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Loss of Function of Parathyroid Hormone Receptor 1 Induces Notch-Dependent Aortic Defects During Zebrafish Vascular Development

Caroline Gray, David Bratt,* Julie Lees,* Marc daCosta, Karen Plant, Oliver J. Watson, Sara Solaymani-Kohal, Simon Tazzyman, Jovana Serbanovic-Canic, David C. Crossman, Bernard D. Keavney, Andrea Haase, Kathryn McMahon, Martin Gering, Henry Roehl, Paul C. Evans, Timothy J.A. Chico

Objective**—**Coarctation of the aorta is rarely associated with known gene defects. Blomstrand chondrodysplasia, caused by mutations in the parathyroid hormone receptor 1 (PTHR1) is associated with coarctation of the aorta in some cases, although it is unclear whether PTHR1 deficiency causes coarctation of the aorta directly. The zebrafish allows the study of vascular development using approaches not possible in other models. We therefore examined the effect of loss of function of PTHR1 or its ligand parathyroid hormone-related peptide (PTHrP) on aortic formation in zebrafish.

Approach and Results**—**Morpholino antisense oligonucleotide knockdown of either PTHR1 or PTHrP led to a localized occlusion of the mid-aorta in developing zebrafish. Confocal imaging of transgenic embryos showed that these defects were caused by loss of endothelium, rather than failure to lumenize. Using a Notch reporter transgenic ([*CSL:Venus*]*qmc61*), we found both PTHR1 and PTHrP knockdown-induced defective Notch signaling in the hypochord at the site of the aortic defect before onset of circulation, and the aortic occlusion was rescued by inducible Notch upregulation.

Conclusions**—**Loss of function of either PTHR1 or PTHrP leads to a localized aortic defect that is Notch dependent. These findings may underlie the aortic defect seen in Blomstrand chondrodysplasia, and reveal a link between parathyroid hormone and Notch signaling during aortic development. **(*Arterioscler Thromb Vasc Biol*. 2013;33:1257-1263.)**

Key Words: aortic coarctation ■ angiogenesis ■ parathyroid hormone ■ zebrafish

Coarctation of the aorta (CoA) arises in 3:10 000 live births, accounting for 5% to 8% of congenital heart defects.[1] Its pathogenesis is unclear and most are sporadic. However, genetic diseases that cause CoA may provide insight into mechanisms of aortic formation.

Isolated CoA is associated with significant heritability,[2] and can be inherited in a Mendelian fashion,[3,4] suggesting an oligogenic or monogenic cause. However, few conditions associated with CoA (eg, Alagille syndrome, Pallister–Hall syndrome, Transaldolase deficiency, Mowat–Wilson syndrome, and Blomstrand chondrodysplasia) are caused by well-validated gene defects (*JAG1*/*NOTCH2*, *GLI3*, *TALDO1*, *ZEB2*, and *PTHR1,* respectively), and penetrance is <100% in these diseases.[5] All these conditions are associated with multiple abnormalities that might indirectly affect aortic construction, making the direct contribution of these genes to aortic formation difficult to test. In addition, many such mutations are lethal perinatally or prenatally in either humans or knockout mice, making assessment of aortic formation challenging.

Blomstrand chondrodysplasia, attributable to mutations in the parathyroid hormone receptor 1 (*PTHR1*[6]), is a rare lethal skeletal disorder, in which ≈50% of infants display CoA.[6–12] The *Pthr1* knockout mouse displays an identical skeletal phenotype and suffers multiple cardiovascular abnormalities, but does not display CoA,[13,14] casting uncertainty over whether *PTHR1* is directly involved in aortic construction. *PTHR1* has 2 ligands: parathyroid hormone (*PTH*) and parathyroid hormone-related peptide (*PTHrP*). PTHrP has been reported to both induce[15] and inhibit angiogenesis[16]; parathyroid hormone (PTH) has not been implicated in vascular development. Impaired PTHrP/PTH1R signaling is therefore a candidate mechanism for the aortic defect seen in Blomstrand chondrodysplasia.

Zebrafish embryos allow detailed visualization of embryonic vascular development. Specific gene knockdown can be achieved using morpholino antisense oligonucleotides,[17] and the embryo's size and milieu provide oxygenation via diffusion in the absence of blood flow.[18] Developing embryos survive for days without cardiac output, blood, or in the presence of severe vascular

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abnormalities.[18,19] This allows differentiation of the direct effects of gene knockdown from indirect effects attributable to other defects. Zebrafish possesses homologs of *PTHR1* and its ligands,[20] although no functional assessment has been performed.

