LRP1-Dependent Endocytic Mechanism Governs the Signaling Output of the Bmp System in Endothelial Cells and in Angiogenesis

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Rationale: Among the extracellular modulators of Bmp (bone morphogenetic protein) signaling, Bmper (Bmp endothelial cell precursor-derived regulator) both enhances and inhibits Bmp signaling. Recently we found that Bmper modulates Bmp4 activity via a concentration-dependent, endocytic trap-and–sink mechanism.

Objective: To investigate the molecular mechanisms required for endocytosis of the Bmper/Bmp4 and signaling complex and determine the mechanism of Bmper’s differential effects on Bmp4 signaling.

Methods and Results: Using an array of biochemical and cell biology techniques, we report that LRP1 (LDL receptor-related protein 1), a member of the LDL receptor family, acts as an endocytic receptor for Bmper and a coreceptor of Bmp4 to mediate the endocytosis of the Bmper/Bmp4 signaling complex. Furthermore, we demonstrate that LRP1-dependent Bmper/Bmp4 endocytosis is essential for Bmp4 signaling, as evidenced by the phenotype of lrp1-deficient zebrafish, which have abnormal cardiovascular development and decreased Smad1/5/8 activity in key vasculogenic structures.

Conclusions: Together, these data reveal a novel role for LRP1 in the regulation of Bmp4 signaling by regulating receptor complex endocytosis. In addition, these data introduce LRP1 as a critical regulator of vascular development. These observations demonstrate Bmper’s ability to fine-tune Bmp4 signaling at the single-cell level, unlike the spatial regulatory mechanisms applied by other Bmp modulators. (Circ Res. 2012;111:564-574.)

Key Words: bone morphogenetic protein ■ low density lipoprotein receptor-related protein 1 ■ endocytosis ■ angiogenesis ■ endothelial cell

Bone morphogenetic proteins (Bmps) are essential for embryonic vascular development, as illustrated by many severe, inherited vascular diseases associated with disrupted Bmp signaling.1 One facet of regulation of Bmp signaling is provided by extracellular molecules that “fine-tune” Bmp signaling by modulating (usually inhibiting) Bmp’s interaction with surface receptors and subsequent downstream signaling events.2 Of these extracellular modulators of Bmp signaling, Bmper (Bmp-binding endothelial cell precursor-derived regulator, the vertebrate ortholog for crossveinless-2 in Drosophila) is a critical determinant of endothelial functions such as differentiation, migration, and angiogenesis.3–6 Bmper can both promote and inhibit Bmp activity,6,7 confounding the development of a working model to explain how Bmper impacts Bmp signaling. However, we recently found that Bmper modulates Bmp4 activity via a concentration-dependent, endocytic trap-and–sink mechanism, with low levels of Bmper promoting and high levels inhibiting Bmp4 signaling, thereby accounting for the biphasic nature of Bmper’s regulation of Bmp4 activity.7 Our previous data suggested that endocytosis of the Bmper/Bmp4 complex may be critical in determining the inhibitory effect of Bmper on Bmp4 function. However, gaps remained in understanding how activation and inhibition of Bmp4 signaling by Bmper were coupled and the mechanism by which the Bmper/Bmp4 complex was endocytosed.

Here we report that LRP1 (LDL receptor-related protein 1) is a novel endocytic receptor for Bmper and a coreceptor of...
Bmp4 that is essential for mediating Bmp4 signaling. A requisite role for LRPI in zebrafish angiogenesis is observed in that knockdown of lrp1 decreases Smad1/5/8 activity and abnormal cardiovascular development. Together, these data demonstrate that LRPI regulates Bmp4-mediated endothelial function and vascular development in vivo and is therefore a bone fide component of the Bmp signaling pathway.

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Generation of Cell Lines

For stable mouse endothelial cell (MEC) cell line construction, MECs were transduced with LRPI or control shRNA lentiviral particles, and positive colonies were screened with puromycin and evaluated by Western blot analysis.

Chemical Cross-Linking in Intact Cells, Immunoblotting, Immunoprecipitation, and Ligand Blotting

Bmp4-treated MECs were crosslinked with dithiobis(succinimidyl-propionate) for immunoprecipitation and matrix-assisted laser desorption/ionization-time of flight analysis. Immunoblotting, immunoprecipitation, and ligand blotting were performed following our previous protocols.7,8

Fluorescence Energy Transfer Experiments

Experiments were performed following a previously published protocol.9 Donor, acceptor, and fluorescence energy transfer images were acquired sequentially using fixed excitation and emission filters, and image processing was performed.

In Vitro Matrigel Tubulogenesis Assay

Endothelial cell tube formation was analyzed with the Matrigel-based tube formation assay.10

Morpholino Injections and Zebrafish Analysis

Morpholino oligonucleotides (MOs) were produced by Gene Tools (Philomath, OR). All MOs were injected into 1 to 4 cell stage embryos as previously described.11 Zebrafish (Danio rerio) vascular development was analyzed following previously published methods.12

Please see the expanded Methods section, available in the online-only Data Supplement for details regarding reagents, cell culture, immunoblotting, ligand blotting, immunofluorescence, and statistical analysis.

Results

LRPI Associates With Bmp4 in MECs

Previously we demonstrated that endocytosis of the Bmp4 precursor is critical for Bmp4-mediated Bmp4 signaling.7 However, the mechanism governing this endocytosis remained unclear. Hypothesizing that Bmp4 may interact with a partner to achieve endocytosis, we used matrix-assisted laser desorption/ionization-time of flight mass spectrometry as an inductive unbiased method of identifying Bmp4-associated proteins in MECs. LRPI, a well-defined endocytic receptor, associated with Bmp4 in MECs (Figure IA; Online Figure IA and Online Table I). Confocal imaging of Bmp4-treated MECs revealed that Bmp4 and LRPI colocalize on the plasma membrane and in intracellular vesicles (Figure IB). Immunoprecipitating for LRPI and immunoblotting for Bmp4 (or the reverse, or Bmp4 ELISA) demonstrated that full-length Bmp4 associates with LRPI in MEC lysates (Figure 1C; Online Figure IB and IC), confirming our matrix-assisted laser desorption/ionization-time of flight and immunofluorescence data. Notably, this association was not affected by the addition of Bmp4 (Online Figure ID and IE). Because Bmp4 may bind Bmp4 through its amino-terminal domain,7,13 its inability to disrupt the LRPI/Bmp4 association suggested that LRPI may bind a different domain of Bmp4 and carboxyl-terminal domain (CTD) fragments of Bmp4 associated with LRPI (Figure 1C; Online Figure ID), indicating that Bmp4 may form a ternary complex with LRPI and Bmp4 through respective associations with its CTD and amino-terminal domain. Consistent with this hypothesis, lysates of Bmp4-treated MECs immunoprecipitated with an anti-LRPI antibody revealed the presence of LRPI/Bmp4 heterocomplexes (Online Figure IF).

Like other members of the LDL receptor family, LRPI is a heterodimer composed of a 515-kDa α chain possessing 4 extracellular ligand binding domains (LBDs) and an 85-kDa membrane-anchored β chain.14 Ligand blotting analysis with full-length LRPI revealed that Bmp4 associates with both the α and β chains of LRPI (Figure 1D). To determine which LBD of LRPI is required for binding to Bmp4, cells were transfected with membrane-containing mini-LRPI receptors (mLRPI-4,5; Figure 1E, left panel) and analyzed via ligand blotting. LBDIII/IV exhibited the strongest association with Bmp4, whereas LBDII did only have a weak association and LBDI did not associate with Bmp4 at all (Figure 1E, right top panel), consistent with what is found with other LRPI ligands such as RAP1.15 However, Bmp4 also binds to the β chain of LRPI, making LRPI unique among other known LRPI ligands. The significance of Bmp4 binding to LRPI’s β chain remains unknown; however, internalized CTD fragments of Bmp4 may bind LRPI and modulate its association with scaffold proteins that participate in LRPI-dependent signaling mechanisms.16 Together, these data identify that LRPI, a recognized endocytic receptor, associates with Bmp4 in MECs and therefore could be responsible for endocytosis of Bmp4/Bmp4 complexes.

LRPI Is Required for Physiological Bmp4 Internalization

Previously, we found that Bmp4 undergoes endocytosis via an unknown mechanism that modulates downstream Bmp4.
Given our observation of a direct interaction between Bmp and the endocytic receptor-LRP1, we hypothesized that LRP1 may act as an endocytic receptor for Bmp and examined cell lysates from LRP1-knockdown (or control) MECs treated with Bmp. The level of internalized Bmp in control MECs increased over time, peaking at 60 minutes and subsequently decreased, probably due to degradative processing, as previously demonstrated (Figure 2A and 2B). LRP1-knockdown MECs also demonstrated a time-dependent increase in Bmp internalization; however, internalization was substantially less than in control MECs (Figure 2B). When cells were treated with increasing doses of Bmp, the presence of cytoplasmic Bmp in control MECs increased over time, peaking at 60 minutes and subsequently decreased, probably due to degradative processing, as previously demonstrated (Figure 2A and 2B). LRP1-knockdown MECs also demonstrated a time-dependent increase in Bmp internalization; however, internalization was substantially less than in control MECs (Figure 2B). When cells were treated with increasing doses of Bmp, the presence of cytoplasmic Bmp in control MECs increased, an effect that was significantly decreased in LRP1-knockdown cells (Figure 2C and 2D). Similar results were observed in mouse embryonic fibroblasts (Online Figure IIA–IID). These data suggest that the majority, but not all, of Bmp is via an LRP1-dependent mechanism and we further explored the role that LRP1 plays in Bmp/Bmp4 endocytosis.

