

ORIGINAL RESEARCH ARTICLE

Orphan G Protein–Coupled Receptor GPRC5B Controls Smooth Muscle Contractility and Differentiation by Inhibiting Prostacyclin Receptor Signaling

BACKGROUND: G protein–coupled receptors are important regulators of contractility and differentiation in vascular smooth muscle cells (SMCs), but the specific function of SMC-expressed orphan G protein–coupled receptor class C group 5 member B (GPRC5B) is unclear.

METHODS: We studied the role of GPRC5B in the regulation of contractility and dedifferentiation in human and murine SMCs in vitro and in iSM-*Gprc5b*-KO (tamoxifen-inducible, SMC-specific knockout) mice under conditions of arterial hypertension and atherosclerosis in vivo.

RESULTS: Mesenteric arteries from SMC-specific *Gprc5b*-KOs showed ex vivo significantly enhanced prostacyclin receptor (IP)–dependent relaxation, whereas responses to other relaxant or contractile factors were normal. In vitro, knockdown of GPRC5B in human aortic SMCs resulted in increased IP-dependent cAMP production and consecutive facilitation of SMC relaxation. In line with this facilitation of IP-mediated relaxation, iSM-*Gprc5b*-KO mice were protected from arterial hypertension, and this protective effect was abrogated by IP antagonists. Mechanistically, we show that knockdown of GPRC5B increased the membrane localization of IP both in vitro and in vivo and that GPRC5B, but not other G protein–coupled receptors, physically interacts with IP. Last, we show that enhanced IP signaling in GPRC5B-deficient SMCs not only facilitates relaxation but also prevents dedifferentiation during atherosclerosis development, resulting in reduced plaque load and increased differentiation of SMCs in the fibrous cap.

CONCLUSIONS: Taken together, our data show that GPRC5B regulates vascular SMC tone and differentiation by negatively regulating IP signaling.

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Clinical Perspective

What Is New?

- Orphan G protein–coupled receptor class C group 5 member B regulates membrane availability of the prostacyclin receptor in human and murine vascular smooth muscle cells.
- In smooth muscle–specific *Gprc5b* knockout mice, increased prostacyclin receptor signaling results in facilitation of vascular relaxation and prevention of smooth muscle dedifferentiation.
- These changes result in protection of smooth muscle–specific *Gprc5b*-knockouts from arterial hypertension and atherosclerosis.

What Are the Clinical Implications?

- Inhibition of the interaction between G protein–coupled receptor class C group 5 member B and the prostacyclin receptor might be beneficial in human arterial hypertension and vascular remodeling.

G protein–coupled receptors (GPCRs) are the largest family of transmembrane receptors in eukaryotes; they transduce signals of numerous physicochemical stimuli, including neurotransmitters, hormones, local mediators, metabolic or olfactory cues, and light.¹ The human genome encodes ≈800 GPCRs, the majority of them olfactory receptors.² For most of the ≈360 nonolfactory GPCRs, the mechanism of activation and biological function are well studied, but >100 GPCRs still remain orphan; that is, their endogenous ligand is unknown.^{3,4}

In the vascular system, the contractile tone of vessels is crucially regulated by GPCRs. Vasoconstrictors such as angiotensin II (AngII), endothelin-1, and sphingosine 1-phosphate act through their respective $G_{12/13}$ - and $G_{q/11}$ -coupled GPCRs to facilitate phosphorylation of the regulatory myosin light-chain MLC20, thereby enhancing actomyosin contractility.^{5,6} In contrast, G_s -coupled GPCRs such as the prostacyclin receptor (IP), the β_2 adrenergic receptor (β_2 AR), or the prostaglandin E2 receptors EP2 and EP4 were shown to induce relaxant effects.^{5–7} As mechanisms underlying G_s -dependent relaxation, cAMP-dependent reduction of intracellular calcium levels, hyperpolarization of the membrane potential, and protein kinase A–dependent interference with RhoA-mediated signaling were suggested.^{8,9} In addition to these immediate effects on vascular contractility, GPCRs modulate smooth muscle cell (SMC) differentiation and inflammatory activation. G_i -coupled GPCRs such as the sphingosine 1-phosphate receptor S1P₁ or the apelin receptor promote SMC proliferation and migration through inhibition of cAMP production, which results in enhanced transactivation of growth factor receptors and induction of mitogen-activated protein

kinase signaling. The G_s -coupled receptors IP and EP2, in contrast, mediate antimigratory and antiproliferative effects through enhancement of cAMP signaling.^{10,11} Activation of the $G_{q/11}$ pathway finally promotes proliferation and dedifferentiation, and activation of the $G_{12/13}$ signaling pathway promotes expression of contractile proteins and differentiation.⁹

To investigate local differences in smooth muscle GPCR expression, we recently performed a single-cell GPCR expression analysis in different types of vascular SMCs and found that the repertoire of GPCRs expressed in aorta and resistance arteries differed strongly: SMCs from resistance arteries expressed significantly more GPCRs than aortic SMCs, among them not only various receptors for peptide hormones with known vaso-regulatory function but also mRNAs encoding different orphan GPCRs, for example, *Gpr19*, *Gpr21*, *Mrgprf*, *Gprc5b*, *Gprc5c*, *Gpr124*, *Gpr126*, or *Lphn1*.¹² Some of these receptors, for example, the class C orphan *Gprc5b*, were also enriched in aortal SMCs that showed signs of spontaneous dedifferentiation,¹² which led us to study the role of this orphan GPCR in SMC biology.

GPCR class C group 5 member B (GPCR5B) is a class C orphan receptor with an expression that is induced by retinoic acid.¹³ *Gprc5b*, the murine gene encoding GPCR5B, is strongly expressed in the central nervous system, and global GPCR5B-deficient mice display various neurological phenotypes such as behavioral abnormalities¹⁴ and altered cortical neurogenesis.¹⁵ In addition, reduced obesity and obesity-associated inflammation have been described in *Gprc5b* knockout (KO) mice.¹⁶ On the basis of mainly in vitro studies, it was suggested that GPCR5B negatively regulates insulin secretion¹⁷ and influences inflammatory cytokine production and fibrotic activity in cardiac fibroblasts.¹⁸ The role of GPCR5B in SMC biology is unknown. Using SMC-specific GPCR5B-deficient mice, we found that GPCR5B critically regulates SMC responsiveness to the vasorelaxant factor prostacyclin by regulating the intracellular localization of the IP receptor.

METHODS

The data, analytical methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Detailed methods are provided in the [Data Supplement](#).

Experimental Animals

Gprc5b^{fl/fl} mice were generated from clone EPD0534_1_A10 (EUCOMM) and intercrossed with *Myh11-CreERT2* mice¹⁹ to generate iSM-*Gprc5b*-KO (tamoxifen-inducible, smooth muscle–specific KOs of *Gprc5b*). Mice were maintained on a C57BL/6J background, and genetically matched Cre-negative *Gprc5b*^{fl/fl} mice were used as controls. Mice were housed under a 12-hour light/dark cycle with free access to food and water and under pathogen-free conditions. Animal

experiments were approved by the Institutional Animal Care and Use Committee of the Regierungspräsidium Darmstadt and in accordance with Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Genotyping for *Gprc5b* was done with the primers 5'-gctgaaggtttctcctct-3' and 5'-aagagacaaccaccagacagg-3', resulting in band sizes of 361 for the wild-type allele and 478 bp for the floxed allele. For atherosclerosis experiments, mice were crossed to the *ApoE*-deficient mouse line²⁰ and kept on high-fat diet (21% butter fat, 1.5% cholesterol; Sniff, Soest, Germany) for 16 weeks. To allow flow cytometric isolation of vascular SMCs, some mice also carried a Cre-dependent fluorescent reporter construct (Rosa26flox-mT-stop-flox-Mg; Jackson Laboratories, stock 007576²¹). For induction of Cre-mediated recombination, mice were treated on 5 consecutive days with 1 mg tamoxifen intraperitoneally.¹⁹

Pressure Myography

Seven days after tamoxifen injection, first- and second-order mesenteric arteries were removed from the mesentery and were mounted between 2 glass micropipettes seated in a pressure myograph chamber (Danish Myo Technology; 114P). The external diameter of the artery was visualized and recorded with a charge-coupled device camera using MyoView software. Arterial segments were pressurized stepwise from 20 to 140 mm Hg. Myogenic tone was expressed as the percentage of passive diameter [(passive diameter–active diameter)/passive diameter×100].

Wire Myography

Seven days after tamoxifen injection, first-order mesenteric arteries (2 mm) were removed from the mesentery, mounted on a conventional myograph setup (610-M, Danish Myo Technology), and kept in Krebs solution. After a 30-minute recovery period and normalization, we determined contractile responses by cumulative administration of the indicated agonists. All segments were exposed to 60 mmol/L K⁺ Krebs solution to elicit a reference contraction. Vessel relaxation in response to cumulative addition of cicaprost, iloprost, isoprenaline, sodium nitroprusside, and acetylcholine was measured before contraction with phenylephrine (10 μmol/L).

Telemetric Blood Pressure Measurements

Measurements were performed in conscious, unrestrained mice with a radiotelemetry system (PA-C10; Data Sciences International) as described previously.¹⁹ For the induction of arterial hypertension, osmotic minipumps releasing AngII for 28 days (2000 ng·kg⁻¹·min⁻¹; Alzet, Cupertino, CA) were implanted subcutaneously on the back of 8- to 14-week-old male mice under anesthesia with isoflurane and intraoperative/postoperative treatment with metamizole. In some cases, on day 6 after tamoxifen injection (day 16 after AngII pump implantation), mice received intraperitoneal injections of the IP antagonist Cay10441 (10 mg/kg, Cayman Chemicals) or vehicle control. The individual changes in systolic blood pressure were evaluated from 30 minutes before (–30) until 60 minutes after (+60) drug application; data are displayed as averages from 30-minute intervals in 5 KO and

5 controls (3 repeated applications of Cay10441 [10 mg/kg] or vehicle per mouse). In the deoxycorticosterone acetate salt model of arterial hypertension, mice were unilaterally nephrectomized, and a deoxycorticosterone acetate pellet (50 mg deoxycorticosterone acetate, 21-day release time, Innovative Research of America, Sarasota, FL) was implanted subcutaneously. After recovery, mice received drinking water containing 1% (wt/vol) NaCl.

Statistical Analyses

Data are presented as mean±SEM. The following statistical tests were used: unpaired or paired Student *t* test for comparisons between 2 groups, 1-way ANOVA with Tukey multiple-comparisons test for multiple groups, 2-way ANOVA with Tukey multiple-comparisons test for comparisons between 2 groups with different treatments, and 2-way repeated-measures ANOVA with Bonferroni or Sidak post hoc test for 2 groups over time. The “n” refers to the number of independent experiments or mice per group. *P* values are indicated as follows: **P*<0.05, ***P*<0.01, and ****P*<0.001.