The zebrafish previously allowed delineation of pathways leading to aortic formation. The morphogen Sonic hedgehog (Shh) is released from the notochord, which induces upregulation of vascular endothelial growth factor (VEGF), in turn inducing signaling via Notch to specify arterial gene expression and aortic development.[21] This pathway is therefore a candidate for spontaneous or inherited abnormalities that give rise to aberrant aortic development. The *Hey2* mutant zebrafish (*gridlock*) has been suggested as a model of CoA[22,23] although *Hey2* knockout mice do not develop CoA.[24] *Hey2* interacts with Notch, although it is less clear whether *Hey2* is downstream[25] or upstream[26] of Notch. However, among human Mendelian diseases associated with CoA, only Alagille syndrome (caused by mutations in the Notch ligand Jagged1 or the Notch2 receptor) is linked to defective Notch pathway.[27] We therefore examined the role of *pthr1* (the zebrafish homolog of *PTHR1*) in aortic development and its relationship to Notch signaling.

We find that *pthr1* knockdown in zebrafish induces a localized aortic occlusion attributable to aberrant endothelial patterning (more distal to that in *gridlock* mutants), implicating PTHR1 directly in aortic construction. This defect is associated with a defect in hypochordal Notch signaling and is rescued by Notch upregulation but not VEGF induction. Knockdown of the PTHR1 ligand *pthrp* induces an identical aortic defect to that seen in *pthr1* morphants.

Our data therefore show for the first time that PTHR1/PTHrP signaling controls Notch signaling to orchestrate correct patterning of the aorta. This is strongly suggestive that the CoA seen in Blomstrand chondrodysplasia is attributable to defective Notch signaling. Our findings illustrate the usability of the zebrafish for the study of congenital vascular defects.

Materials and Methods

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

Results

We first examined *pthr1* expression during zebrafish embryonic development. By reverse transcriptase polymerase chain reaction (rt-PCR) of whole embryo RNA, we found *pthr1* was expressed from as early as 1 day postfertilization (dpf) to at least 5 dpf (Figure 1A), despite the absence of mineralized bone at these stages. Using whole mount in situ hybridization, we observed ubiquitous *pthr1* expression (Figure 1B). By using combined fluorescent in situ hybridization with anti-green fluorescent protein (GFP) immunostaining in *Fli1:GFP* transgenics (Figure 1C), we found that most *pthr1* expression was detected in somitic muscle without obvious vascular expression, although in situ hybridization is relatively insensitive. To determine whether *pthr1* is expressed in endothelial cells in zebrafish, we dissociated *Flk1:GFP-NLS* embryos that express GFP in endothelial cells. We then used fluorescent automated cell sorting to sort fluorescent endothelial cells from nonfluorescent cells and examined expression of *pthr1*. Figure 1D shows that the GFP+ve cells expressed the endothelial marker VE-Cadherin by rt-PCR, whereas GFP−ve cells did not, confirming our ability to isolate relatively pure populations of endothelial cells. Figure 1E shows that these fluorescent automated cell sorting-sorted endothelial cells do indeed express *pthr1*, although at lower overall levels than in whole embryo RNA. We conclude from these studies that *pthr1* is expressed in endothelium during zebrafish development, in keeping with previous studies showing *pthr1* expression in cultured endothelial cells,[28,29] but that most *pthr1* expression is nonvascular.

Having confirmed *pthr1* is expressed during development, we examined the effect of reducing *pthr1* expression using antisense morpholinos (MO). Injection of a splice-blocking MOs targeting the boundary between intron 1 and exon 2 induced a truncated transcript at 2 dpf (Figure 2A), although this effect was incomplete. Sequencing the aberrant transcript revealed the splice-blocking MO-induced partial deletion of exon 2 of *pthr1*, leading to variable alterations, including major abnormalities in the predicted protein sequence or a premature stop codon (Figure 2B). These would be highly likely to induce significant or complete loss of function of *pthr1*.

When we examined the effect of *pthr1* knockdown on general embryonic development, neither the splice-blocking MOs nor the translation-blocking MOs targeting the *pthr1* start site altered general embryo morphology. This is in keeping with the fact that skeletal ossification does not commence until ≈7 dpf, and chondrodysplastic effects of *pthr1* would not be likely to appear until these stages.