We used confocal microscopy to investigate whether LRP1 is involved in the previously reported internalization and transport of Bmp to endosomes. Following Bmp internalization, both LRP1 and Bmp were detected in a subset of vesicles expressing EEA-1 and Rab-7 (endosome markers; Online Figure IIE and IIF). LRP1-knockdown MECs contained far fewer Bmp-containing EEAs-1 and Rab-7 positive endosomal vesicles compared to control MECs (Figure 2E and data not shown). Similar results were found in LRP1 null mouse embryonic fibroblasts (Online Figure IIG). Further subcellular fractionation experiments confirmed this localization (Online Figure IIH). These data collectively indicate that LRP1 influences the endosomal localization of Bmp and acts as an endocytic receptor for Bmp. We have previously shown that Bmp is internalized in a complex together with Bmp4 and Bmp receptors and that endocytosis of this holocomplex is critical for modulating Bmp4 signaling. Therefore, we next investigated whether the interaction between LRP1 and Bmp receptors could influence Bmp4-dependent Smad1/5/8 signaling in endothelial cells.

LRP1 Is Associated With Bmp Receptor Type IB/Activin-Like Kinase Receptor 6

LRP1 functions as a coreceptor for ligands such as transforming growth factor-β and NMDA. Given our data demonstrating that LRP1 is an endocytic receptor for Bmp, we hypothesized that LRP1 may act as a coreceptor of Bmp receptors. There are multiple Bmps (Bmp2, 4, and 6) and Bmp type I receptors (activin-like kinase receptor [ALK]1, 2, 3, and 6) in MECs and specific siRNA knockdown of ALK2,
3, and 6 (but not ALK1) inhibited Bmp4-induced Smad1/5/8 phosphorylation (Online Figure IIIA–IIIC). Given that our previous data identified ALK6 as a Bmp4 type I receptor that mediates Bmp-dependent endothelial migration and angiogenesis,10 we used ALK6 as a representative Bmp type I receptor in the following experiments. Fluorescence energy transfer imaging analysis demonstrated the close proximity of cyan fluorescent protein-tagged ALK6 (Figure 3A) and yellow fluorescent protein-tagged mLRP2 in cytoplasmic vesicles and plasma membranes of transfected cells (Figure 3B), indicating that LRP1 and ALK6 could physically interact in cells. Coimmunoprecipitation of lysates from cells transfected with a wild-type ALK6 plasmid (ALK6-WT; Figure 3A) and each mLRP construct (Figure 1E) confirmed this association (Figure 3C). Additionally, when cells were transfected with a LRP1/H9252 construct (Figure 3A), the LRP1 and ALK6 association persisted (Figure 3D), indicating that this association likely occurs through the LRP1/H9252 chain. Because Bmper could bind to this region of LRP1 (Figure 1D), this interesting observation suggests that both Bmper and Bmps influence LRP1 signaling, thereby initiating cross-talk between the LRP1 and Bmp signaling pathways.

To determine the domains of ALK6 involved in the interaction with LRP1, ALK6 deletion mutants were constructed (Figure 3A). Both ALK6-CTD and ALK6-protein kinase domain coimmunoprecipitated with mLRP2, indicating that the cytoplasmic region of ALK6 is required for the association of ALK6 with mLRP2 (Figure 3E). However, the association of mLRP2 with ALK6-CTD and protein kinase domain was decreased in comparison to mLRP2 binding to ALK6-WT, suggesting that CTD of ALK6 is required but not sufficient for the association with mLRP2. Similar results were observed with the interaction of ALK6 and mLRP4 (Online Figure IIID). Collectively, we conclude that the intracellular domains and possible membrane region of ALK6 are required for the association with LRP1. Because the protein kinase domain of ALK6 is critical for downstream Bmp-mediated Smad1/5/8 phosphorylation,19 next we tested whether the ALK6 and LRP1 interaction was involved in Bmp4-regulated Smad1/5/8 signaling induced by Bmper.

**Endocytosis of the Bmper/Bmp4 Complex Is Required for Both Stimulatory and Inhibitory Regulation of Bmp4 Signaling by Bmper**

We previously demonstrated that endocytosis of the Bmper/Bmp4 complex is required for the inhibitory effect of Bmper on Bmp4 signaling,7 but whether endocytosis of the Bmper/Bmp4 complex is also required more generally for Bmper-dependent Bmp4 signaling regulation remained unclear. Therefore, we used 3 endocytosis inhibitors—chlorpromazine, chloroquine (CQ), and bafilomycin A1—in MECs and evaluated the subsequent effect on Bmp4-dependent phosphorylation of Smad1/5/8. Treatment of MECs with Bmp4 alone or in the presence of substoichiometric concentrations of Bmper increased Smad1/5/8 phosphorylation (Figure 4A, lanes 2, 4, 12, and 14; Online Figure IVA), consistent with our previous observations that substoichiometric ratios of Bmper to Bmp4 enhances Bmper-dependent Bmp4 signaling.7 However, when cells were pretreated with chlorpromazine to prevent clathrin-mediated endocytosis, the level of Smad1/5/8 phosphorylation induced by either Bmp4 alone or
Bmp4 plus Bmper in substoichiometric concentrations was substantially decreased (Figure 4A, lanes 7 and 9; Online Figure IVB), indicating that Bmper-mediated enhancement of Bmp4 signaling requires endocytosis of the Bmper/Bmp4 complex. Next we investigated whether Rab4-mediated rapid recycling is required for Bmper’s promoting effect on Smad1/5/8 phosphorylation. Rab4 siRNA knockdown resulted in a significant decrease in Smad1/5/8 phosphorylation induced by Bmp4 alone or Bmp4 plus Bmper in substoichiometric concentrations (Figure 4B, Online Figure IVC). This suggests that the Rab4-mediated rapid recycling route is required for both Bmp4 activity and the promoting effect of Bmper at stoichiometric concentrations. This observation, combined with our previous data demonstrating that endocytosis is also necessary for the inhibition of Bmp4 signaling caused by suprastoichiometric concentrations of Bmper in the Bmper/Bmp4 complex,7 indicates that endocytosis is required for all aspects of Bmper-mediated regulation of Bmp4 signaling.

We next examined the effect that CQ and bafilomycin A1 had on Bmper-mediated Bmp4 signaling. Treatment of cells with CQ did not affect the ability of Bmp4 alone or Bmp4 plus Bmper in substoichiometric concentrations to induce Bmp4-mediated Smad1/5/8 phosphorylation (Figure 4A, lanes 17 and 19; Online Figure IVD). However, CQ treatment relieved the inhibitory effect of Bmp4 in the presence of suprastoichiometric concentrations of Bmper, resulting in augmented Smad1/5/8 phosphorylation (Figure 4A, compare lanes 15 and 20). A similar effect on Smad1/5/8 phosphorylation was obtained with bafilomycin A1 pretreatment (Online Figure IVE). Because CQ and bafilomycin A1 prevent endosomal acidification and inhibit endosome fusion and lysosomal degradation, our data suggest that the inhibition of Bmp signaling by suprastoichiometric concentrations of Bmper involves the lysosomal degradation of the Bmper/Bmp4/Bmp receptor (BMPR) complex, and not simply endocytosis of the signaling complex as previously thought.7 Collectively, these data demonstrate that endocytosis is a crucial process linked to Bmper’s ability to both activate and inhibit Bmp4 signaling. In addition, the inhibitory effect of suprastoichiometric concentrations of Bmper on Bmp4 signaling may involve the lysosomal degradation of the Bmper/Bmp4 signaling complex.

LRP1 Is Required for Bmper/Bmp4-Dependent Signaling

We next examined the role that LRP1 plays in Bmper-dependent Bmp4 signaling. Following treatment with Bmp4 and Bmper, Smad1/5/8 phosphorylation was evaluated in LRP1-knockdown MECs. Not surprisingly, LRP1-knockdown inhibited Bmper and Bmp4 internalization in these cells (Figure 4C) and decreased Smad1/5/8 phosphorylation induced by Bmp4 alone and by Bmp4 plus Bmper in substoichiometric concentrations (Figure 4C, lanes 7, 9; Online Figure IVF), supporting that endocytosis is required for Bmper-dependent enhancement of Bmp4 signaling. Interestingly, LRP1-knockdown in MECs...
LRP1 competes with BMPRII for association with ALK6. In (Figure 4D, comparing lane 6 to lane 1), suggesting that the dance of ALK6/BMPRII heterodimers in the basal state decreased LRP1 protein level resulted in a greater abundance was similar to that seen under basal conditions (Figure 4D, lane 5). Surprisingly, in LRP1-knockdown MECs, the inhibitory effect of suprastoichiometric concentrations of Bmper (Figure 4D, lanes 7–10, compared to lane 6). These data demonstrate the pivotal role that LRP1 plays in Bmper-mediated Bmp4 signaling. Lysates of MECs treated with Bmp4 and LRP1 (15 min) were analyzed by Western blotting. C, LRP1 is required for Bmp4/Bmp internalization and Bmpr-dependent Bmp downstream signaling. Lysates of MECs transfected with control (Ctrl) or Rab4 siRNA and treated with Bmp4 and Bmper (15 min) were analyzed by Western blotting. D, Lysates of MECs treated with Bmp4 and Bmper for 15 min were immunoprecipitated with the mouse anti-LRP1 antibody and analyzed by Western blotting. E, The association of LRP1 and ALK6 is regulated on the different treatment of Bmp4 and Bmper. Lysates of MECs following Bmp4 and Bmper treatments for 15 min were immunoprecipitated with the mouse anti-LRP1β antibody and analyzed by Western blotting.