RESULTS

Vascular Tone Regulation in iSM-*Gprc5b*-KO Mice

Mice in which exon 2 of the *Gprc5b* gene was flanked with loxP sites were generated from embryonic stem cell clone EPD0534_1_A10 (EUCOMM) and bred to *Myh11*-Cre/ERT2 mice, which express the tamoxifen-inducible Cre/ERT2 fusion protein under control of the SMC-specific *Myh11* promoter in all vascular regions.¹⁹ Western blotting of lysates prepared from the smooth muscle layer of mesenteric arteries showed a clear reduction of GPCR5B immunoreactivity in tamoxifen-treated *Myh11*-Cre/ERT2-positive *Gprc5b*^{fl/fl} mice (henceforth iSM-*Gprc5b*-KO; Figure 1A). To allow isolation of SMCs by fluorescence-activated cell sorting, control mice and iSM-*Gprc5b*-KOs were further bred to the Cre reporter line *Gt(ROSA)26Sor^{tm4(ACB-tdTomato-EGFP)}Luo 21*, and in vivo recombination was confirmed by mRNA sequencing in enhanced green fluorescent protein (EGFP)-expressing SMCs from skeletal muscle vasculature (Figure 1B). To investigate vascular contractility after SMC-specific inactivation of *Gprc5b*, we performed pressure and wire myography in mesenteric arteries 7 to 14 days after induction with tamoxifen. Myogenic tone in pressure myography was normal (Figure 1C), and contractile responses to the α₁-adrenergic agonist phenylephrine and the thromboxane A₂ analog U46619, acting on the TP receptor, were normal (Figure 1D and 1E). Vessel relaxation in response to the 2 prostacyclin analogs cicaprost and iloprost, both acting on the IP receptor, was significantly enhanced (Figure 1F and 1G), whereas relaxation induced by the β₂-adrenergic agonist isoproterenol or the nitric oxide donor sodium nitroprusside was not

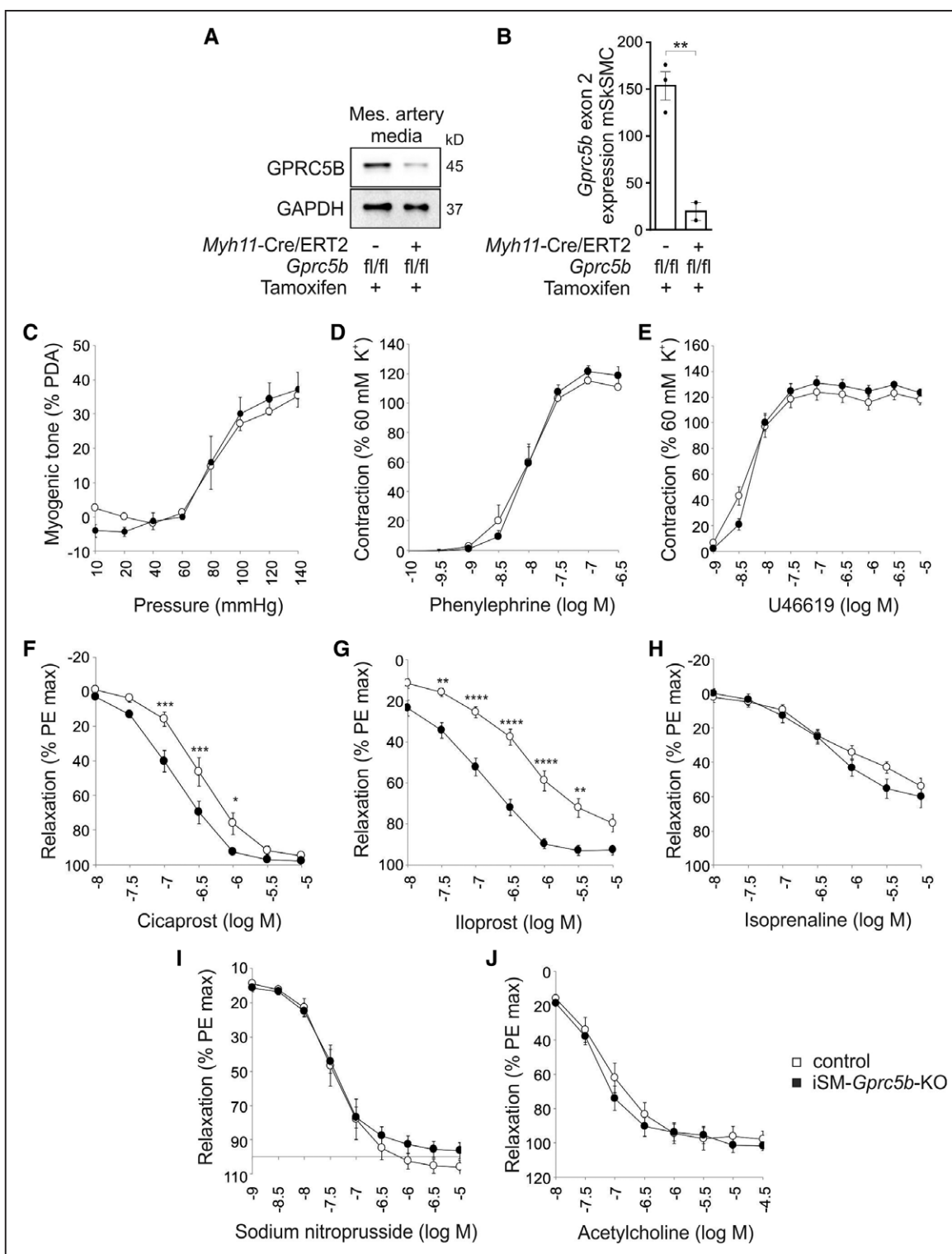


Figure 1. Vascular reactivity in iSM-*Gprc5b*-KO (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockout) mice.

A, G protein-coupled receptor class C group 5 member B (GPCR5B) immunoreactivity in the media of mesenteric arteries obtained 7 to 14 days after the end of tamoxifen induction from control mice (*Gprc5b*^{fl/fl}) and iSM-*Gprc5b*-KOs (*Myh11-Cre/ERT2*; *Gprc5b*^{fl/fl}). GAPDH was used as loading control. **B**, Library size-normalized counts detected by mRNA sequencing in enhanced green fluorescent protein (EGFP)-expressing smooth muscle cells sorted from skeletal muscles of tamoxifen-treated control mice and iSM-*Gprc5b*-KOs bred to a Cre-dependent EGFP reporter line. **C**, Myogenic contraction in response to increased pressure in first-order mesenteric arteries from control mice and iSM-*Gprc5b*-KOs ($n=4$). **D** through **I**, Dose-response curves of different vasoconstrictors (**D** and **E**) or vasodilators (**F** through **I**) were determined by wire myography in first- and second-order mesenteric arteries from control mice and iSM-*Gprc5b*-KOs. Numbers of mice tested were $n=7$ (**D**), $n=6$ (**E**), $n=4$ (**F**), $n=7$ (**G**), $n=4$ (**H**), and $n=3$ (**I**) (1–5 vessels per mouse). **J**, Endothelium-dependent relaxation induced by acetylcholine ($n=4$). All values are expressed as percentages of reference contraction induced by 60 mmol/L K⁺ or 10 μ mol/L phenylephrine (PE). Data are mean \pm SEM; differences between genotypes were analyzed with the unpaired Student *t* test (**B**) or 2-way repeated-measures ANOVA and Bonferroni post hoc test (**C** through **J**). PDA indicates passive diameter. * $P<0.05$. ** $P<0.01$. *** $P<0.001$. **** $P<0.0001$.

altered (Figure 1H and 1I). In addition, endothelium-dependent relaxation induced by acetylcholine was not altered (Figure 1J). Taken together, these data suggest a selective enhancement of IP-mediated relaxation in GPCR5B-deficient SMCs.

Enhanced IP-Mediated Signaling in GPCR5B-Deficient SMCs

IP receptor-mediated SMC relaxation depends on $G\alpha_s$ -mediated activation of adenylyl cyclase isoforms and consecutive cAMP/protein kinase A-dependent reduction of intracellular calcium levels, cellular hyperpolarization, and suppression of RhoA/ROCK-mediated inhibition of myosin phosphatase.^{6,8,9} Together, these changes result in reduced MLC20 phosphorylation and, consecutively, reduced actomyosin contractility. To test whether IP receptor-mediated cAMP production was altered in GPCR5B-deficient SMCs, we performed siRNA-mediated knockdown in human aortic

SMCs (hAoSMCs; Figure 2A). Basal and isoprenaline-induced cAMP production did not differ between knockdown and control, whereas iloprost-induced cAMP production was enhanced after GPCR5B knockdown (Figure 2B). We next investigated whether enhanced IP receptor-mediated cAMP production was sufficient to reduce MLC20 phosphorylation (Figure 2C and 2D). Stimulation of hAoSMCs with thromboxane A_2 receptor agonist U46619 resulted in increased MLC20 phosphorylation in both control and GPCR5B knockdown cells, and this effect was strongly reduced in knockdown cells by concomitant iloprost application. In control cells, in contrast, iloprost caused only a mild reduction of U46619-induced MLC20 phosphorylation (Figure 2C and 2D). These data suggest that GPCR5B deficiency results in a selective enhancement of IP receptor signaling in murine and human SMCs. This enhanced signaling was not caused by increased expression of IP receptor on mRNA or protein level in hAoSMCs or in murine SMCs (Figure 2E–2G).

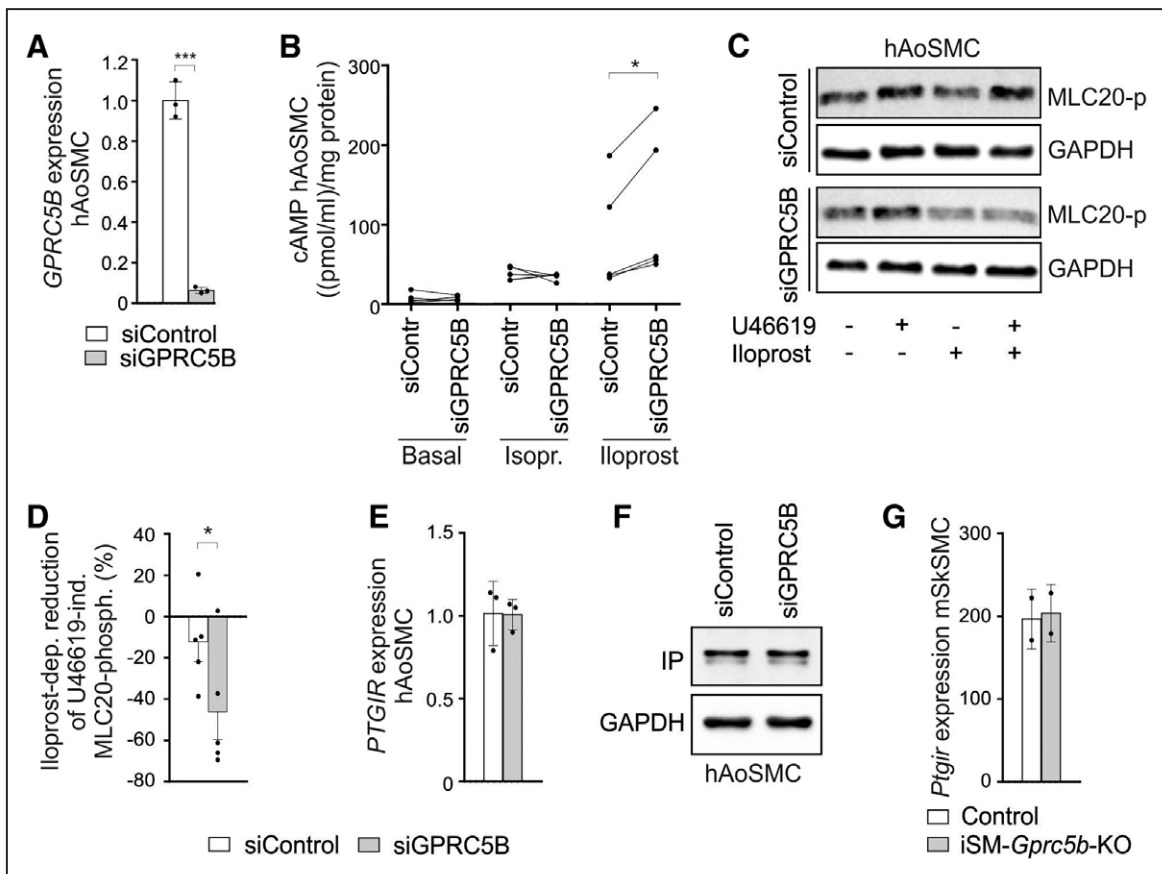


Figure 2. Enhanced prostacyclin receptor (IP)-mediated signaling after siRNA-mediated G protein-coupled receptor class C group 5 member B (GPCR5B) knockdown (siGPCR5B) in smooth muscle cells (SMCs).