When we examined vascular development in *pthr1* morphants immediately after circulation is established, we found ≈50% of *pthr1* morphants had a localized occlusion in the mid-aorta, at the 17th somite (situated 10±2 intersegmental vessels from the end of aorta) apparent immediately after onset of circulation. The aortic occlusion seen in *pthr1* morphants is more distal than that in *gridlock* mutants, which manifests at the junction between the paired dorsal aortae.[23]

Both start-blocking, or splice-blocking, *pthr1* MO induced the same aortic defect, which was never observed in control morphants or uninjected embryos. This phenotype was particularly obvious when the circulation was well established at 2 dpf. Figure 2C shows digital motion angiograms from 2 dpf *pthr1* and control morphants, demonstrating the site of aortic occlusion. Movies I–IV in the online-only Data Supplement show light and digital subtraction movies of representative 2 dpf control and *pthr1* morphants (see the online-only Data Supplement).

The aortic defect seen in *pthr1* morphants was seen in both splice-blocking and translation-blocking MO with similar penetrance (49±8%; n=4 groups of 30–40 embryos/group). Although <100% penetrance is normal with MO because these are rarely 100% efficient and only partially reduce target gene expression, we examined the reason for this incomplete penetrance. When we injected embryos with the splice-blocking *pthr1* morpholino, morphants with no aortic abnormality did not have aberrant *pthr1* transcripts on rt-PCR (Figure I in the online-only Data Supplement), suggesting the incomplete penetrance was attributable to inefficient *pthr1* knockdown.

We next attempted to determine the nature of the aortic occlusion induced by *pthr1* knockdown. We used double *Fli1:GFP/GATA1:dsRED* transgenics expressing GFP in the endothelium and dsRED in erythrocytes. Examination of

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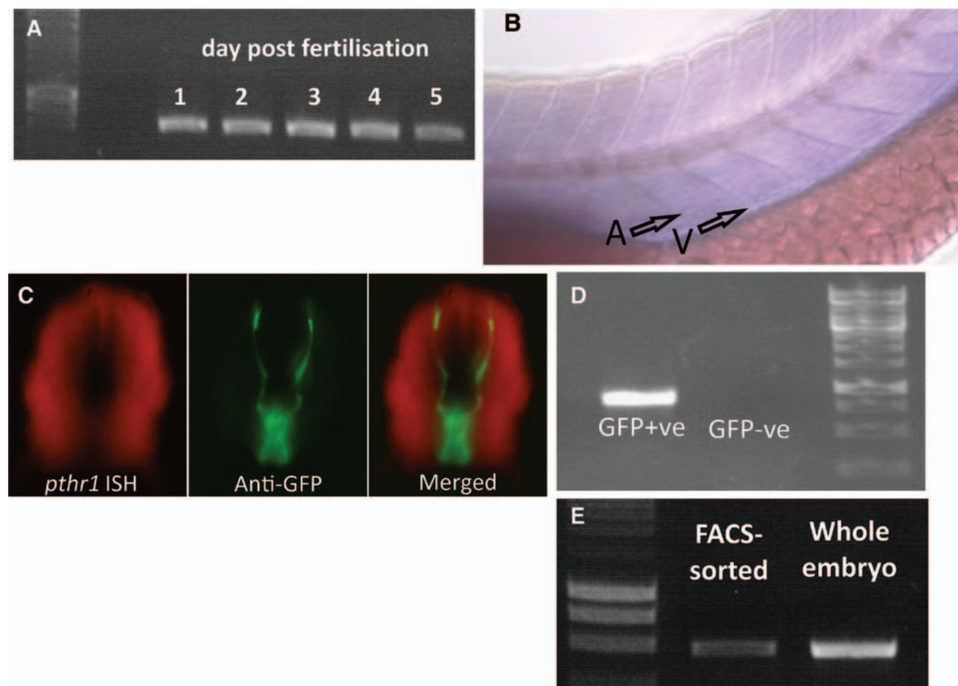