To activate Bmp signaling, BMPRI/II heterodimers must form before Smad1/5/8 phosphorylation occurs. Having established that LRP1 is required for Bmper-dependent regulation of Bmp4 signaling, we tested the involvement of LRP1 in the regulation of ALK6/BMPRII heterodimerization. ALK6/BMPRII heterodimers were detected in control MECs under basal conditions (Figure 4D, lane 1) with the level increasing steadily with the addition of Bmp4, or Bmp4 plus substoichiometric concentrations of Bmper (Figure 4D, lanes 2 and 4; Online Figure IVG). In contrast, when cells were treated with Bmp4 alone or suprastoichiometric ratios of Bmper to Bmp4, the level of ALK6/BMPRII heterodimerization was similar to that seen under basal conditions (Figure 4D, lane 5). Surprisingly, in LRP1-knockdown MECs, the decrease of LRP1 protein level resulted in a greater abundance of ALK6/BMPRII heterodimers in the basal state (Figure 4D, comparing lane 6 to lane 1), suggesting that LRP1 competes with BMPRII for association with ALK6. In contrast, when LRP1-knockdown MECs were treated with Bmp4, Bmper, or Bmp4 and both low and high concentrations of Bmper, ALK6/BMPRII heterodimers were decreased in abundance (Figure 4D, lanes 7–10, compared to lane 6). These data suggest that LRP1 regulates the interaction of ALK6 with BMPRII under both basal and stimulated conditions—blocking the association of the receptors in the absence of Bmp4 or Bmper and promoting receptor interaction once stimulation with Bmp4 and/or Bmper at substoichiometric concentrations occurs. Other Bmp type II receptors (eg, activin receptor type II) showed different behavior in response to LRP1 (Online Figure IVH). In addition, the binding of ALK6 and BMPRII was similar but not the same in mouse embryonic fibroblasts (Online Figure IVI). Because exogenous mLrps and ALK6 were associated in HEK293 cells (Figure 3), we wanted to know whether they form a complex in MECs and, if so, how Bmper/Bmp4 affect this association. Immunoprecipitation assays using MEC lysates confirmed that ALK6 and LRP1 were associated in MECs. Whereas their interaction was inhibited by Bmp4, it was increased by treatment with Bmper alone, or Bmp4 and Bmper at both sub- and suprastoichiometric concentrations (Figure 4E, Online Figure IVJ), suggesting that the LRP1 and ALK6 association is dynamically regulated by Bmp4 and Bmper, which may explain the differential effects of Bmper at sub- or suprastoichiometric concentrations on Smad1/5/8 activation.

**Figure 4.** LDL receptor-related protein 1 (LRP1)-mediated endocytosis is required for the bone morphogenetic protein endothelial cell precursor-derived regulator (Bmper)-dependent regulation of bone morphogenetic protein (Bmp)4 downstream signaling. A, Endocytosis is required for both promoting and inhibiting Bmp function of Bmper. Mouse endothelial cells (MECs) were pretreated with chlorpromazine (CPM) or chloroquine (CQ) and then treated with Bmp4 (0.6 nmol/L) and Bmper for 15 min (chlorpromazine [CPM]) or 120 min (CQ) at 10 nmol/L (Bmper alone); 0.3 nmol/L (BB-sub [substoichiometric Bmper]; 10 nmol/L (BB-supra [Bmp suprastoichiometric]). Cell lysates were analyzed by Western blotting. B, Rab4 is required for Smad1/5/8 phosphorylation induced by Bmp4 and substoichiometric Bmper. Lysates of MECs transfected with control (Ctrl) or Rab4 siRNA and treated with Bmp4 and Bmper (15 min) were analyzed by Western blotting. C, LRP1 is required for Bmp4/Bmp internalization and Bmpr-dependent Bmp downstream signaling. Lysates of LRP1-knockdown or control MECs treated with Bmp4 and Bmper (30 min) were analyzed by Western blotting. D, Lysates of MECs treated with Bmp4 and Bmper for 15 min were immunoprecipitated with an anti-Bmp receptor (BMPRII) antibody and analyzed by Western blotting. E, The association of ALK6/BMPRII heterodimers was similar but not the same in mouse embryonic fibroblasts (Online Figure IVI). Because exogenous mLrps and ALK6 were associated in HEK293 cells (Figure 3), we wanted to know whether they form a complex in MECs and, if so, how Bmper/Bmp4 affect this association. Immunoprecipitation assays using MEC lysates confirmed that ALK6 and LRP1 were associated in MECs. Whereas their interaction was inhibited by Bmp4, it was increased by treatment with Bmper alone, or Bmp4 and Bmper at both sub- and suprastoichiometric concentrations (Figure 4E, Online Figure IVJ), suggesting that the LRP1 and ALK6 association is dynamically regulated by Bmp4 and Bmper, which may explain the differential effects of Bmper at sub- or suprastoichiometric concentrations on Smad1/5/8 activation.
LRP1 Regulates Bmpr/Bmp4-Dependent Endothelial Migration and Angiogenesis

Our data demonstrating that LRP1 is required for the BMP-mediated Bmp4 signaling module suggests that LRP1 could influence physiological outcomes of BMP-mediated Bmp4 signaling such as endothelial migration and angiogenesis. In Boyden chamber migration assays, both Bmp4 and Bmper enhanced endothelial migration in MECs whereas a BMP-neutralizing antibody blocked Bmp4-induced cell migration (Online Figure IVK and IVL), consistent with previous reports.2,21 However, LRP1 knockdown in MECs completely inhibited migration induced by Bmp4, Bmper alone, or Bmp4 plus a substoichiometric concentration of Bmper. Furthermore, LRP1 knockdown in MECs relieved the inhibition on cell migration caused by the combined treatment of Bmp4 plus suprastoichiometric concentrations of Bmper, consistent with our finding that the inhibition of Smad1/5/8 phosphorylation induced by high concentrations of Bmp4 was also relieved in the absence of LRP1 (Figure 4C). To study the role of LRP1 in angiogenesis, we performed an in vitro Matrigel tubulogenesis assay. Similar to the effects on endothelial migration, LRP1 knockdown in MECs blocked tube formation induced by Bmp4, Bmper, or the cotreatment of Bmp4 plus Bmper at the substoichiometric concentrations (Figure 4F). However, LRP1-knockdown MECs demonstrated increased tube formation on the treatment of Bmp4 and Bmper at suprastoichiometric concentrations (Figure 4F). These data establish that the biochemical model we constructed through in vitro analysis holds true in a physiologically relevant cellular setting, demonstrating that LRP1 is a critical determinant of BMP-mediated Bmp4 signaling events.

LRP1 Is Necessary for Cardiovascular Development in Zebrafish

The fact that Bmp4/Bmp signaling pathways are essential for vascular development in zebrafish,3,12 along with our observations of a clear reliance of Bmp-mediated Bmp4 signaling on LRP1, prompted us to test whether LRP1 may also play an important role in Bmp4-dependent cardiovascular development. The spatiotemporal expression of lrp1 during zebrafish embryonic development was examined. Weak lrp1a expression was observed at 12 hours postfertilization (hpf), whereas a stronger, symmetrical expression signal could be detected at the lateral dorsal aorta at 24 hpf (Online Figure VA). Interestingly, the expression pattern of lrp1a closely paralleled that of bmp,5 in that lrp1a was expressed in structures that have Bmp and vasculogenic activity, such as lateral dorsal aorta and dorsal longitudinal anastomotic vessel.

To determine the importance of lrp1a in vasculogenesis, we used lrp1a-specific MOs to knock down lrp1a during zebrafish embryonic development. lrp1a knockdown efficiently decreased embryonic levels of lrp1a RNA as determined by RT-PCR (Online Figure VB) and resulted in an abnormal vascular phenotype, illustrated by delayed dorsal and intersegmental vessel formation, fewer vascular branches within the caudal vein plexus, and a large swollen vascular lumen with ectopically-placed Kdr7 cells (Figure 5B), which have also been described for bmper morphants.5 Additionally, lrp1a morphants demonstrated disrupted blood flow and a slower or stopped heart beat (dsRed images in Online Figure VC and Table III). Increased doses of lrp1a MOs resulted in a higher percentage of affected embryos (increasing from 75%–100%) at 24 hpf (Online Table II). This dose-dependent effect of the lrp1a MO was specific to the knockdown of lrp1a RNA and not due to activation of the p53-dependent cell death pathway22 (Online Figure VC–VF, Tables II–III and Movies I–III). Knockdown of the second lrp1 gene (lrp1b, ENSDART00000088208, chr. 23), either alone or with lrp1a resulted in a similar vascular phenotype, suggesting that the lrp1 genes possess redundant functions (Online Figure VG).

Next, we investigated whether the vascular defect of lrp1 morphant fish is cell-autonomous by performing cell transplantation assays. Control or lrp1 MO-injected donor cells were transplanted into wild-type recipient embryos. We observed that both control and lrp1 MO-injected cells contributed to blood, endothelial structures (dorsal aorta, cardinal vein, caudal vein plexus, and intersegmental vessel) and other structures (somite, notochord, etc.) similarly (data not shown), suggesting that lrp1 MO did not affect cell differentiation during development. lrp1 MO-injected cells were excluded from the tip cell position within venous network located in the caudal vein plexus and participated in fewer ventral sprouting events (Figure 5C and 5D, Online Figure VH). However, there was no obvious defect in the injected cell’s ability to contribute to the tip cell within the intersegmental vessel, which is predominantly arterial in nature at this developmental stage. Bmp signaling was recently reported to regulate the ventral sprouting from the axial vein.12 This finding, together with our data, suggests that LRP1, similar to Bmp, regulates vein development in a cell-autonomous fashion. The cardiovascular defects observed in LRPI morphant fish might be due to nonautonomous effects of LRPI and reflect the autonomous requirement of LRPI in venous endothelial function.