A, Efficiency of GPCR5B knockdown (kd) in human aortic SMCs (hAoSMCs) was evaluated by quantitative reverse transcription-polymerase chain reaction (all data normalized to respective control; $n=3$). **B**, cAMP production in control and GPCR5B-kd hAoSMCs under basal conditions and after stimulation with isoprenaline (Isopr.; $1 \mu\text{mol/L}$) or iloprost ($1 \mu\text{mol/L}$; $n=4$). **C** and **D**, Representative Western blot (**C**) and statistical evaluation (**D**) of MLC20 phosphorylation in control and GPCR5B-kd hAoSMCs after addition of the thromboxane A_2 analog U46619 ($1 \mu\text{mol/L}$) for 1 minute and consecutive treatment with iloprost ($1 \mu\text{mol/L}$) for 3 minutes; GAPDH was used as loading control ($n=5-7$). **E** and **F**, mRNA (**E**) and protein (**F**) levels of prostacyclin receptor IP in control and GPCR5B-kd hAoSMCs ($n=2-3$). **G**, mRNA levels of *Ptgir* (encoding IP) in primary SMCs from skeletal muscle (mSkSMC) of control mice and iSM-*Gprc5b*-KO (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockout; $n=2$). Data are mean \pm SEM; comparisons between genotypes were performed with unpaired (**A**, **D**, **E**, and **G**) or paired (**B**) Student *t* test. *n* Indicates number of independent experiments (**A**–**F**) or individual mice (**G**); and siContr, sample treated with scrambled control siRNA. * $P<0.05$. *** $P<0.001$.

GPCR5B Controls IP Membrane Localization in Human Embryonic Kidney Cells

Given that total IP receptor levels were not altered in GPCR5B-deficient SMC, we next investigated whether GPCR5B modulates intracellular IP receptor trafficking. To do so, human embryonic kidney (HEK) 293 cells were transfected with plasmids encoding HA-tagged IP receptor (HA-IP), and membrane expression of HA-IP was detected by ELISA in nonpermeabilized cells after knockdown or overexpression of GPCR5B. We found that knockdown of GPCR5B enhanced HA-IP membrane abundance, whereas overexpression of GPCR5B decreased it (Figure 3A). Overexpression of other randomly chosen GPCRs such as HCA2 or P2Y10 did not affect IP surface expression (Figure 3B), and GPCR5B overexpression did not affect cell viability (data not shown). Immunofluorescence staining in permeabilized cells overexpressing either HA-IP alone or in combination with Myc-tagged GPCR5B (GPCR5B-Myc) showed that, in the absence of overexpressed GPCR5B, the IP signal was found mainly in or close to the membrane, here identified by wheat germ agglutinin, whereas in cells coexpressing GPCR5B, the HA-IP signal was shifted to intracellular regions (Figure 3C and 3D). This altered cellular trafficking did not affect total IP protein levels in HEK cells (Figure 3E and 3F), and the kinetic of iloprost-induced internalization of remaining membrane-targeted HA-IP was not changed (Figure 3G). GPCR5B seems to be cointernalized with IP in response to iloprost treatment (Figure 3H). To further define the subcellular localization of intracellular GPCR5B and IP, we performed costainings with the Golgi marker GM130, endoplasmic reticulum (ER) marker calnexin, and endosome marker EEA1. We found that both GPCR5B-Myc and HA-IP partially colocalized with the Golgi apparatus and ER, whereas colocalization with endosomes was less prominent (Figure 3I and 3J). To investigate how coexpression of GPCR5B altered intracellular localization of IP, we determined the Manders coefficients²² for colocalization with the different organelle markers in HA-IP single-transfected and HA-IP/GPCR5B-Myc double-transfected cells. We found that coexpression of GPCR5B significantly increased colocalization of HA-IP with the Golgi marker GM130 and ER marker calnexin but not with endosome marker EEA1 (Figure 3K), suggesting that the presence of GPCR5B results in retention of IP receptor in Golgi and ER.

GPCR5B Controls IP Membrane Localization in SMCs

To test whether enhanced membrane localization of the IP receptor was also observed in GPCR5B-deficient SMC, we determined surface IP expression in

nonpermeabilized hAoSMCs using an antibody directed against extracellular loop 3 of the IP receptor. In line with our findings in HEK cells, we found that GPCR5B-deficient hAoSMCs showed increased membrane staining compared with control cells (Figure 4A and 4B). We also investigated whether similar changes were observed in resistance vessels of iSM-*Gprc5b*-KO mice. Immunohistochemical analysis in small-diameter arteries from skeletal muscle showed that the media of iSM-*Gprc5b*-KOs, here identified by Cre reporter-mediated EGFP expression, showed significantly increased immunoreactivity of extracellular loop 3 of the IP receptor (Figure 4C and 4D).

GPCR5B Regulates IP Trafficking by Physical Interaction

We next tested whether GPCR5B modulates IP receptor trafficking by physical interaction. As tentative first evidence, we observed that GPCR5B-Myc and HA-IP signals localized to the same compartment with a high Manders coefficient, indicating colocalization (Figure 5A). We then investigated whether GPCR5B could be coimmunoprecipitated with IP. Western blotting of proteins precipitated by anti-HA beads (HA pull-down in Figure 5B) showed a Myc signal of the expected size in lysates of cells coexpressing HA-IP and GPCR5B-Myc, whereas no Myc signal was detected in cells coexpressing a randomly chosen other orphan GPCR, in this case the Myc-tagged adhesion GPCR GPR133 (Figure 5B). A more systematic analysis of interactions between prostanoid receptors and class C orphans showed that HA-IP pull-down resulted in coimmunoprecipitation not only of GPCR5B but also of GPCR5C, the second smooth muscle-expressed member of the GPCR5 subfamily (Figure 5C, left). In contrast, GPR156, another class C orphan GPCR with lower homology to GPCR5B, was not coprecipitated with IP (Figure 5C, left). We also investigated whether GPCR5B and GPCR5C interacted with other prostanoid receptors expressed in SMCs, for example, the PGE₂ receptor subtype EP2 (Figure 5C, right). Although HA pull-down was equally efficient in EP2-HA-expressing cells, the amount of coimmunoprecipitated GPCR5B was clearly reduced, and no coimmunoprecipitation of GPCR5C was observed (Figure 5C, right). These data suggest that in the HEK293 overexpression system, GPCR5B physically interacts with the prostacyclin IP receptor and, although with lower efficiency, with other prostanoid receptors such as the EP2 receptor. To further corroborate these interaction data, we used dual-color fluorescence recovery after photobleaching²³ to test whether immobilization of the IP receptor, but not of other GPCRs such as β_2 AR, affected lateral mobility of GPCR5B. To do so, HEK293 cells were transfected with GPCR5B-mCitrine and either mTurq2-IP or CFP- β_2 AR, and a large fraction of N-terminally

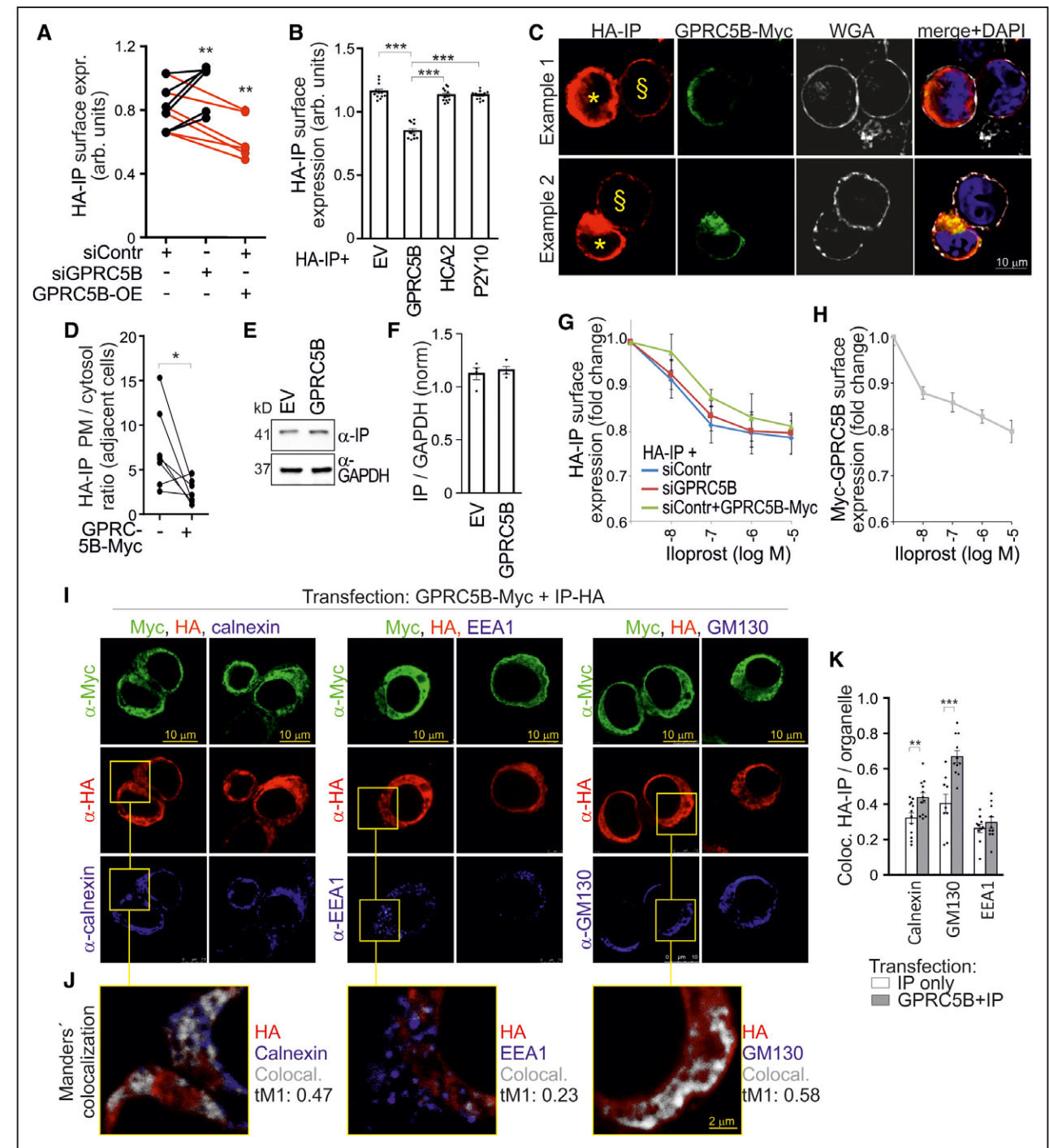


Figure 3. G protein-coupled receptor (GPCR) class C group 5 member B (GPCR5B) controls prostacyclin receptor (IP) membrane availability in human embryonic kidney (HEK) cells.

