24.

A more detailed protocol is provided in [Supplemental Information](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.04.013>.

## AUTHOR CONTRIBUTIONS

J.Z. designed and performed the experiments, analyzed the data, and wrote the paper. B.E.M. and M.D.P. performed the high-throughput screening. L.-W.G. and C.K. designed, and B.W. performed, rat balloon injury experiments. M.E.B. and

W.J.B. designed, and M.E.B. and Y.Z. performed, kidney capsule experiments. S.W. provided technical support for cell culture. B.D. provided technical support for mouse experiments. M.F. wrote the algorithms for high-throughput screening. J.A.T. designed the experiments and revised the manuscript.

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