Bmpr4 knockdown in zebrafish leads to diminished levels of Smad1/5/8 phosphorylation and a dorsaled phenotype consisting of defects in hematopoiesis and vascular patterning, reflecting the role that Bmp-mediated Bmp signaling plays during embryo gastrulation and vascular development.23,5 Because the pattern of lrp1a in the developing zebrafish embryo mirrors that of bmper, we examined the effect of lrp1a knockdown on Bmp-mediated Bmp signaling in lrp1a MO-injected Tg(kdrl:EGFP) embryos using immunohistochemical localization of Smad1/5/8 phosphorylation. In wild-type fish, LRPI expression was localized to the dorsal aorta, caudal vein, and caudal artery, whereas the expression of LRPI protein was significantly reduced in lrp1a morphant fish (Figure 5E). In wild-type fish, the signal for phosphorylated Smad1/5/8 was mainly localized to the dorsal longitudinal anastomotic vessel, dorsal aorta, caudal artery, and some at intersegmental vessel and caudal vein (Figure 5F). In contrast, lrp1a knockdown decreased Smad1/5/8 phosphorylation in these regions (Figure 5F), indicating that LRPI, similar to Bmp, is required for Bmp-dependent events in vascular development.
The data presented in this report is a continuation of our previous work designed to elucidate the molecular mechanisms involved in Bmp4 regulation of Bmp signaling. Here we present data supporting a model in which LRP1 acts as an endocytic receptor for Bmper, facilitating the formation and internalization of the Bmp4/BMPR signaling complex. Receptor endocytosis plays a critical role not only in the control of receptor protein levels at the cell surface and in the regulation of signaling pathways.24 Similar to the case of endocytosis of transforming growth factor-β1 and epidermal growth factor,24 we believe that, in the case of Bmp4 signaling, clathrin-coated pits and early endosomes are signaling compartments, whereas late endosomes and lysosomes are sites where signaling is eventually blocked. Furthermore, our data support a model in which the magnitude and rate of LRP1-dependent endocytosis and the association of LRP1 and Bmp receptors, regulated by the Bmper:Bmp4 stoichiometric ratio, are critical factors determining the endocytic route of the Bmp4 signaling complex.

Previous studies have demonstrated that regulators of Bmp signaling modulate Bmp signaling using a spatial gradient effect that covers the distance of many cells.25 In contrast, our data suggest that the mechanism for Bmper's regulation of Bmp4 signaling operates at the single cell level and involves a negative feedback loop within the same cell. For example, when Bmp4 is released it is bound by extracellular Bmper that initially issubstoichiometric. Bmp4 together with Bmper binds to BMPRs and is subsequently endocytosed and recycled via an LRP1-dependent mechanism, which promotes the activation of Bmp4 signaling. Bmp signaling results in a plethora of cellular responses, including upregulation of Bmper expression.26 As Bmp4 signaling continues, more Bmper is released into the extracellular environment until the intercellular concentration of Bmper eventually exceeds that of Bmp4. When this happens, the endocytosed Bmper/Bmp4/ALK6 complex is routed to lysosomes where it is degraded, thereby resulting in inhibition of Bmp4 signaling. In this way, each cell involved in the Bmp4 signaling event responds in a tightly controlled manner.

Discussion

The data presented in this report is a continuation of our previous work designed to elucidate the molecular mechanisms involved in Bmp regulation of Bmp signaling. Here we present data supporting a model in which LRP1 acts as an endocytic receptor for Bmper, facilitating the formation and internalization of the Bmp4/BMPR signaling complex. Receptor endocytosis plays a critical role not only in the control of receptor protein levels at the cell surface and in the regulation of signaling pathways.24 Similar to the case of endocytosis of transforming growth factor-β and epidermal growth factor,24 we believe that, in the case of Bmp4 signaling, clathrin-coated pits and early endosomes are signaling compartments, whereas late endosomes and lysosomes are sites where signaling is eventually blocked. Furthermore, our data support a model in which the magnitude and rate of LRP1-dependent endocytosis and the association of LRP1 and Bmp receptors, regulated by the Bmper:Bmp4 stoichiometric ratio, are critical factors determining the endocytic route of the Bmp4 signaling complex.

Previous studies have demonstrated that regulators of Bmp signaling modulate Bmp signaling using a spatial gradient effect that covers the distance of many cells.25 In contrast, our data suggest that the mechanism for Bmper’s regulation of Bmp4 signaling operates at the single cell level and involves a negative feedback loop within the same cell. For example, when Bmp4 is released it is bound by extracellular Bmper that initially is substoichiometric. Bmp4 together with Bmper binds to BMPRs and is subsequently endocytosed and recycled via an LRP1-dependent mechanism, which promotes the activation of Bmp4 signaling. Bmp signaling results in a plethora of cellular responses, including upregulation of Bmper expression.26 As Bmp4 signaling continues, more Bmper is released into the extracellular environment until the intercellular concentration of Bmper eventually exceeds that of Bmp4. When this happens, the endocytosed Bmper/Bmp4/ALK6 complex is routed to lysosomes where it is degraded, thereby resulting in inhibition of Bmp4 signaling. In this way, each cell involved in the Bmp4 signaling event responds in a tightly controlled manner.
Although the focus of this report has been the effect of LRP1 on Bmp4 signaling, it is entirely possible that Bmp4/Bmp may also have an effect on LRP1 signaling. More than 40 ligands have been identified for LRP1, encompassing multiple cellular functions such as the regulation of lipid metabolism, cell migration, blood-brain barrier integrity, and neuronal homeostasis.14 Our observation that both Bmp4 and ALK6 bind to the β chain of LRP1, which is responsible for ligand uncoupling,14 raises the interesting possibility that Bmp4 and Bmps may influence the signaling mechanisms carried out by LRP1. This is an intriguing thought, especially given the evidence that LRP1 regulates atherosclerosis via modulation of PDGF and transforming growth factor-β receptor functions.14 A potential interaction or competition between previously identified LRP1 signaling pathways and Bmp-mediated pathways remains a topic of future research.

We have demonstrated that LRP1, through its effect on Bmp signaling, is essential for cardiovascular development in zebrafish. The expression pattern of lrp1 in developing embryos is remarkable for several reasons. lrp1 is expressed in regions known for their high Bmp/Bmp activity and areas of known vasculogenesis.5 This pattern places lrp1 at a time and location in which it could interact with Bmp4 and therefore mediate Bmp/Bmp signaling. This observation is confirmed by the similar phenotype of lrp1- and bmper-deficient fish. Knockdown of either lrp1 or bmper leads to a similar abnormality in vascular development, such as the compromised caudal vein plexus and aberrant intersegmental vessels. However, bmper morphants also exhibit a reduced number of gata1 expressing hematopoietic precursor cells and circulating blood cells.5 Whereas these cell types were distributed in a disorganized pattern in lrp1 morphants, the actual cell number remained similar to wild-type embryos. The subtle differences in the expression pattern and knockdown phenotype of LRP1, compared to Bmp4, could be attributed to the activation of LRP1 by its other ligands.

Collectively, our data suggest that LRP1 plays a requisite role in Bmp4-mediated regulation of Bmp4 signaling by acting as an endocytic receptor for Bmp4 and mediating the endocytosis of the Bmp4/Bmp/BMPR complex. Based on these observations, we propose the following working model to explain the role of LRP1 in the regulation of Bmp4 signaling (Figure 6). In the absence of ligand, ALK6 and LRP1 are associated, blocking the assembly of an active BMPRII/ALK6 complex. In the presence of Bmp4 (A, middle), ALK6 dissociates from LRP1 and heterodimerizes with BMPRII.20 This Bmp4/BMPRII/ALK6 receptor complex is sequestered within endosomes where Bmp signaling occurs.20 B, When the concentration of Bmp4 is substoichiometric, Bmp4/LRP1 forms a transient holocomplex of Bmp4/ALK6/BMPRII, which promotes Rab4-dependent endocytic fast recycling and enhances downstream Bmp signaling (D, red route). C, When the concentration of Bmp4 is suprastoichiometric, the association of LRP1 with ALK6 increases, but that of ALK6 with BMPRII decreases. The LRP1-dependent endocytosis of a transient Bmp4/Bmp/ALK6/LRP1 holocomplex leads to the degradation of the Bmp4/Bmp signaling complex and termination of Bmp signaling activity (E, green route).

Figure 6. A schematic model shows how LDL receptor-related protein 1 (LRP1) is required for bone morphogenetic protein (Bmp)/4/bone morphogenetic protein endothelial cell precursor-derived regulator (Bmper) signaling. A, left. In the absence of ligand, activin-like kinase receptor (ALK)6 and LRP1 are associated, blocking the assembly of an active Bmp receptor (BMPRII/ALK6 complex. In the presence of Bmp4 (A, middle), ALK6 dissociates from LRP1 and heterodimerizes with BMPRII.20 This Bmp4/BMPRII/ALK6 receptor complex is sequestered within endosomes where Bmp signaling occurs.20 B, When the concentration of Bmp4 is substoichiometric, Bmp4/LRP1 forms a transient holocomplex of Bmp4/ALK6/BMPRII, which promotes Rab4-dependent endocytic fast recycling and enhances downstream Bmp signaling (D, red route). C, When the concentration of Bmp4 is suprastoichiometric, the association of LRP1 with ALK6 increases, but that of ALK6 with BMPRII decreases. The LRP1-dependent endocytosis of a transient Bmp4/Bmp/ALK6/LRP1 holocomplex leads to the degradation of the Bmp4/Bmp signaling complex and termination of Bmp signaling activity (E, green route).
components of the Bmpr/Bmp4 signaling complex are recycled back to the cell membrane (Figure 6D, red route), where they would be available for future signaling events. This pathway would explain the ability of substoichiometric concentrations of Bmpr to activate Bmp4 signaling. When the concentration of Bmpr is suprastoichiometric, the association of LRPI with ALK6 increases, but that of ALK6 with BMPR1I decreases (Figure 6C). The LRPI-dependent endocytosis of a transient Bmpr/Bmp4/ALK6/LRPI holocomplex leads to the degradation of the Bmpr/Bmp4 signaling complex and early termination of Bmp4 signaling activity (Figure 6E, green route).