A and **B**, ELISA-based detection of HA-tagged IP receptor in the plasma membrane of HEK293 cells after siRNA-mediated knockdown (siGPCR5B) or overexpression (OE) of GPCR5B (**A**) or after OE of other GPCRs such as HCA2 or P2Y10 (**B**; $n=6-12$). **C**, HEK293 cells cotransfected with HA-IP and GPCR5B-Myc were first subjected to plasma membrane (PM) staining with wheat germ agglutinin (WGA), permeabilized, and stained with anti-Myc and anti-HA antibodies as well as DAPI for nuclei. **C**, Exemplary photomicrographs showing the intracellular localization of HA-IP in cells expressing HA-IP alone (§) or in combination with GPCR5B-Myc (*). **D**, Statistical evaluation of the HA-IP PM-to-cytosol ratio in GPCR5B-negative and -positive cells in **C** ($n=7$ view fields with neighboring IP alone/IP+GPCR5B cells; total number of cells quantified, 17). **E** and **F**, Levels of endogenous IP receptor in HEK293 cells overexpressing GPCR5B (**E**, exemplary blot; **F**, statistical evaluation of $n=4$). **G**, Internalization of surface HA-IP 40 minutes after addition of different concentrations of iloprost in control HEK293 cells or after knockdown/OE of GPCR5B ($n=8$). **H**, Internalization of an N-terminally Myc-tagged GPCR5B 40 minutes after addition of different concentrations of iloprost in HEK cells transfected with Myc-GPCR5B and HA-IP ($n=6$). **I**, HEK293 cells transfected with both HA-IP and GPCR5B-Myc were stained with anti-Myc and anti-HA antibodies and the indicated organelle markers. **J**, Visualization of colocalization between IP-HA (red) and the respective organelle marker (blue). Gray indicates colocalization; the tM1 value indicates the Manders coefficient for colocalization of IP within the organelle marker (1.0 indicates full colocalization). **K**, Statistical evaluation of Manders coefficients indicating colocalization between HA-IP and different organelle markers in HEK cells expressing either HA-IP alone or both HA-IP and GPCR5B-Myc (1.0 indicates complete overlay; $n=9-12$ per group). Data are mean \pm SEM; comparisons between genotypes were performed with paired (**A** and **D**) or unpaired (**K**) Student *t* test or 1-way ANOVA (**B**). EV indicates empty vector. * $P<0.05$. ** $P<0.01$. *** $P<0.001$.

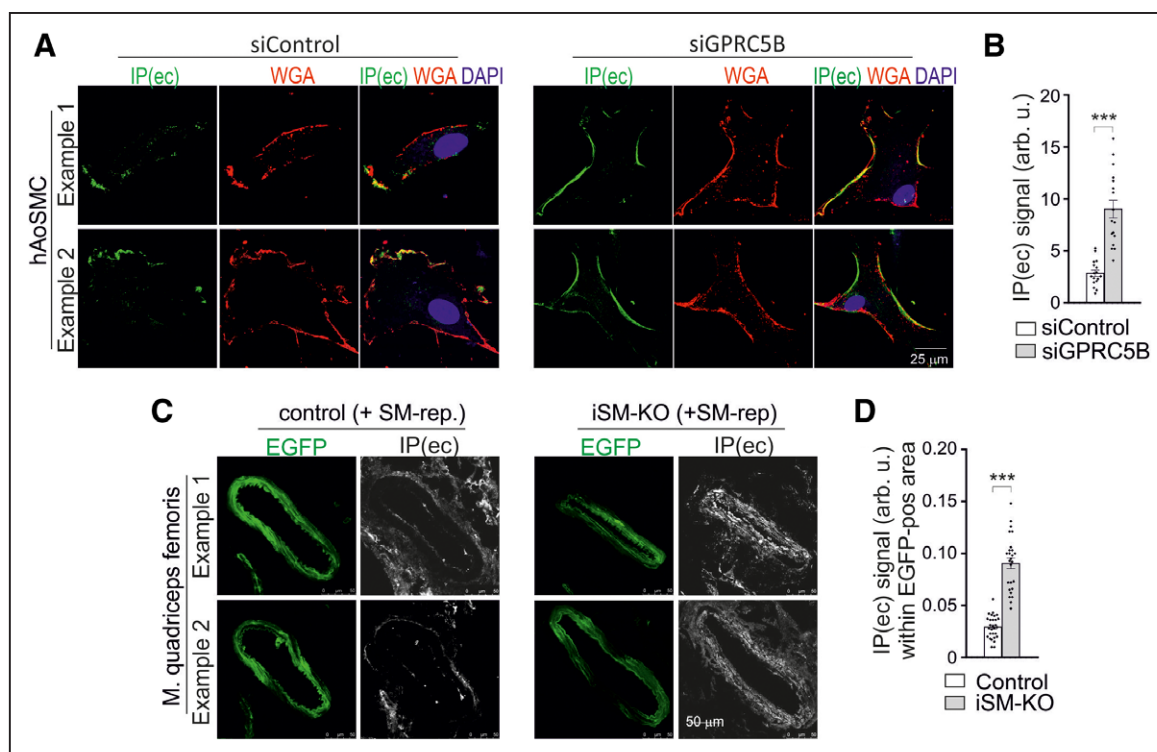


Figure 4. G protein-coupled receptor class C group 5 member B (GPCR5B) controls prostacyclin receptor (IP) membrane availability in smooth muscle cells (SMCs).

A and B, Antibody-mediated detection of the extracellular domain of the endogenous IP receptor [IP(ec)] in nonpermeabilized human aortic SMCs (hAoSMCs) treated with control siRNA or GPCR5B-specific siRNA (wheat germ agglutinin [WGA] staining indicates plasma membrane, DAPI nucleus). **A,** Exemplary photomicrographs. **B,** Statistical evaluation ($n=17$). **C and D,** Antibody-mediated detection of IP(ec) in nonpermeabilized sections of murine skeletal muscle (SM) from control mice and iSM-*Gprc5b*-KOs (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockouts) bred to a Cre-dependent EGFP reporter line (confocal scans of regions at least 5 μm away from upper and lower cut surface). **C,** Exemplary photomicrographs. **D,** Statistical evaluation of IP(ec) immunoreactivity within enhanced green fluorescent protein (EGFP)-positive areas ($n=6$ –13 vessels from 3 mice each). Data are mean \pm SEM; comparisons between genotypes were performed with unpaired Student *t* tests. *** $P < 0.001$.

mTurq2- and CFP-tagged receptors were immobilized with polyclonal antibodies recognizing these GFP variants. After photobleaching of a defined membrane area, lateral mobility of immobilized and nonimmobilized receptors was measured as the recovery of mTurq2/CFP and yellow fluorescent protein fluorescence. As expected, recovery of mTurq2-IP or CFP- β_2 AR was markedly reduced after antibody-mediated immobilization (Figure 5D). Recovery of GPCR5B-mCitrine, however, was clearly reduced after immobilization of mTurq2-IP, whereas immobilization of CFP- β_2 AR had only a little effect on GPCR5B-mCitrine lateral mobility (Figure 5E). Together with the coimmunoprecipitation data, these data support a specific physical interaction between GPCR5B and IP receptor.

Relevance of SMC GPCR5B in Arterial Hypertension

We next investigated whether increased IP-mediated relaxation in iSM-*Gprc5b*-KOs was sufficient to alter vascular tone in vivo. To test this, control mice and not-yet-induced KO mice were implanted with telemetric catheters, and basal blood pressure was determined

for 3 days. After this, GPCR5B deficiency in SMCs was induced by tamoxifen treatment on 5 consecutive days. This resulted in a mild hypotension in iSM-*Gprc5b*-KOs compared with tamoxifen-treated controls, which was quickly compensated for (Figure 6A). To investigate whether loss of GPCR5B in SMCs was able to ameliorate hypertensive disease, we used 2 models, implantation of AngII-releasing miniosmotic pumps (Figure 6B) and subcutaneous implantation of a pellet releasing the murine aldosterone analog deoxycorticosterone acetate in combination with unilateral nephrectomy and salt load via the drinking water (Figure 6C). Of note, the KO is induced in these models after establishment of hypertensive disease, which allows us to study the effect of GPCR5B inactivation in a therapeutic setting. To do so, we first monitored the development of arterial hypertension in control mice and not-yet-induced KO mice for 5 days and then induced recombination by tamoxifen. In both models, GPCR5B inactivation resulted in a significant reduction of blood pressure compared with control, suggesting that loss of GPCR5B in SMCs indeed facilitates relaxation in hypertensive disease. Although 3 weeks of hypertension is a rather short observation period, we found

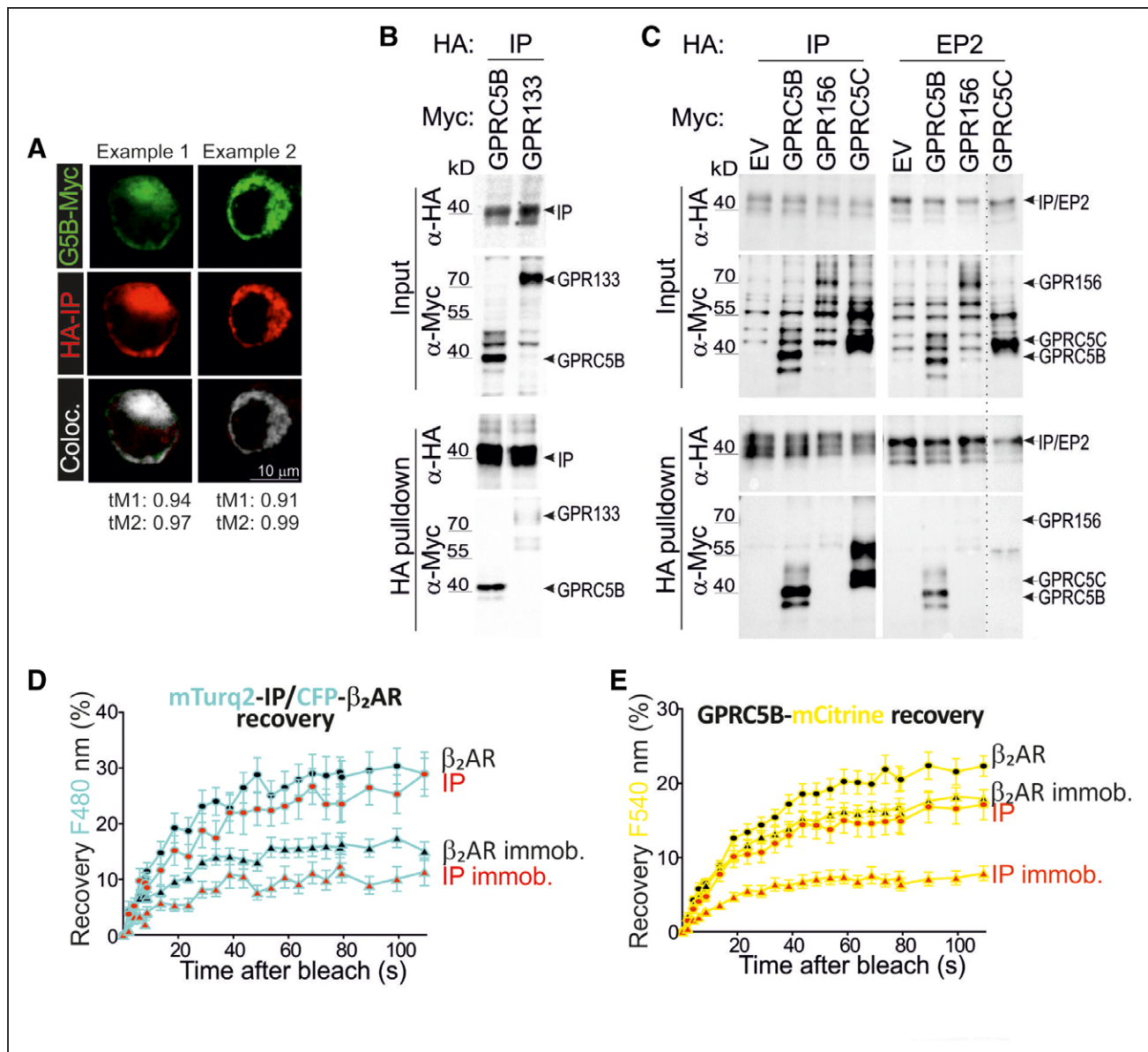


Figure 5. GPCR5B regulates IP trafficking by physical interaction.