We therefore propose that the different components of the transient Bmp4/Bmpr receptor holocomplex may be the determining factor in deciding which endocytic sorting routes are used in the presence of sub- versus suprastoichiometric concentrations of Bmpr. Bmpr was recently reported to preferentially regulate Bmp9/ALK1 signaling in endothelial cells.27 Whether LRPI is required for Bmpr-modulated Bmp9/ALK1 signaling and how Bmp9 regulates Bmp4/Bmpr/BMPR/LRPI complex formation needs future investigation. Moreover, the mechanisms behind the receptor-dependent endocytic sorting described remain unknown. The different components of the transient holocomplex may recruit different scaffolding proteins, thereby influencing the intracellular route of receptor complex processing. Alternatively, intracellular routing of the Bmpr/Bmp4 signaling complex may be regulated by posttranslational modification of LRPI, which may occur differentially depending on which components comprise the Bmpr/Bmp4 receptor complex. For example, LRPI contains an NPxY motif in its cytoplasmic tail that lies proximal to the plasma membrane. This NPxY motif is a sorting nexitin 17-binding motif that help sort LRPI-contained endosomes during the receptor recycling process. If this motif is mutated, LRPI-containing endosomes cannot be recycled and become targets of lysosomal degradation.28 LRPI endocytosis can also be regulated by a cAMP-dependent protein kinase A-mediated serine phosphorylation on its cytoplasmic tail.29 It is possible that the different Bmpr/Bmp4 receptor complexes formed in the presence of high and low concentrations of Bmpr may influence either the phosphorylation of the LRPI cytoplasmic tail or the recruitment of endosome sorting proteins such as sorting nexitin 17, which in turn may result in differential intracellular sorting routes. Although additional work is needed to fully elucidate the exact mechanism for the different sorting processes by which LRPI regulates Bmpr/Bmp4 signaling, LRPI-dependent endocytosis is clearly critical for all aspects of Bmpr-mediated Bmp4 signaling, and the stoichiometric ratio of Bmpr to Bmp4 is a key to determine whether Bmp4 signaling is activated or inhibited.

Acknowledgments
We thank the University of North Carolina’s (UNC) Michael Hooker Proteomics Center for help with protein characterization, the UNC Zebrafish Aquaculture core facility for help with zebrafish experiments, and the UNC Microscopy Services Laboratories for help with immunohistochemistry and in situ hybridization experiments.

Sources of Funding
This work was supported in part by NIH grant R01-HL061656 (C.P.).

Disclosures
None.

References

### Novelty and Significance

**What Is Known?**
- Bmp (bone morphogenetic protein) is an essential regulator of endothelial function.
- Bmper (Bmp endothelial cell precursor-derived regulator) is an important extracellular modulator of Bmp and regulates Bmp4 activity via a concentration-dependent, endocytic trap-and-sink mechanism.

**What New Information Does This Article Contribute?**
- LRP1 (LDL receptor-related protein 1) acts as an endocytic receptor for Bmper and a co-receptor of Bmp4.
- LRP1-dependent endocytosis governs the signaling output of the Bmper/Bmp4 system in endothelial cells and in angiogenesis in vivo.
- Bmper, and possibly other Bmp modulators, functions both extracellularly and intracellularly to regulate Bmp4 function, allowing regulation of Bmp4 at the single cell level.

The Bmper/Bmp signaling axis is an important signaling pathway that regulates endothelial function in both health and disease. In this study, we identified LRP1 as a novel endocytic receptor of Bmper and a co-receptor of Bmp4 and demonstrated that the LRP1-dependent endocytosis of Bmper/Bmp4 receptor complex is a crucial molecular mechanism for both promoting and inhibiting Bmp4 activity by Bmper. Previous studies have demonstrated that regulators of Bmp signaling, such as Chordin, Noggin and Gremlin, modulate Bmp signaling using a spatial gradient effect that covers the distance of many cells. In contrast, our data indicates that Bmper, and possibly these other Bmp modulators, can work at the single cell level; not only binding Bmp4 and influencing its interaction with receptors, but also internalizing with the receptor complex to regulate intracellular signaling. In addition, our data introduce LRP1 as a novel regulator of endothelial function and vessel development. The knowledge gained from this study will provide crucial mechanistic information concerning blood vessel formation in health and disease and offers novel therapeutic targets and strategies against angiogenesis-related diseases such as blood vessel ischemic disease, cancer, and other related diseases.
LRP1-Dependent Endocytic Mechanism Governs the Signaling Output of the Bmp System in Endothelial Cells and in Angiogenesis

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Circ Res. 2012;111:564-574; originally published online July 9, 2012;
doi: 10.1161/CIRCRESAHA.112.274597

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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Supplemental Material

Methods

Reagents. LRP1 minireceptor constructs (mLRP1~4) were generated as described\(^1\). Each mLRP contains an HA epitope followed by a signal peptide, one of the four ligand-binding domains (LBD) and the identical carboxyl-terminal region to that of endogenous LRP1\(\beta\). ALK6 cDNA was kindly provided by Dr. Attisano (Univ. of Toronto, Toronto, CA). ALK6 and mLRP2 cDNAs were cloned into pMFP(YFP/CFP)-N vector (with YFP/CFP at the C-terminal). Flag-tagged LRP1\(\beta\) was cloned from mLRP4 as a template into the pCMV-Tag2B vector, with the flag tag at amino-terminus (Invitrogen). The constructs of wild type ALK6 (ALK6-WT, a.a. 1~502, with a Flag, HA or CFP tag at C-terminus), its deletion mutants- ALK6-CTD (a.a. 146~502, containing both GS domain and protein kinase domain and a Flag tag at the C-terminus) and ALK6-PKD (a.a. 203~502, containing protein kinase domain only and a Flag tag at the C-terminus), and LRP1\(\beta\) (containing a Flag-tag at the C-terminus) are listed in Figure 3A.

Recombinant human Bmp4, Bmper protein, anti-Bmper, Bmp, ALK6 and BMPRII antibodies were obtained from R&D Systems (Minneapolis, MN). BMPRII was detected as multiple bands with different motility possibly due to differential glycosylation, different mRNA splice variants resulting in truncated forms of receptors or other modifications\(^2\). Anti-pSmad1/5/8 antibody was purchased from Cell Signaling (Danvers, MA). Anti-LRP1 \(\alpha\) chain and \(\beta\) chain rabbit antibodies were generated as described\(^3\), and the mouse antibodies against LRP1\(\alpha\) and \(\beta\) were purchased from American Diagnostica (Stamford, CT).

Chlorpromazine (CPM), chloroquine (CQ) and bafilomycin A1 (BafA1) were purchased from Sigma (St. Louis, MO). Chlorpromazine is a cationic amphipathic molecule that inhibits the budding process of clathrin-coated vesicles (previously shown to be involved in Bmper/Bmp endocytosis\(^4\)) and the formation
of early endosomes. Chloroquine is a lysosomotropic agent that prevents endosomal acidification and inhibits fusion of endosomes and lysosomal degradation. Bafilomycin A1 is a specific inhibitor of vacuolar-type H(+)−ATPase that inhibits acidification and protein degradation in lysosomes. The treatments of CPM, CQ and BafA1 used to block endocytosis in MECs are 50 μmol/L for 30 minutes, 200 μmol/L for 30 minutes and 100 nmol/L for 6 hours, respectively.

Cell culture and generation of cell lines. MECs (mouse endothelial cells) and HEK293 cells were grown in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 68.6 mol/L streptomycin). LRP1 knockout mouse embryonic fibroblasts (MEFs; PEA 13, CRL-2216) and control MEFs (MEF-1, CRL-2214) were purchased from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 68.6 mol/L streptomycin). For stable MEC cell line construction, 50~70% confluent MECs in 6 well plates were transduced with 8 ul of LRP1 shRNA lentiviral particles (sc-40102-V) or control shRNA lentiviral particles (sc-108080) (Santa Cruz Biotechnology, Santa Cruz, CA). The positive colonies were screened with puromycin and evaluated by Western blot analysis with anti-LRP1 antibodies.

Chemical cross-linking in intact cells and immunoprecipitation. MECs were treated with Bmper at 5 nmol/L for 1.5 hours and then washed with cold PBS for 3 times. Cells were incubated with 2 mmol/L dithiobis(succinimidylpropionate) (DSP; Pierce) for crosslinking on ice for 30 min, and terminated with the addition of Tris-HCl (pH 7.5) to 20 mmol/L. Cells were harvested with lysis buffer (1% Triton X-100, 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L Na3VO4 and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 16,000g. Equal amounts of protein were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A/G plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to pull down Bmper-associated antibody complexes following previously described methods. Proteins were separated by SDS-PAGE and stained by Coomassie blue. The interested protein bands were recovered for protein identification by matrix-assisted laser
desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis performed by UNC Michael Hooker Proteomics Center. This immunoprecipitation and MALDI-TOF analysis (Figure 1A) have been performed twice and LRP1 was identified in both experiments.