A, Localization of G protein-coupled receptor class C group 5 member B (GPCR5B)-Myc (green) and HA-prostacyclin receptor (IP; red) in double-transfected human HEK293 cells; colocalization of both is shown in white. Manders' colocalization coefficients tM1 and tM2 are shown next to each panel (1.0 indicates complete overlay). **B**, Western blot detection of HA and Myc signals in lysates of human embryonic kidney (HEK) 293 cells expressing HA-IP (40 kDa) in combination with GPCR5B-Myc (44 kDa) or Myc-GPR133 (96 kDa) without (input) and after immunoprecipitation of HA-IP (HA pull-down). **C**, Western blot detection of HA and Myc signals in lysates of HEK293 cells expressing HA-IP (left) or EP2-HA (right) in combination with empty vector, GPCR5B-Myc (44 kDa), GPR156 (89 kDa), or GPCR5C-Myc (53 kDa) without (input) and after immunoprecipitation of HA-IP (HA pull-down). Dotted line indicates border between membranes. **D** and **E**, Coimmobilization experiments using dual-color fluorescence recovery after photobleaching were performed in HEK cells expressing GPCR5B-mCitrine and either mTurquoise(mTurq2)-IP (red symbols) or CFP-β₂ adrenergic receptor (β₂AR; black symbols) as indicated. Fluorescence recovery after photobleaching was determined for mTurq2-IP/CFP-β₂AR (**D**) and for GPCR5B-mCitrine (**E**) in the presence (triangles) or absence (circles) of antibodies mediating immobilization of IP or β₂AR (n=22–29 cells per group). Data are mean±SEM.

preliminary evidence for reduced end-organ damage: Hypertension-induced cardiac hypertrophy, here determined as the heart weight–body weight ratio, was reduced in trend in iSM-*Gprc5b*-KOs (Figure 6D). There was no significant difference in body weight between the groups (control, 28.7±1.45 g; iSM-*Gprc5b*-KO, 26.3±1 g). However, because end-organ damage was not the primary aim of this investigation, further studies are required for a final assessment of this aspect. To

investigate to what degree enhanced IP signaling contributes to these changes, we tested the effect of the IP antagonist Cay10441 on blood pressure in control and iSM-*Gprc5b*-KOs 16 days after induction of AngII-dependent hypertension (Figure 6E and 6F). We found that intraperitoneal injection of 10 mg/kg Cay10441, but not of vehicle, significantly increased blood pressure in iSM-*Gprc5b*-KOs but had no effect on control mice (Figure 6F). These data show that enhanced IP

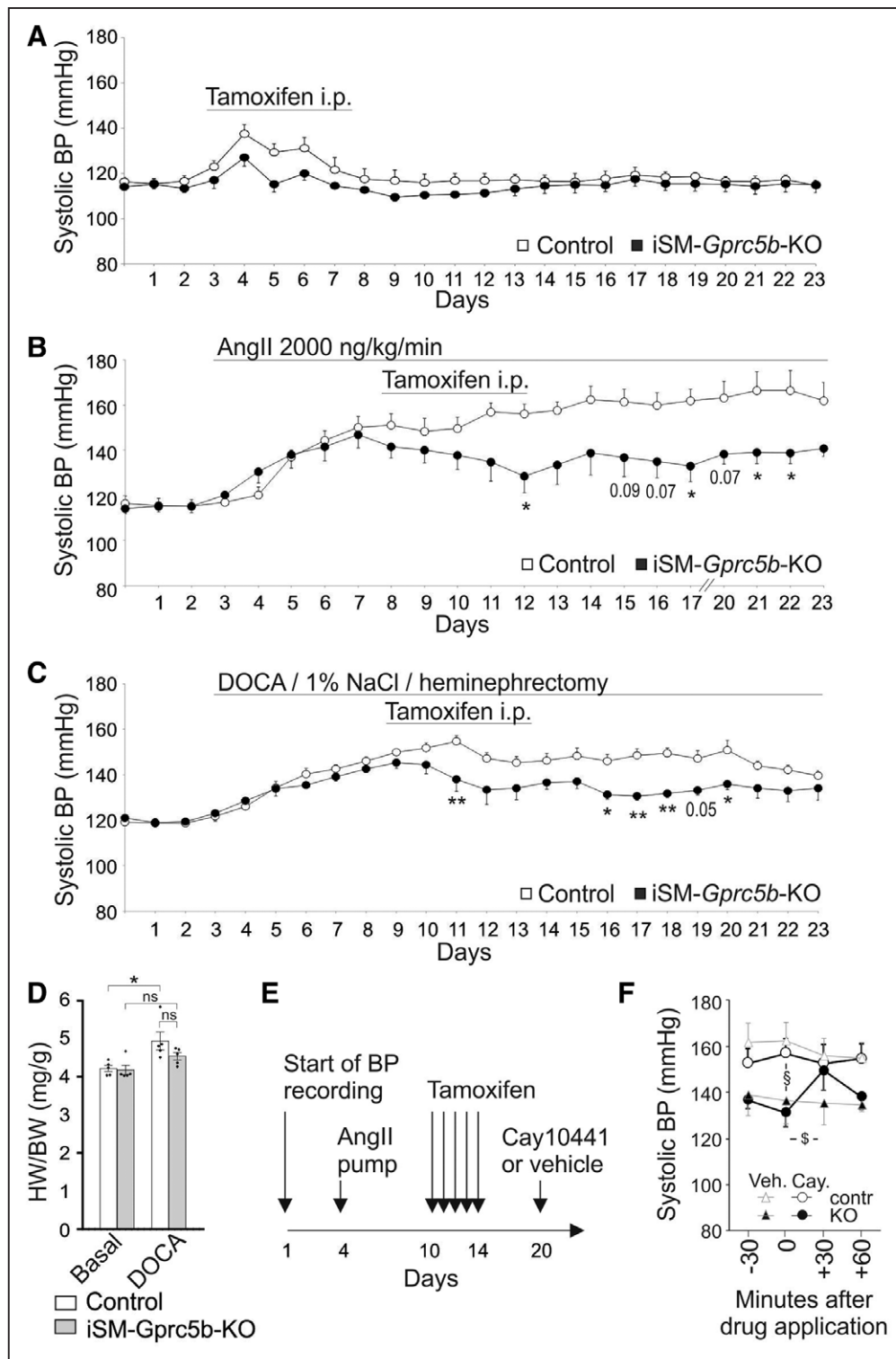


Figure 6. Telemetric blood pressure (BP) measurement in iSM-*Gprc5b*-KOs (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockouts).

A through **C**, Telemetric recording of BP in control and iSM-*Gprc5b*-KO mice before and after knockout induction with tamoxifen under basal conditions (**A**; n=12 controls, 10 KOs), after implantation of angiotensin II (AngII)-releasing miniosmotic pumps (**B**; n=11 controls, 6 KOs; data for days 18 and 19 are missing because of system failure), or after implantation of a deoxycorticosterone acetate (DOCA)-releasing pellet in combination with unilateral nephrectomy and exposure to 1% NaCl in drinking water (**C**; n=8 controls, 10 KOs). Data are mean±SEM; differences between genotypes were analyzed with 2-way repeated-measures ANOVA and Bonferroni post hoc test. **D**, Heart weight/body weight ratio in control and KO mice in the basal state and 3 weeks after induction of the DOCA/salt hypertension model (n=5) (2-way ANOVA with Tukey multiple-comparisons test). **E** and **F**, Effect of IP antagonist Cay10441 (10 mg/kg intraperitoneally [i.p.]) on systolic BP in control mice and iSM-*Gprc5b*-KOs (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockouts) 16 days after implantation of AngII-releasing pumps. Experimental design (**E**) and statistical evaluation (**F**) of BP from 30 minutes before (−30) until 60 minutes after (+60) application of Cay10441 (circles) or vehicle (triangles) in control mice (white symbols) or iSM-*Gprc5b*-KOs (black symbols) (n=5 mice each). Significances according to 2-way ANOVA with Tukey multiple-comparisons test: by genotype: control-Cay vs KO-Cay at t=0: 0.04 (\$); by treatment: KO-Cay t=0 vs t=+30: 0.0049 (\$). *P<0.05. **P<0.01.

signaling is a major contributor to the observed amelioration of hypertensive disease in iSM-*Gprc5b*-KOs.

Relevance of GPCR5B in Smooth Muscle Differentiation

The IP receptor not only mediates relaxation but also induces differentiation of SMCs from a synthetic, proliferative phenotype to a more quiescent, contractile phenotype.²⁴ We investigated the expression of marker genes for proliferation and contractility in hAoSMCs and found that GPCR5B-deficient hAoSMCs showed enhanced expression of contractile genes such as *ACTA2* (encoding α -smooth muscle actin) and *TAGLN* (encoding smooth muscle protein 22 α) and reduced the expression of proliferative markers such as *MKI67*, *PCNA*, and *CCNA2* (Figure 7A). To test whether this could be attributed to an increased IP receptor signaling, we analyzed gene expression in GPCR5B-deficient SMCs in the presence of the IP receptor antagonist Cay10441. Inhibition of IP receptor largely normalized expression of contractile markers such as *ACTA2* and *TAGLN* (Figure 7B), suggesting that enhanced IP signaling underlies increased differentiation in GPCR5B-deficient hAoSMCs. Cay10441 did not have a clear effect on the expression of cell cycle genes (data not shown), but determination of cell numbers 24 hours after seeding of equal cell numbers showed that reduced proliferation of GPCR5B-deficient hAoSMCs was corrected by the IP antagonist (Figure 7C). To investigate whether increased SMC differentiation was also observed in vivo, we performed mRNA sequencing in fluorescence-activated cell sorting–sorted vascular SMCs from control mice and iSM-*Gprc5b*-KO mice with SMC-specific EGFP expression. Single-cell expression analyses previously showed that the frequency of *Gprc5b* expression is very low in aortal SMCs but high in resistance arteries from mesenteric and skeletal vasculature¹² (Figure 7D), which led us to use SMCs from skeletal muscle for this analysis. Gene Ontology term analysis showed that expression of contraction-related genes was clearly altered in GPCR5B-deficient skeletal muscle SMCs (Figure 7E). In particular, markers of SMC differentiation such as *Acta2* or *Tagln* but also various myosin heavy- and light-chain isoforms and molecules involved in fiber organization such as *Actn1*, *Flna*, *Des*, *Tpm1/2*, and *Dstn* were increased (Figure 7F). In addition, we observed reduced expression of genes indicative of inflammatory activation such as *Icam1*, *Ptgs1/2* (encoding COX1/2), or *Nfkb1* or proteoglycans such as *Lum* or *Dcn* (Figure 7F). Proliferation markers such as *Mki67*, *Pcna*, and *Ccna2* were not reliably detected in freshly isolated SMCs. In further support of the hypothesis that *Gprc5b* expression is correlated with reduced SMC differentiation, we found that, in freshly isolated murine aortic SMCs,¹² the rare *Gprc5b*-positive SMCs

have reduced expression of *Myh11* and *Acta2* compared with *Gprc5b*-negative aortic SMCs (Figure 7G). Taken together, these data show that, in both human and murine SMCs, GPCR5B is a regulator of SMC differentiation.