**Immunoblotting, immunoprecipitation and ligand blotting analysis.** After appropriate treatments, cells were extensively washed 3 times with PBS and harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L Na3VO4 and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 16,000g. Equal amounts of protein were incubated with a specific antibody overnight at 4 °C with gentle rotation. Protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to pull down the antibody complexes following previously described methods. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Ligand blotting was performed following the previously described method. All the immunoblotting, immunoprecipitation and ligand blotting experiments have been performed at least three times.

**Immunofluorescence.** Cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature. After 3 washes with PBS, the cells were sequentially treated with 0.2% Triton X-100 for 5 min (for permeabilization), with 5% boiled serum for 1 hour (for blocking), then with the primary antibody overnight in the blocking solution. After 3 washes, cells were incubated in the dark with second antibody conjugated with Alexa Fluor 488, 568 or 647 (Molecular Probes, Eugene, OR) in blocking solution for 90 min at 37 °C. After 3 washes in PBS, the cells were counterstained with DAPI and the fluorescent signal visualized by fluorescence microscopy or confocal laser scanning microscopy. All the immunofluorescence experiments have been performed at least three times.

**Bmper ELISA assay.** Each step of each assay was conducted by room temperature incubation with the indicated reagents unless specified. Between each step, plates were washed 3 times in TBS containing 0.1% Tween-20 (TBST). Data points were generally in triplicate. 96-well ELISA plates were coated at
4°C overnight with 25 µg total cell lysates of MECs or LRP1Δ MEFS as the negative control. Following the coating, ELISA wells were incubated with the unlabeled Bmper (50–500 nmol/L) in binding buffer (1x TBST, 5 mM CaCl2, 5% milk) for 1 hour. Then, the wells were incubated with biotinylated Bmper in the binding buffer for 3 hours at 4°C. The bound biotinylated Bmper was detected by addition of avidin-HRP and then TMB substrate.

Subcellular fractionation. MECs (four 10-cm dishes for each sample) were treated with 5 nmol/L Bmper for 30 minutes and washed with cold PBS for three times. Then, they were detached by scraping off in ice-cold phosphate-buffered saline. Subcellular fractionation was performed following the previous publication. Specifically, cells were pelleted and resuspended in homogenization buffer (10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 0.25 M sucrose, supplemented with a protease inhibitor mixture), and then disrupted by 15 passages through a 25-gauge needle. Nuclei and unbroken cells were spun down by centrifugation at 3000 g for 10 minutes. Postnuclear supernatants were centrifuged at 200,000 × g for 1 hour in a Beck Coulter Optima LE-80k-ultracentrifuge (SW60 Ti rotor, 44129 rpm), and the membrane pellet was resuspended in 400 µl of homogenization buffer. All steps were carried out at 4°C. For fractionation, Optiprep (60%, wt/vol, Sigma), a ready-made solution of iodixanol (5,5′-[(2-hydroxy-1-3-propanediyl)-bis(acetylamino)]bis[N,N′-bis(2,3-dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide)], was used. Separation of different membrane compartments was achieved by centrifugation in three step (10–20–30%; wt/vol) iodixanol gradients. Each one-third of the post-nuclear supernatant was mixed with Optiprep (60%, wt/vol) iodixanol and homogenization buffer to generate solutions of 10, 20, or 30% iodixanol. Equal volumes of these three solutions were layered into centrifuge tubes and samples were centrifuged at 353,000 g (58626 rpm, SW60 Ti rotor) for overnight at 4°C. Sequential 400 µl fractions were collected from the top of the gradient, and proteins were subjected to SDS-PAGE and immunoblotting.

RNA interference. The siRNA oligos against mouse Rab4, ALK1, ALK2, ALK3 and ALK6 were
designed using the BLOCK-iT™ RNAi search engine (Life technologies, Grand Island, NY, USA). The oligos used were the following: Rab4: forward 5'-CAUAGGAGUGGAUUUUGGCUCAAAAG-3', reverse 5'-CUUUGAGCCAAAUUCCACUCCUAUG-3'; ALK1: forward 5'-AAUGCAACACUGCAAGGUUACUUGUG-3, reverse 5'-CAAGAGUAACUUUGCAGUGUUGCAUU-3; ALK2: forward 5'-AUGACUGCCAGGCCCAAAUCUGCUA-3', reverse 5'-UAGCAGAUUUGGCCUGGCAGUCAU-3; ALK3: 5'-CUGUUCUUUGAUAGGCGAGCAGCAUCCGAU-3', reverse 5'-AUCGGAUGCGCAGCAUCCGA-3; ALK6: forward 5'-ACGGCUCCUUAUGACUAUCUGA-3', reverse 5'-UUCAGAUAGCAUAAAGGGAGCCCU-3; and negative control duplex (medium GC, Life technologies). MECs were plated at 2 × 10⁵ cells per 6-well plate. The following day, the siRNA oligos were transfected using Lipofectamine RNAiMAX (Life technologies) following the manufacturer’s instructions, and the optimal concentrations of duplexes for knockdown were determined by titration analysis. RNA from confluent T-75 flasks of HUVEC was isolated using RNeasy plus mini kit (Qiagen, Valencia, CA, USA).

RT-PCR. The RNA was reverse transcribed into cDNAs with iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) using specific pairs of primers for Bmp2, 4, 6 and Bmper (designed for Taqman real-time PCR, Life technologies), ALK1 (forward primer: 5'-CTTGGGGAGCCTCAGAGGGG and reverse primer: GGTGGGCTTCAGCAATCAGGA-3'), ALK2, 3 and 6 (purchased from Santa Cruz Biotechnology), or Lrp1(a(e1i1)) (forward primer: 5'-GACGTGTGATGGATGGGACGAG-3' and reverse primer: 5'-CTGCTTCAGAAGAGTGTAGTTT-3), LRPla(e3i3) (forward primer: 5'-GCATGATCAGACGCATGAAC-3' and reverse primer: 5'-CCACACGGACAGCTCAGTG-3'), Lrp1b (forward primer: 5'-GATGGGACACGGACTGC-3' and reverse primer: 5'-CTCCATTTCACCAGTG-3'). PCR with Platinum™ PCR supermix (Life technologies) was performed in a Mastercycler EP PCR machine (Eppendorf, Hamburg, Germany) with the following program: 3 min 95°C to activate the polymerase followed by 40 cycles of 45 s 95°C, 45 s 55°C, and 45 s 72°C. GAPDH was the housekeeping gene used for internal control of MECs and zebrafish tissue.
**FRET experiments.** Experiments were performed following a previously published protocol\textsuperscript{10}. Cells grown on coverslips were rinsed briefly with PBS, prefixed with 2% paraformaldehyde for 10 min, and then rinsed three times with PBS. The coverslips were imaged with confocal microscopy using an inverted microscope and a 40x dry lens under a series of CFP, YFP and FRET channels (Olympus FV500 Confocal Laser Scanning Microscope). The control cells with CFP-ALK6 or YFP-mLRP2 were used to normalize the CFP and YFP signals. Specifically, donor, acceptor and FRET images were acquired sequentially using fixed excitation and emission filters. After background subtraction and shade correction, FRET efficiency analysis was performed using ImageJ software (W.S. Rasband, ImageJ, NIH, Bethesda, MD) and the PixFRET plug-in\textsuperscript{11}. Bleed-through coefficients were calculated using fret/donor or fret/acceptor image stacks captured from cells expressing only the donor or acceptor respectively. The PixFRET plug-in renders pixel-by-pixel FRET efficiency values (shown as percentage) and typical images are shown as normalized FRET (NFRET). The FRET experiments have been repeated four times.

**Boyden chamber assay.** Boyden chamber assays were performed as previously described\textsuperscript{8}. The lower chambers of the apparatus were filled with DMEM with or without Bmp4 and Bmp7 and then covered with the collagen-coated filter and the upper chambers. Cells were incubated in the upper chamber for 6 h at 37 °C, cells present on the lower surface were fixed, stained and identified with the 10X objective lens on a Nikon Eclipse TS100 inverted microscope.

**In vitro Matrigel angiogenesis assay.** Endothelial cell tube formation was analyzed with the matrigel-based tube formation assay\textsuperscript{8}. Chilled 24-well plates were coated with growth factor–reduced matrigel (Becton Dickinson) that was polymerized at 37°C for 30 min. MECs were plated at equal numbers into each Matrigel coated well. After 6 h of incubation in the absence or presence of Bmp4 and Bmp7, the formation of tubes was photographed with a camera (Eclipse TS100; Nikon) and a 10x objective lens. Images were quantified with ImageJ.
Morpholino sequences and injections. Morpholino phosphorodiamidate oligonucleotides (MOs) were produced by Gene Tools (Philomath, OR). The MOs were designed to inhibit splicing of the \textit{lrp1a} (ENSDART00000011592) and \textit{lrp1b} RNA (ENSDART00000088208): \textit{lrp1a} MO: ATCGGTGTTCATACCAGAGGTGGAT, targeting the first exon-intron splicing site of \textit{lrp1a} mRNA; \textit{lrp1a(e3i3)} MO: GAGACACACATGCAGCTGACCTGCA, targeting the third exon-intron splicing site of \textit{lrp1a} mRNA. \textit{lrp1b} MO: GTGTGTGCCTTACTACAAAGCTTCT, targeting the seventh exon-intron splicing site of \textit{lrp1b} mRNA. All MOs were prepared and injected as previously described\textsuperscript{12}. One to four cell stage embryos were injected with the indicated amount of either \textit{lrp1} MO or standard MO (Gene Tools) and raised at 28.5 °C until analyzed. \textit{lrp1a} and \textit{lrp1b} knockdown was analyzed by PCR with reverse transcribed cDNA of those fish morphants.