Role in Atherosclerosis

During atherosclerosis development, dedifferentiated SMCs migrate into the growing plaque, where they may exert both beneficial (stabilization of the fibrous cap) and detrimental (contribution to inflammation) effects.²⁵ Because GPCR5B seems to play a role in the regulation of SMC differentiation, we investigated atherosclerosis development in iSM-*Gprc5b*-KOs. In murine atherosclerosis models, plaque development is found largely in aorta and large arteries, which in healthy young mice express *Gprc5b* only rarely. However, during aging and in response to atherosclerosis development in apolipoprotein E-deficient mice, the frequency of *Gprc5b*-expressing cells increases also in murine aortic SMCs (Figure 8A). To investigate how altered differentiation and proliferation in iSM-*Gprc5b*-KOs affect atherosclerosis development, we bred mice to the apolipoprotein E-deficient mouse strain and studied aortic plaque development after 16 weeks of a high-fat diet. We found that the plaque area determined by Oil Red O staining was in trend reduced for total aorta and significantly reduced in the aortic arch (Figure 8B–8D). In addition, plaques of the innominate artery were significantly smaller in iSM-*Gprc5b*-KOs than in controls (Figure 8E and 8F), and immunoreactivity for the smooth muscle differentiation marker α -smooth muscle actin was increased in deficient mice (Figure 8G). In line with this, mRNA sequencing of fluorescence-activated cell sorting–isolated murine aortic SMCs from aortas of early atherosclerotic mice showed that GPCR5B-deficient SMCs express higher levels of contractile markers such as *Acta2*, *Tagln*, or *Smtn* (Figure 8H). Levels of IP expression were not altered in aortas of atherosclerotic iSM-*Gprc5b*-KOs (data not shown).

DISCUSSION

Our data show that GPCR5B, a class C orphan GPCR, modulates SMC contractility and differentiation by negatively regulating IP receptor trafficking, probably by physical interaction and intracellular retention.

Numerous coimmunoprecipitation studies using tagged receptors indicated that GPCRs can form homodimers or heterodimers, and it was suggested that dimerization may affect both signaling and trafficking.^{26,27} Although for most class A receptors the functional relevance of dimerization is unclear, there is no doubt that class C receptors exist and function as stable dimers: Metabotropic glutamate receptors

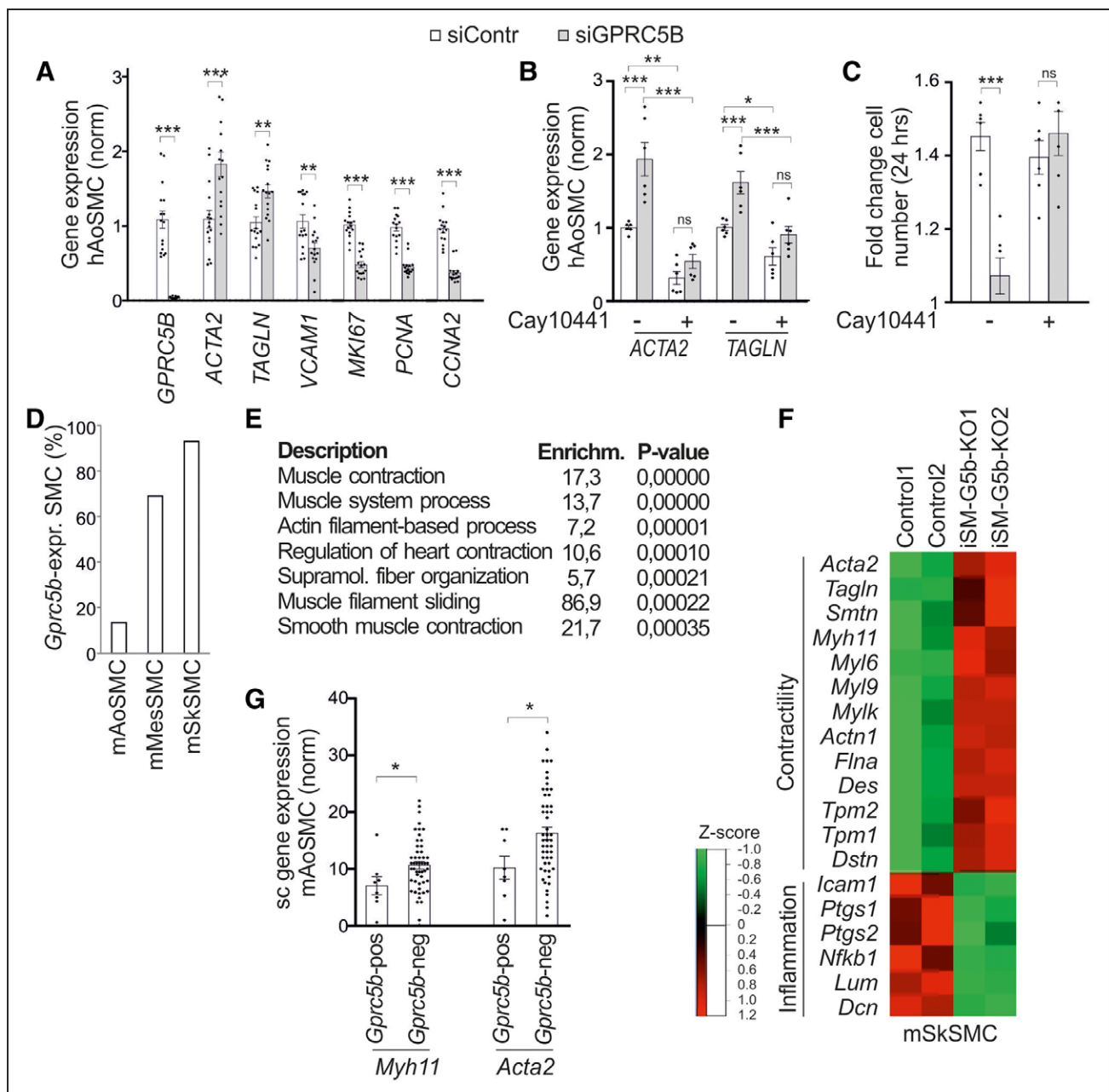


Figure 7. Enhanced differentiation and reduced proliferation in G protein-coupled receptor class C group 5 member B (GPCR5B)-deficient vascular smooth muscle cells (SMCs).

A and B. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) gene expression analysis in control and GPCR5B knockdown human aortic SMCs (hAoSMCs) under basal conditions (**A**) or after addition of 1 $\mu\text{mol/L}$ Cay10441 (**B**) ($n=6$; all data normalized to respective control). **C.** Fold change in cell numbers within 24 hours after seeding of equal numbers of control and GPCR5B-kd hAoSMCs in the presence or absence of 1 $\mu\text{mol/L}$ Cay10441 ($n=6$; all data normalized to respective control). **D.** Frequency of *Gprc5b*-expressing cells in murine aortic SMCs (mAoSMCs) and SMCs from the murine skeletal (mSkSMCs) or mesenteric (mMesSMCs) vasculature as judged by single-cell (sc) RT-PCR ($n=29$, 57, and 60 cells). **E and F.** Heat map of library size-normalized counts detected by mRNA sequencing in enhanced green fluorescent protein (EGFP)-expressing SMCs sorted from skeletal muscles of tamoxifen-treated control mice and iSM-*Gprc5b*-KO mice (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockouts) bred to a Cre-dependent EGFP reporter line ($n=2$ per group; data are normalized to respective control). **E.** Top 7 changes according to GO term analysis. **F.** Heat map showing Z score (column)–normalized gene expression of selected contractility- and inflammation-related genes. **G.** Expression of differentiation markers *Myh11* and *Acta2* in *Gprc5b*-positive and -negative mAoSMCs as judged by single-cell RT-PCR ($n=60$ cells; data are reanalyzed from Kaur et al¹² and shown after normalization to the geometric mean of reference genes). Data are mean \pm SEM; comparisons between genotypes were performed with unpaired Student *t* test (**A**, **C**, and **G**) or 2-way ANOVA with Tukey multiple-comparisons test (**B**). *n* Indicates number of independent experiments (**A** through **C**), individual cells (**D** and **G**) or individual mice (**E** and **F**); siContr, sample treated with scrambled control siRNA; and siGPCR5B, siRNA-mediated GPCR5B knockdown. * $P<0.05$. ** $P<0.01$. *** $P<0.001$.

and the calcium-sensing receptor form homodimers, whereas GABA_B and sweet and umami taste receptors are obligatory heterodimers.^{26,27} Dimerization of class

C receptors is mediated by distinct structural features, for example, by the very large N-terminus containing a Venus flytrap motif, which mediates both ligand

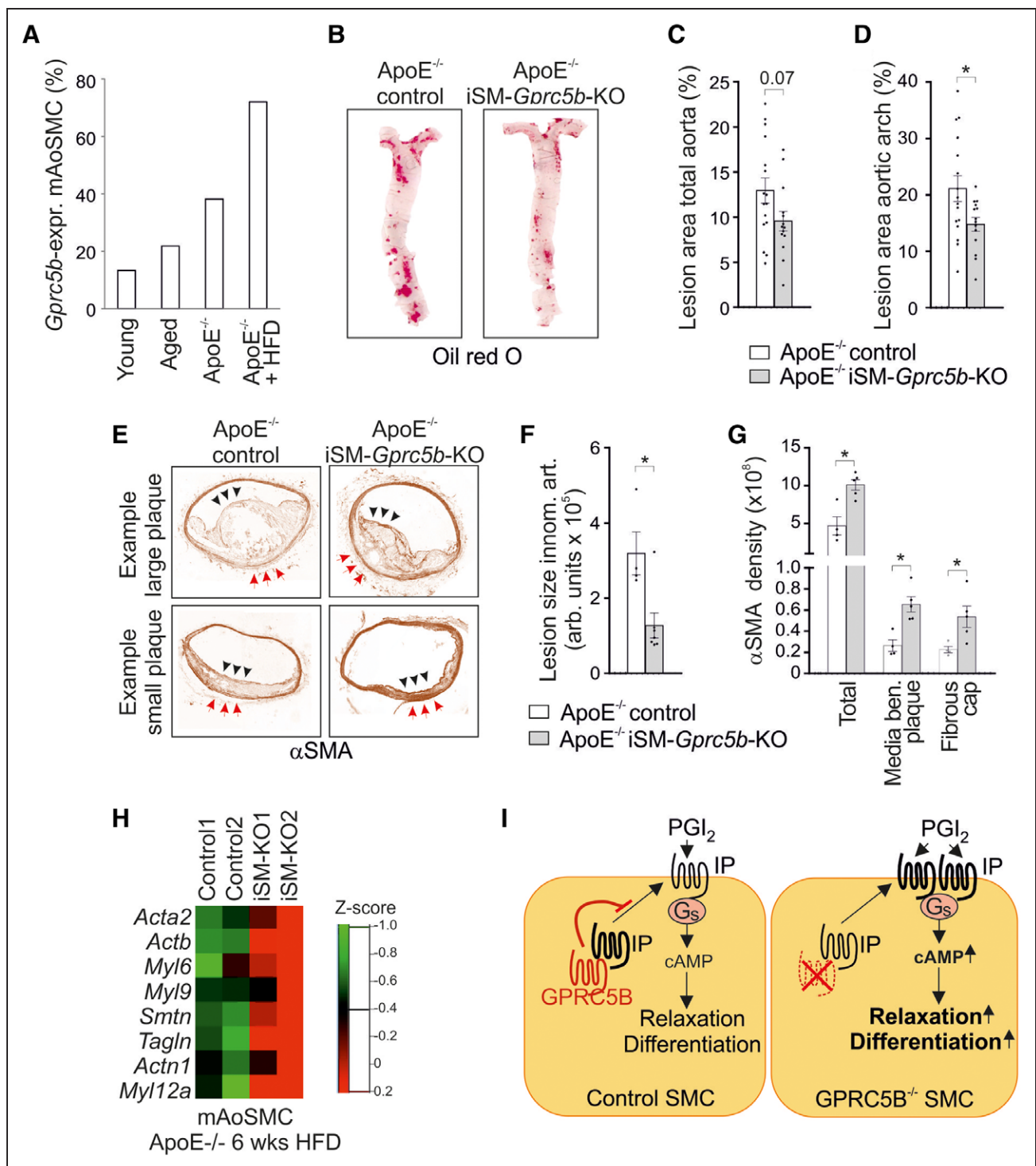


Figure 8. Reduced atherosclerosis in iSM-*Gprc5b*-KO (tamoxifen-inducible, smooth muscle-specific *Gprc5b* knockout) mice.