Raising and staging of embryos. Zebrafish (\textit{Danio rerio}) embryos were obtained from the University of North Carolina, Chapel Hill (UNC-CH) Zebrafish Aquaculture Core Facility and raised as previously described\textsuperscript{13}. Embryos were staged using standard morphologic criteria prior to analysis\textsuperscript{14}. Blood vessel nomenclature was applied according to published recommendations\textsuperscript{15}. 0.003% phenylthiourea (PTU, Sigma) was added to embryo cultures before 24 hours post fertilization (hpf) to block pigment formation of the embryos. \textit{Tg(kdrl:EGFP)}\textsuperscript{843}, \textit{Tg(myl7:EGFP-HSHRas)}\textsuperscript{883}, \textit{Tg(kdrl:HSHRas-mCherry)}\textsuperscript{896} and \textit{Tg(kdrl:EGFP)}\textsuperscript{883};\textit{Tg(gata1:dsRed)}\textsuperscript{ad2} were used to study vascular development (\textit{kdrl} in green or cherry), hematopoiesis (\textit{gata1} in red) and heart development (\textit{myl7} in green). Phenotypic images were taken with fluorescent microscopy and pseudo-colored with Adobe Photoshop.

Whole-mount in situ hybridization. To study the expression pattern of \textit{lrp1} during embryogenesis, whole-mount \textit{in situ} hybridization was performed as described previously\textsuperscript{16}. Zebrafish embryos were fixed at different developmental stages in 4% paraformaldehyde. Embryos were hybridized with an antisense \textit{lrp1}-specific probe with the corresponding sense probe used as a negative control. The \textit{lrp1} dig labeled
probe was synthesized with Sp6 or T7 RNA polymerase (Roche) using pCRII-TOPOII (Invitrogen) that contained a fragment of lrp1 between Sp6 and T7 priming sites as a template.

*Cell transplantation.* Transplantations were performed as described previously\textsuperscript{17,18}. Embryos were injected in the yolk at the one cell stage with the following: 5% rhodamine dextran (w/v; 10,000 Da molecular mass; Molecular Probes), phenol red in HEPES buffer and mixed with p53 MO and standard MOs (for control donor) or *lrp1a and b* MOs (for LRP1 MO donor). Between the sphere and germ ring stages, 5-50 donor cells were isochronically transplanted into each recipient wild type *Tg(kdrl:EGFP)s843* embryos. Transplantations were performed under 40 to 60X magnification using a Hamilton syringe with a micrometer drive and micromanipulator in a standard petri dish containing a bed of 3% agarose in 30% Danieau with 150 individual embryo wells (Mold available from Adaptive Science Tools) at room temperature. Donor cells were targeted to the presumptive mesendoderm at the ventral margin. Embryos were allowed to recover for one hour post-transplantation at room temperature and then washed three times with 10% Danieau solution and placed at 28.5°C. Transplant recipients were permitted to develop until 48 hpf. Donor contribution to caudal vein plexus and other tissues was quantitated at 32 hpf by examining embryos using fluorescent microscopy. Representative images were taken using the whole mount immunohistochemistry method described previously\textsuperscript{19}. Briefly, chick anti-GFP primary antibody (Abcam, Cambridge, MA, USA) and donkey anti-chick-488 secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) were used to boost GFP signal after storing embryos in methanol and then imaged with confocal microscopy at 34 hpf.

*Whole-mount staining for zebrafish embryo.* To stain LRP1, Bmp and phosphorylated Smad1/5/8 in zebrafish embryo, whole-mount staining was performed. The fish embryos were fixed with 4% paraformaldehyde overnight at 4°C. After washing with PBS-tween (0.1%), the embryo was blocked with 5% normal goat serum containing 10 mg/ml BSA and 0.3% Triton-X for 1 hour. Fish embryos were then incubated with primary antibodies diluted in blocking solution overnight. After washing, secondary
antibodies were incubated for 4 hours. The embryos were washed again and prepared for imaging with confocal microscopy.

Statistical analysis. Data are shown as mean ± SD for 3 to 4 separate experiments. Differences were analyzed with Student’s t-test. Values of $P \leq 0.05$ were considered statistically significant.