A, The frequency of *Gprc5b* expression in murine aortic smooth muscle cells (mAoSMCs) from young mice (3 months), aged mice (16 months), young apolipoprotein E (ApoE)-deficient mice, and ApoE^{-/-} mice kept for 16 weeks on high fat diet (HFD) was determined by single-cell reverse transcription–polymerase chain reaction (data reanalyzed from Kaur et al¹²). **B** through **D**, Oil Red O staining of aortas from ApoE^{-/-} control mice and ApoE^{-/-} iSM-*Gprc5b*-KO mice after 16 weeks of Western-type diet (3 independent experiments with n=14–16 in total). Exemplary photomicrograph (**B**) and statistical evaluation of plaque area in total aorta (**C**) and aortic arch only (**D**). **E** through **G**, α-smooth muscle actin (αSMA) staining in innominate arteries of ApoE^{-/-} control mice and ApoE^{-/-} iSM-*Gprc5b*-KO mice after 16 weeks of Western-type diet (samples from experiment 3 in **B** through **D**). **E**, Exemplary photomicrographs. **F**, evaluation of plaque size. **G**, Statistical evaluation of αSMA staining in total vessel, media underlying plaque, and fibrous cap (n=4–5). **H**, Heat map showing Z score (column)–normalized gene expression of selected contractility-related genes as detected by mRNA sequencing in enhanced green fluorescent protein (EGFP)-expressing smooth muscle cells (SMCs) sorted from aortas of tamoxifen-treated control mice and iSM-*Gprc5b*-KOs bred to the ApoE^{-/-} line and a Cre-dependent EGFP reporter line after 6 weeks on Western-type diet. **I**, Graphical summary; for details, see text. Data in **C**, **D**, **F**, and **G** are mean±SEM. PGI₂ indicates prostaglandin I₂. Comparisons between genotypes were performed with unpaired Student *t* test. *P<0.05.

binding and dimerization of N-termini. In addition, metabotropic glutamate receptors and calcium-sensing receptors contain a cystein-rich domain between Venus flytrap and the heptahelical domain, which allows formation of disulfide bonds within the homodimer.²⁶ GABA_B receptors, which do not contain a cystein-rich domain, also dimerize through their C-terminal coiled-coil domains, thereby masking an RXR motif in the GABA_{B1} receptor that would trap the receptor in the ER.²⁸ In contrast with these prototypical class C GPCRs, class C orphan receptors such as GPCR5B have a very short N-terminus and lack both Venus flytrap motif and full cystein-rich domain. GPCR5B was nevertheless classified as a glutamate-like/class C receptor because its transmembrane domains show a 26% homology with mGluR2.²⁹ Other structural features also support class C classification: GPCR5B contains in its short N-terminus 2 cysteine residues that are analogs of the 2 cysteines closest to the membrane in the cystein-rich domain of metabotropic glutamate receptors and calcium-sensing receptor.³⁰ However, the class C classification is not undisputed; GPCR5B was originally rather classified as "other 7-transmembrane proteins" because it could not be included in any of the classic GPCR families with sufficient certainty.² Our data suggest that GPCR5B is able to undergo dimerization or oligomerization with certain receptors such as IP, but whether the conserved cysteines in the extracellular domain of GPCR5B play a role in this context is unclear.

Although GPCR5B dimerization has so far not been studied, various previous studies suggested that the IP, which belongs to GPCR class A, may dimerize: Coimmunoprecipitation of differentially tagged IP in COS-7 indicated that the receptor homodimerizes³¹ and that this depends on disulfide bonds between cysteines in different locations.³¹ Disruption of the disulfide bonds resulted in reduced receptor expression and membrane localization.³¹ Furthermore, coexpression of an IP variant that is retained in the ER [IP(R212C)]³² leads to retention of wild-type IP in the ER.³³ Together, these data suggest that IP homodimerization may be required for proper receptor trafficking. Our data suggest that the presence of GPCR5B reduces the ability of the IP receptor (as monomer or dimer) to traffic to the plasma membrane. The reason might be that GPCR5B interferes with IP homodimerization by allowing GPCR5B/IP heterodimerization. In line with this notion, we found that GPCR5B-Myc and HA-IP largely colocalize in both the plasma membrane and the cytosol. The structural motifs mediating class A dimerization are not understood. Structural studies suggest that (in contrast with class C) class A GPCRs dimerize through their transmembrane domain,^{34,35} but the specific sites mediating IP dimerization are unknown. It is also unclear whether GPCR5B/IP interaction is ligand dependent or independent. Our data show that GPCR5B and IP interact in

the absence of an exogenous ligand, but it is still possible that SMC-autonomous ligands play a role, either SMC-produced prostacyclin^{36,37} or an as-yet-unknown autocrine activator of GPCR5B.

Prostacyclin, also known as prostaglandin I₂ (PGI₂), is the major arachidonic acid metabolite produced in endothelium and SMCs of arteries and veins; it is also produced by microvessels.^{36,37} Prostacyclin exerts its numerous effects through activation of the G_s-coupled receptor IP, resulting in adenylyl cyclase-dependent cAMP production. In SMCs, IP-dependent cAMP production was shown to induce relaxation in various types of human and rodent arteries.^{38,39} How cAMP elevation results in SMC relaxation is not fully understood, but cAMP/protein kinase A-dependent inhibition of RhoA, reduction of intracellular calcium levels, and cAMP-independent activation of hyperpolarizing MaxiK (BK) channels have been suggested.^{8,40} The relevance of PGI₂-IP signaling for the regulation of systemic blood pressure is complex: Genetic deletion of the IP receptor does not result in relevant blood pressure changes,⁴¹ and application of IP receptor antagonist Cay10441/Ro3244794 does not alter mean arterial blood pressure.^{42,43} However, pharmacological stimulation of IP receptors via the stable analog iloprost results in arterial hypotension in healthy mice⁴³ and significantly lowered blood pressure in hypertensive mongrel dogs.⁴⁴ Furthermore, patients with pregnancy-induced hypertension and some patients with essential hypertension have reduced endogenous synthesis of PGI₂,^{36,45} and mice lacking prostaglandin synthase, a key enzyme in formation of PGI₂, showed hypertension and chronic kidney disease.⁴⁶ These data suggest that although PGI₂ exerts important antihypertensive effects, loss of the IP receptor can be compensated for by other receptors, for example, EP4, another SMC-expressed prorelaxant GPCR that is activated by PGI₂.^{47,48}

Our data indicate that IP receptor-mediated responses to exogenous and endogenous PGI₂ are facilitated in the absence of GPCR5B, resulting in an antihypertensive effect in vivo. It is possible that additional mechanisms exist through which GPCR5B regulates vascular function, but the finding that the IP antagonist Cay10441 strongly reduced the beneficial effect of GPCR5B deficiency indicates that enhanced IP receptor signaling is a major contributing factor. However, more complex changes such as abnormalities in salt sensitivity cannot be excluded and require further investigation. In addition to the role of GPCR5B in contractility regulation, our data point to a facilitation of other IP-mediated effects such as an increased SMC differentiation and reduced proliferation. This is in line with previous studies showing that IP agonists can inhibit vascular SMC migration and proliferation^{10,11} and induce SMC differentiation through the G_s/protein kinase A/cAMP-responsive element binding protein pathway.²⁴ Reduced

PGI₂ signaling has been suggested to contribute to the development of chronic vascular remodeling processes such as atherosclerosis or neointima formation by facilitating the phenotypic switch from contractile SMCs to the more proliferative, migratory, and synthetic dedifferentiated phenotype.⁴⁹ In line with this, we found reduced atherosclerosis development after SMC-specific inactivation of GPCR5B, and this was associated with a preservation of a differentiated SMC phenotype. This included an increased α -smooth muscle actin expression not only in the media underlying the atherosclerotic plaque but also in the fibrous cap covering the plaque. α -Smooth muscle actin-positive cells of the fibrous cap were shown to be derived mainly from media SMCs⁵⁰ and are believed to form an atheroprotective layer associated with plaque stability.²⁵ Our findings therefore suggest that inactivation of GPCR5B in SMCs not only prevents plaque growth by reducing SMC dedifferentiation but also may prevent plaque rupture by reinforcing the fibrous cap.

GPCR5B is in SMCs of the healthy murine aorta barely expressed but strongly upregulated during atherosclerotic dedifferentiation.¹² Even in the normal aorta, a small subpopulation of SMCs exist that show signs of dedifferentiation, and these cells are enriched in GPCR5B.¹² These data suggest that GPCR5B is upregulated during SMC dedifferentiation and then perpetuates dedifferentiation by inhibiting IP-mediated protective effects. Although such a positive feedback loop of dedifferentiation might be beneficial after acute vascular damage, it is certainly counterproductive when chronically activated. Of note, the IP receptor also is upregulated in dedifferentiating vascular SMCs,¹² but our data suggest that GPCR5B-mediated inhibition of IP membrane availability counteracts the putative beneficial effects of enhanced IP expression. Pharmacological approaches inhibiting the upregulation of GPCR5B or its interaction with the IP receptor may be of therapeutic use in the treatment of chronic vascular diseases such as atherosclerosis. In addition, inactivation of GPCR5B is beneficial in murine arterial hypertension, and our data suggest that this phenotype is at least partly caused by the facilitation of IP signaling. However, SMCs of resistance arteries show, in contrast with aortal SMCs, a high basal expression of GPCR5B, indicating that GPCR5B here exerts a constitutive repression of IP-mediated effects.

CONCLUSIONS

Our data show that GPCR5B plays an important role in the regulation of smooth muscle contractility and differentiation, at least partly as a result of regulation of membrane availability of other receptor systems such as the IP receptor.

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Disclosures

None.

REFERENCES

1. Rosenbaum DM, Rasmussen SG, Kobilka BK. The structure and function of G-protein-coupled receptors. *Nature*. 2009;459:356–363. doi: 10.1038/nature08144
2. Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families: phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*. 2003;63:1256–1272. doi: 10.1124/mol.63.6.1256
3. Sriram K, Insel PA. G protein-coupled receptors as targets for approved drugs: how many targets and how many drugs? *Mol Pharmacol*. 2018;93:251–258. doi: 10.1124/mol.117.111062
4. Ngo T, Kufareva I, Coleman JL, Graham RM, Abagyan R, Smith NJ. Identifying ligands at orphan GPCRs: current status using structure-based approaches. *Br J Pharmacol*. 2016;173:2934–2951. doi: 10.1111/bph.13452
5. Brinks HL, Eckhart AD. Regulation of GPCR signaling in hypertension. *Biochim Biophys Acta*. 2010;1802:1268–1275. doi: 10.1016/j.bbdis.2010.01.005
6. Wirth A, Offermanns S. G-protein-coupled receptors in smooth muscle In: *Muscle: Fundamental Biology and Mechanisms of Disease*. New York, NY: Elsevier; 2012:1145–1153.
7. Wilson RJ, Giles H. Piglet saphenous vein contains multiple relaxatory prostanoid receptors: evidence for EP4, EP2, DP and IP receptor subtypes. *Br J Pharmacol*. 2005;144:405–415. doi: 10.1038/sj.bjp.0706088
8. Tanaka Y, Koike K, Toro L. MaxiK channel roles in blood vessel relaxations induced by endothelium-derived relaxing factors and their molecular mechanisms. *J Smooth Muscle Res*. 2004;40:125–153. doi: 10.1540/jsmr.40.125
9. Althoff TF, Offermanns S. G-protein-mediated signaling in vascular smooth muscle cells: implications for vascular disease. *J Mol Med (Berl)*. 2015;93:973–981. doi: 10.1007/s00109-015-1305-z
10. Zucker TP, Bönisch D, Hasse A, Grosser T, Weber AA, Schrör K. Tolerance development to antimitogenic actions of prostacyclin but not of

- prostaglandin E1 in coronary artery smooth muscle cells. *Eur J Pharmacol*. 1998;345:213–220. doi: 10.1016/s0014-2999(98)00022-3
11. Blindt R, Bosserhoff AK, vom Dahl J, Hanrath P, Schrör K, Hohlfeld T, Meyer-Kirchdrath J. Activation of IP and EP(3) receptors alters cAMP-dependent cell migration. *Eur J Pharmacol*. 2002;444:31–37. doi: 10.1016/s0014-2999(02)01607-2
 12. Kaur H, Carvalho J, Looso M, Singh P, Chennupati R, Preussner J, Günther S, Albarrán-Juárez J, Tischner D, Classen S, et al. Single-cell profiling reveals heterogeneity and functional patterning of GPCR expression in the vascular system. *Nat Commun*. 2017;8:15700. doi: 10.1038/ncomms15700
 13. Robbins MJ, Michalovich D, Hill J, Calver AR, Medhurst AD, Gloger I, Sims M, Middlemiss DN, Pangalos MN. Molecular cloning and characterization of two novel retinoic acid-inducible orphan G-protein-coupled receptors (GPCR5B and GPCR5C). *Genomics*. 2000;67:8–18. doi: 10.1006/geno.2000.6226
 14. Sano T, Kim YJ, Oshima E, Shimizu C, Kiyonari H, Abe T, Higashi H, Yamada K, Hirabayashi Y. Comparative characterization of GPCR5B and GPCR5CLacZ knockin mice; behavioral abnormalities in GPCR5B-deficient mice. *Biochem Biophys Res Commun*. 2011;412:460–465. doi: 10.1016/j.bbrc.2011.07.118
 15. Kurabayashi N, Nguyen MD, Sanada K. The G protein-coupled receptor GPCR5B contributes to neurogenesis in the developing mouse neocortex. *Development*. 2013;140:4335–4346. doi: 10.1242/dev.099754
 16. Kim YJ, Sano T, Nabetani T, Asano Y, Hirabayashi Y. GPCR5B activates obesity-associated inflammatory signaling in adipocytes. *Sci Signal*. 2012;5:ra85. doi: 10.1126/scisignal.2003149
 17. Soni A, Amisten S, Rorsman P, Salehi A. GPCR5B a putative glutamate-receptor candidate is negative modulator of insulin secretion. *Biochem Biophys Res Commun*. 2013;441:643–648.
 18. von Samson-Himmelstjerna FA, Freundt G, Nitz JT, Stelter F, Luedde M, Wieland T, Frey N, Hippe HJ. The orphan receptor GPCR5B modulates inflammatory and fibrotic pathways in cardiac fibroblasts and mice hearts. *Biochem Biophys Res Commun*. 2019;514:1198–1203. doi: 10.1016/j.bbrc.2019.05.038
 19. Wirth A, Benyó Z, Lukasova M, Leutgeb B, Wettschurek N, Gorbey S, Orsy P, Horváth B, Maser-Gluth C, Greiner E, et al. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med*. 2008;14:64–68. doi: 10.1038/nm1666
 20. Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci USA*. 1992;89:4471–4475.
 21. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. *Genesis*. 2007;45:593–605. doi: 10.1002/dvg.20335
 22. Manders EMM, Verbeek FJ, Aten JA. Measurement of colocalization of objects in dual-colour confocal images. *J Microsc*. 1993;169:375–382.
 23. Dorsch S, Klotz KN, Engelhardt S, Lohse MJ, Bünemann M. Analysis of receptor oligomerization by FRAP microscopy. *Nat Methods*. 2009;6:225–230. doi: 10.1038/nmeth.1304
 24. Fetalvero KM, Shyu M, Nomikos AP, Chiu YF, Wagner RJ, Powell RJ, Hwa J, Martin KA. The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol Heart Circ Physiol*. 2006;290:H1337–H1346. doi: 10.1152/ajpheart.00936.2005
 25. Bennett MR, Sinha S, Owens GK. Vascular smooth muscle cells in atherosclerosis. *Circ Res*. 2016;118:692–702. doi: 10.1161/CIRCRESAHA.115.306361
 26. Gurevich VV, Gurevich EV. How and why do GPCRs dimerize? *Trends Pharmacol Sci*. 2008;29:234–240. doi: 10.1016/j.tips.2008.02.004
 27. Milligan G. G protein-coupled receptor hetero-dimerization: contribution to pharmacology and function. *Br J Pharmacol*. 2009;158:5–14. doi: 10.1111/j.1476-5381.2009.00169.x
 28. Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron*. 2000;27:97–106. doi: 10.1016/s0896-6273(00)00012-x
 29. Bräuner-Osborne H, Krosgaard-Larsen P. Sequence and expression pattern of a novel human orphan G-protein-coupled receptor, GPCR5B, a family C receptor with a short amino-terminal domain. *Genomics*. 2000;65:121–128. doi: 10.1006/geno.2000.6164
 30. Lagerström MC, Schiöth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov*. 2008;7:339–357. doi: 10.1038/nrd2518
 31. Giguère V, Gallant MA, de Brum-Fernandes AJ, Parent JL. Role of extracellular cysteine residues in dimerization/oligomerization of the human prostacyclin receptor. *Eur J Pharmacol*. 2004;494:11–22. doi: 10.1016/j.ejphar.2004.04.041
 32. Arehart E, Stitham J, Asselbergs FW, Douville K, MacKenzie T, Fetalvero KM, Gleim S, Kasza Z, Rao Y, Martel L, et al. Acceleration of cardiovascular disease by a dysfunctional prostacyclin receptor mutation: potential implications for cyclooxygenase-2 inhibition. *Circ Res*. 2008;102:986–993. doi: 10.1161/CIRCRESAHA.107.165936
 33. Ibrahim S, Tetruashvili M, Frey AJ, Wilson SJ, Stitham J, Hwa J, Smyth EM. Dominant negative actions of human prostacyclin receptor variant through dimerization: implications for cardiovascular disease. *Arterioscler Thromb Vasc Biol*. 2010;30:1802–1809. doi: 10.1161/ATVBAHA.110.208900
 34. Carrillo JJ, López-Giménez JF, Milligan G. Multiple interactions between transmembrane helices generate the oligomeric alpha1b-adrenoceptor. *Mol Pharmacol*. 2004;66:1123–1137. doi: 10.1124/mol.104.001586
 35. Lee SP, O'Dowd BF, Rajaram RD, Nguyen T, George SR. D2 dopamine receptor homodimerization is mediated by multiple sites of interaction, including an intermolecular interaction involving transmembrane domain 4. *Biochemistry*. 2003;42:11023–11031. doi: 10.1021/bi0345539
 36. Frölich JC. Prostacyclin in hypertension. *J Hypertens Suppl*. 1990;8:S73–S78.
 37. Smith WL. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Annu Rev Physiol*. 1986;48:251–262. doi: 10.1146/annurev.ph.48.030186.001343
 38. Baxter GS, Clayton JK, Coleman RA, Marshall K, Sangha R, Senior J. Characterization of the prostanoid receptors mediating constriction and relaxation of human isolated uterine artery. *Br J Pharmacol*. 1995;116:1692–1696. doi: 10.1111/j.1476-5381.1995.tb16393.x
 39. Jones RL, Qian YM, Wise H, Wong HN, Lam WL, Chan HW, Yim AP, Ho JK. Relaxant actions of nonprostanoid prostacyclin mimetics on human pulmonary artery. *J Cardiovasc Pharmacol*. 1997;29:525–535. doi: 10.1097/00005344-199704000-00015
 40. Hofmann F. Smooth muscle tone regulation. In: Offermanns S, Rosenthal W, eds. *Encyclopedic Reference of Molecular Pharmacology*. Heidelberg, Germany: Springer-Verlag; 2004:870–875.
 41. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature*. 1997;388:678–682. doi: 10.1038/41780
 42. Höcherl K, Schmidt C, Kurt B, Bucher M. Activation of the PGI(2)/IP system contributes to the development of circulatory failure in a rat model of endotoxic shock. *Hypertension*. 2008;52:330–335. doi: 10.1161/HYPERTENSIONAHA.108.112029
 43. Guo Y, Tukay DN, Wu WJ, Zhu X, Book M, Tan W, Jones SP, Rokosh G, Narumiya S, Li Q, et al. The COX-2/PGI2 receptor axis plays an obligatory role in mediating the cardioprotection conferred by the late phase of ischemic preconditioning. *PLoS One*. 2012;7:e41178. doi: 10.1371/journal.pone.0041178
 44. Villa E, Morales ME, Martínez J, de la Fuente A, Hurtado A, Santirso RA, Ruilope LM, Sancho JM, García-Robles R. Acute effects of iloprost on blood pressure, heart rate, renal hemodynamics, diuresis, natriuresis, plasma renin activity and plasma norepinephrine in uninephrectomized hypertensive mongrel dogs. *J Hypertens Suppl*. 1991;9:S352–S353.
 45. Fitzgerald DJ, Entman SS, Mulloy K, Fitzgerald GA. Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. *Circulation*. 1987;75:956–963. doi: 10.1161/01.cir.75.5.956
 46. Yokoyama C, Yabuki T, Shimomishi M, Wada M, Hatae T, Ohkawara S, Takeda J, Kinoshita T, Okabe M, Tanabe T. Prostacyclin-deficient mice develop ischemic renal disorders, including nephrosclerosis and renal infarction. *Circulation*. 2002;106:2397–2403. doi: 10.1161/01.cir.0000034733.93020.bc
 47. Davis TL, Sharif NA. Pharmacological characterization of ([3H]-prostaglandin E2) binding to the cloned human EP(4) prostanoid receptor. *Br J Pharmacol*. 2000;130:1919–1926. doi: 10.1038/sj.bjp.0703525
 48. Davis RJ, Murdoch CE, Ali M, Purbrick S, Ravid R, Baxter GS, Tilford N, Sheldrick RL, Clark KL, Coleman RA. EP4 prostanoid receptor-mediated vasodilatation of human middle cerebral arteries. *Br J Pharmacol*. 2004;141:580–585. doi: 10.1038/sj.bjp.0705645
 49. Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA. Prostanoids in health and disease. *J Lipid Res*. 2009;50(suppl):S423–S428. doi: 10.1194/jlr.R800094-JLR200
 50. Albarrán-Juárez J, Kaur H, Grimm M, Offermanns S, Wettschurek N. Lineage tracing of cells involved in atherosclerosis. *Atherosclerosis*. 2016;251:445–453. doi: 10.1016/j.atherosclerosis.2016.06.012