Online Figure I. LRP1 is associated with Bmpr. (A) LRP1 was expressed in MECs. Lysates of cells were analyzed by Western blotting with an anti-LRP1α antibody. The expression of LRP1 was abundant in MEC and LRP1+/+ MEFs, with no LRP1 protein detected in LRP1−/− MEFs, indicating the specificity of the anti-LRP1 antibody. (B) Lysates of MECs were immunoprecipitated with an anti-Bmpr antibody or goat control IgG and analyzed by Western blotting with anti-LRP1 antibody (the upper panel). The lower panel demonstrates the internalized Bmpr detected in the total cell lysates. (C) Competitive inhibition of Bmpr binding to LRP1–containing MEC Lysates. Competitive binding ELISA was performed in order to determine whether unlabeled Bmpr competes with biotinylated Bmpr for the binding of LRP1–containing MEC lysates. The reaction with lysates of LRP1−/− MEFs was used as a negative control. (D) Lysates of MECs treated with Bmpr and Bmp4 were immunoprecipitated with anti-LRP1 antibody and analyzed by Western blotting with anti-Bmpr antibody (the upper panel). The lower panel demonstrates the expression levels of LRP1α. N.S. means non-specific. (E) Lysates of MECs treated with Bmpr and Bmp2 or 6 were immunoprecipitated with anti-LRP1 antibody and analyzed by Western blotting with biotinylated anti-Bmpr antibody and avidin-HRP (the upper panel). The lower panel demonstrates the expression levels of LRP1α. N.S. means non-specific. (F) LRP1 is complexed with Bmp4. Lysates of MECs treated with Bmp4 were immunoprecipitated with an anti-LRP1 antibody or mouse IgG control, and then analyzed with Western blotting with the anti-Bmp4 antibody.
Online Figure II. LRP1 is required for Bmper endocytosis. (A) Non-denatured cell lysates from cells treated with Bmper for different time periods were analyzed for Bmper protein level by Western blotting with an anti-Bmper antibody. (B) Quantification of Bmper protein band intensity in A. *, compared to non-treated wild-type MEFs, \( P<0.02, n=3 \); #, compared to LRP1 null MEFs treated similarly, \( P<0.05, n=3 \) independent experiments. (C) Non-denatured cell lysates from cells treated with increasing doses of Bmper were analyzed for Bmper protein level by western blotting with an anti-Bmper antibody. (D) Quantification of Bmper protein band intensity in C. *, compared to non-treated wild-type MEFs, \( P<0.05 \); #, compared to LRP1 null MEFs treated similarly, \( P<0.02, n=3 \) independent experiments. (E) Bmper-treated MECs (15 minutes) were analyzed by confocal imaging for the co-localization of Bmper (green), LRP1 (red) and EEA1 (purple). Colocalization of these three proteins is indicated by blue
particles (arrow). These are the representative images of three independent experiments. (F) MECs treated with Bmper for 90 minutes were analyzed by confocal imaging for the co-localization of Bmper (green), LRP1 (red) and Rab7 (purple). Colocalization of these three proteins is indicated by blue particles (arrow). These are the representative images of three independent experiments. (G) MEFs treated with Bmper for 5 minutes were analyzed by confocal imaging for the co-localization of Bmper (green), LRP1 (red) and EEA1 (purple). Colocalization of these three proteins results in blue immunofluorescence, as indicated by the arrows. Scale bars: 5 µm. (H) Subcellular fractionation experiments were performed to confirm the location of Bmper and LRP1 in MECs. Eleven serial membrane fractions prepared from 5 nmol/L Bmper-treated control (Ctrl sh-MEC) or LRP1 shRNA stably transfected MECs (LRP1 sh-MEC) were fractionated on a self-generated Optiprep gradient (10%, 20%, 30%) and immunoblotted with antibodies against proteins-Bmper and LRP1α, and that enriched in plasma membrane (FLK1); early endosomes (EEA1); or late endosomes (Rab7). Optiprep gradient fractionation resulted in Bmper and LRP1α mainly cofractionating with Flk1 (plasma membrane marker), EEA1 (early endosome marker) and Rab7 (late endosome marker). In LRP1-knockdown MECs, we observed considerably less Bmper localized in plasma membrane and endosomal fractions.
Online Figure III. ALK2, 3 and 6 are required for Smad1/5/8 activation induced by Bmp4. (A) The total RNA of MECs was reverse transcribed to cDNA and detected for the RNA levels of Bmp2, 4, 6, Bmper using their specific primers. GAPDH was used as a housekeeping gene control. (B) MECs were transfected with control (Ctrl) siRNA, alk1, alk2, alk3 or alk6 siRNAs. The total RNA obtained from these MECs were used for RT-PCR analysis with the specific primers of ALK1, 2, 3 and 6. GAPDH was used as an internal control. (C) MECs were transfected with alk1, 2, 3 or 6 siRNAs, or control siRNAs, and then treated with Bmp4 to activate Smad1/5/8. The cell lysates were immunoblotted with the antibodies against phospho-Smad1/5/8 and Smad1. (D) Lysates of HEK 293 cells transfected with Flag-ALK6-CTD/PKD and HA-mLRP4 were immunoprecipitated with an anti-Flag antibody and analyzed by Western blotting with an anti-HA antibody to detect mLRP4. The total cell lysates were also blotted with antibodies as indicated to show the equal loading of overexpressed proteins.
Online Figure IV. LRP1-mediated endocytosis is required for the Bmp4-dependent regulation of Bmp4 downstream signaling. (A) MECs were treated for indicated time periods with Bmp4 (0.6 nmol/L) or subjected to the "BB-sub" treatment in which the ratio of Bmpr (0.3 nmol/L) to Bmp4 (0.6 nmol/L) concentration is substoichiometric. The "BB-supra" treatment is the treatment in which the ratio of Bmpr (10 nmol/L) to Bmp4 (0.6 nmol/L) concentration is suprastoichiometric. Cell lysates were analyzed by Western blotting with antibodies for phosphorylated Smad1/5/8 (pSmad1/5/8) and Smad1. *, compared to MECs at control condition, P<0.05. **, P<0.05. n.s., not significant. n=3. (B) Quantitative analysis of Figure 4A "CPM" experiment (upper panel). *, compared to MECs at control condition, P<0.05. **, P<0.05. #, compared to MECs treated with Bmp4 alone, P<0.05. **, P<0.05. n=3. (C) Quantitative analysis of Figure 4B. *, compared to MECs at control condition, P<0.05. **, P<0.05. #, compared to MECs treated with Bmp4 alone, P<0.05. **, P<0.05. n=3. (D) Quantitative analysis of Figure 4A "CQ" experiment (lower panel). *, compared to same MECs at control condition, P<0.05. #, compared to MECs treated with Bmp4 alone, P<0.05. **, P<0.05. n=3. (E) MECs were pretreated with 100 nmol/L bafilomycin A1...
(BafA1) for 6 hours and then subjected to the indicated treatments of Bmp4 and Bmper for 2 hours. The cell lysates were analyzed by Western blotting with antibodies for pSmad1/5/8 and Smad1. *, compared to same MECs at control condition, $P<0.05$. #, compared to MECs treated with Bmp4 alone, $P<0.05$. **, $P<0.05$. $n=3$. (F) Quantitative analysis of Figure 4C. *, compared to same MECs at control condition, $P<0.05$. #, compared to MECs treated with Bmp4 alone, $P<0.05$. **, $P<0.05$. $n=3$. (G) Quantitative analysis of Figure 4D. *, compared to same MECs at control condition, $P<0.05$. **, $P<0.05$. $n=3$. (H) The role of LRP1 in the regulation of ALK6 binding to other Bmp type II receptors such as the activin receptor type II (ACVRII) was examined. Lysates of MECs treated with Bmp4 (0.6 nmol/L) and Bmper (0.3 nmol/L or 10 nmol/L) for 15 minutes were immunoprecipitated with an anti-ACVRII antibody and analyzed by Western blotting with anti-ALK6 and ACVRII antibodies. Interestingly, the pattern of ALK6 binding to ACVRII was opposite from that of ALK6 and BMPRII (Figure 4D). At the basal state, ALK6 was associated with ACVRII, however the level of their binding significantly decreased in LRP1-knockdown MECs. Additionally, the binding of ALK6 and ACVRII was promoted upon Bmp4 and Bmper treatment in LRP1-knockdown MECs. Therefore, we speculate that LRP1 is required for the dynamic balance of Bmp receptors on the membrane. (I) Lysates of MEFs treated with Bmp4 (0.6 nmol/L) and Bmper (0.3 nmol/L or 10 nmol/L) for 15 minutes were immunoprecipitated with an anti-BMPR II antibody and analyzed by Western blotting with anti-ALK6 and BMPRII antibodies. The binding of ALK6 and BMPRII was similar in MEFs, except samples treated with Bmper alone demonstrated no change in the association of ALK6 and BMPRII in MECs (Figure 4D) but an increased association in MEFs, suggesting that the expression levels of Bmp receptors and LRP1 possess subtle difference between MEFs and MECs. (J) Quantitative analysis of Figure 4E. *, compared to MECs at control condition, $P<0.05$. $n=5$. (K) MECs were subjected to the Boyden chamber migration analysis with Bmp4 (0.6 nmol/L) and Bmper as chemoattractants. *, compared to the same type of MECs at control condition, $P<0.001$. #, compared to the same type of MECs treated with Bmp4, $P<0.001$. $n=4$. (L) MECs were subjected to the Boyden chamber migration analysis with Bmp4 (4 nmol/L) as chemoattractants, and neutralizing Bmp4 antibody (10 µg/mL) was used to co-treat cells and block Bmp4 activity. *, compared to MECs at control condition, $P<0.05$. #, compared to MECs treated with Bmp4, $P<0.05$. $n=3$. 
Online Figure V. LRP1 is required for vascular development in zebrafish. (A) RNA expression of *lrp1a* in a whole-mount zebrafish embryo, analyzed by in-situ hybridization using a *lrp1a*-specific antisense probe. The sense probe of *lrp1a* was used as a negative control. Scale bar: 100 µm. (B) PCR analysis of zebrafish embryo RNAs demonstrates the knockdown of *lrp1a* or *lrp1b* or both with their specific MOs. (C) Loss of *lrp1a* and *p53* results in a similar vascular phenotype to the loss of *lrp1a*, suggesting there is no off-targeting effect with *lrp1a* MO. lateral views of *Tg(kdrl:EGFP)*1643; *Tg(gata1:dsRed)*162 fish embryo tails at 48 hpf were presented. The arrowhead represents the blood circulation with a changed route; the arrow represents the disrupted intersegmental vessel. Scale bar: 100 µm. (D) PCR analysis of zebrafish embryo RNAs demonstrates the knockdown of *lrp1a* after injection of *lrp1a* MO targeting the e1i1 splice site were confirmed with an alternative MO -lrp1a(e3i3), targeting the e3i3 splice site. It suggests that *lrp1a* MO is specific. Images are lateral views of *Tg(kdrl:EGFP)*1643; *Tg(gata1:dsRed)*162 zebrafish embryo tails at 24 hpf. Scale bar: 100 µm. (G) Loss of *lrp1a*, *lrp1b* or both results in a similarly disrupted vascular phenotype. Images are lateral views of *Tg(kdrl:EGFP)*1643 zebrafish embryo tails at 24 hpf. The arrowheads represent the causal vein plexus with branches; the arrows represent filopodia located on the front edge of vessel plexus. Scale bar: 100 µm. (H) The wild-type recipient embryos of *Tg(kdrl:EGFP)*1643 were transplanted with control or *lrp1* MO-injected donor cells (labeled with rhodamine-dextran). At 32 hpf, images of lateral views were taken with fluorescent microscopy. White arrowheads represent the donor cells located at cardinal vein plexus. Scale bar: 75 µm. LDA, lateral dorsal aorta. CCV, common cardinal vein. ACV, anterior cardinal vein. Se, intersegment vessel. DLAV, dorsal longitudinal anastomotic vessel. DA, dorsal aorta. CV, caudal vein. CA, caudal artery. PCV, posterior cardinal vein.
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Notes:
1 = Number of peptides that match the theoretical digest of the primary protein identified.
2 = Score of the quality of the peptide-mass fingerprint match.
3 = Score of the quality of MS/MS peptide fragment ion matches only.
4 = Significant score threshold. A hit with an "MS & MS/MS score" above this value is considered a significant identification (p<0.05) for the given database. Only hits with "MS and MSMS scores" above this value are reported.
### Online Table II. Dose effects of *lrp1a MO*

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<td>35/46 (75 %)</td>
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<tr>
<td>1.84</td>
<td>0/48 (0 %)</td>
<td>44/46 (95 %)</td>
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<td>3.68</td>
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<th>Dose (pmol)</th>
<th>24 hpf</th>
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<td>22/41 (54 %)</td>
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<tr>
<td>3.68</td>
<td>0/42 (0 %)</td>
<td>29/29 (100 %)</td>
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Abnormal: Delayed formation of dorsal vessels & intersegmental vessel, caudal vein plexus with fewer branches, and a swollen vascular lumen with ectopically placed Kdr<sup>+</sup> cells.
### Online Table III. Comparison of the effects with *lrp1a* MO or *lrp1a* MO + *p53* MO

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<td><em>lrp1a+p53</em></td>
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<td>1.84</td>
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Abnormal: Changed or stopped blood flow; delayed formation of dorsal vessels & intersegmental vessels; the caudal vein plexus with less branches, and a swollen vascular lumen with ectopically placed Kdr<sup>+</sup> cells; slower or stopped heart beat.
Online Movie I. Blood circulation of Tg(kdrl:EGFP)\textsuperscript{s843}; Tg(gata1:dsRed)\textsuperscript{sD2} fish embryos at 48 hpf without injection of MOs. 25 time lapse images were recorded with 100 miniseconds per image using fluorescent microcopy and stacked into the animation movies by ImageJ.

Online Movie II. Blood circulation of Tg(kdrl:EGFP)\textsuperscript{s843}; Tg(gata1:dsRed)\textsuperscript{sD2} fish embryos at 48 hpf injected with lrp1a MO. 25 time lapse images were recorded with 100 miniseconds per image using fluorescent microcopy and stacked into the animation movies by ImageJ.

Online Movie III. Blood circulation of Tg(kdrl:EGFP)\textsuperscript{s843}; Tg(gata1:dsRed)\textsuperscript{sD2} fish embryos at 48 hpf injected with lrp1a + p53 MOs. 25 time lapse images were recorded with 100 miniseconds per image using fluorescent microcopy and stacked into the animation movies by ImageJ.