Figure 1. *pthr1* is ubiquitously expressed during embryonic development but is expressed in isolated endothelium. **A**, rt-PCR for *pthr1* of whole embryo RNA in 1-5d postfertilization embryos, demonstrating *pthr1* expression at all time points. **B**, Whole mount *pthr1* in situ hybridization in 2 dpf embryo, showing trunk. Head is to the left. A indicates site of aorta; and V, site of cardinal vein. **C**, Combined fluorescent in situ hybridization for *pthr1* and anti-green fluorescent protein (GFP) immunostaining in 2 dpf *Fli1:GFP* transgenic embryo sectioned at mid-trunk. **D**, rt-PCR for *VE-Cadherin* expression in fluorescent automated cell sorting (FACS)-sorted GFP+ve and GFP−ve cells from 5 dpf *Flk1:GFP-NLS* transgenics. **E**, rt-PCR for *pthr1* in FACS-sorted endothelial cells from 5 dpf *Flk1:GFP-NLS* transgenics, confirming endothelial *pthr1* expression.

pthr1 morphant embryos revealed that compared with control morphants (Figure 2D), *pthr1* knockdown induced a blind-ended aorta with localized loss of endothelium at the site of occlusion (Figure 2E) extending 1 to 2 somites. These data indicate that the aortic defect induced by *pthr1* knockdown is not caused by a failure to lumenize, but rather a primary failure of formation of aortic endothelium at this site. We next performed immunostaining for active Caspase 3, in control and *pthr1* morphant *Flk1:GFP-NLS* embryos at 24 hpf. We colocalized active Caspase 3 with endothelial nuclei but detected no difference in endothelial apoptosis to account for the aortic defects seen in *pthr1* morphants (Figure 2F).

These data confirmed *pthr1* directly plays a role in aortic development. We next attempted to determine the mechanism of this contribution. Specifically, we set out to test whether *pthr1* sits in the same pathway as Sonic hedgehog, VEGF, and Notch.

We first examined whether *pthr1* knockdown impairs aortic formation via a reduction in hedgehog signaling. During vascular development, Sonic hedgehog is secreted from the notochord, which induces VEGF expression.[21] To examine hedgehog signaling, we examined expression of the hedgehog receptor *ptch1* in control or *pthr1* morphants. Because *ptch1* expression is upregulated by hedgehog, its expression is a useful readout of hedgehog signaling. As previously described, we detected *ptch1* expression by in situ hybridization in the neural tube of control morphants, responding to Shh. However, we did not detect any reduction in *ptch1* expression in *pthr1* morphants, either by in situ hybridization (Figure 3A) or by qPCR (Figure 3B), suggesting that the effect of *pthr1* knockdown on aortic formation is not mediated by a reduction in hedgehog signaling.

We next attempted to determine the relationship of PTHR1 with VEGF signaling, which lies downstream of Shh signaling.[21] Incubation of *gridlock* mutants in the VEGF inducer GS4012 completely prevents development of the aortic blockage seen in these mutants.[30] We therefore incubated *pthr1* morphant embryos with the same VEGF inducer in identical conditions ([25 umol/L] immediately postfertilization), but this did not suppress the morphant phenotype (Figure 3C).

Next, we examined whether Notch signaling was impaired in *pthr1* morphants. We used a *Tg(CSL:Venus)qmc61* transgenic expressing the yellow fluorescent protein derivative Venus at sites of Notch signaling during development. Figure 4A shows representative images from 24 hpf control and *pthr1* morphant *Tg(CSL:Venus)qmc61* embryos (just before the onset of circulation, before aortic occlusions could be observed). Control morphants demonstrate Notch signaling in the hypochord (thick arrow) along the length of the embryo. The hypochord is a transient structure sitting immediately between the notochord and the developing aorta, and is believed to contribute to aortic development.[31,32]

Compared with control morphants, *pthr1* morphant embryos (Figure 4B) frequently demonstrated defects in Notch signaling in the hypochord (thin arrow). When we quantified the percentage of embryos with such a defect in a blinded manner, we found that defective Notch signaling could be identified in 58% of PTHR1 morphants (n=40), whereas only 5% (1 embryo from 20) of control morphants had any interruption in hypochordal Notch signaling (Figure 4C). We observed *pthr1* morphants during the following 24 h, and whereas 79% of 2 dpf embryos that had a defect in Notch signaling at 24 hpf (before onset of circulation) had an aortic occlusion at the same site of earlier defective Notch signaling, no *pthr1* morphant with normal Notch signaling (and no control morphant) displayed abnormal aortic development (Figure 4D).

These data suggested that *pthr1* knockdown induces defects in hypochord Notch signaling that could explain the localized aortic defect. We therefore attempted to rescue the defective aortic phenotype in *pthr1* morphants by upregulating Notch signaling. We used a heatshock:Gal4 / UAS:NICD transgenic in which heatshock drives expression of the Notch intracellular domain

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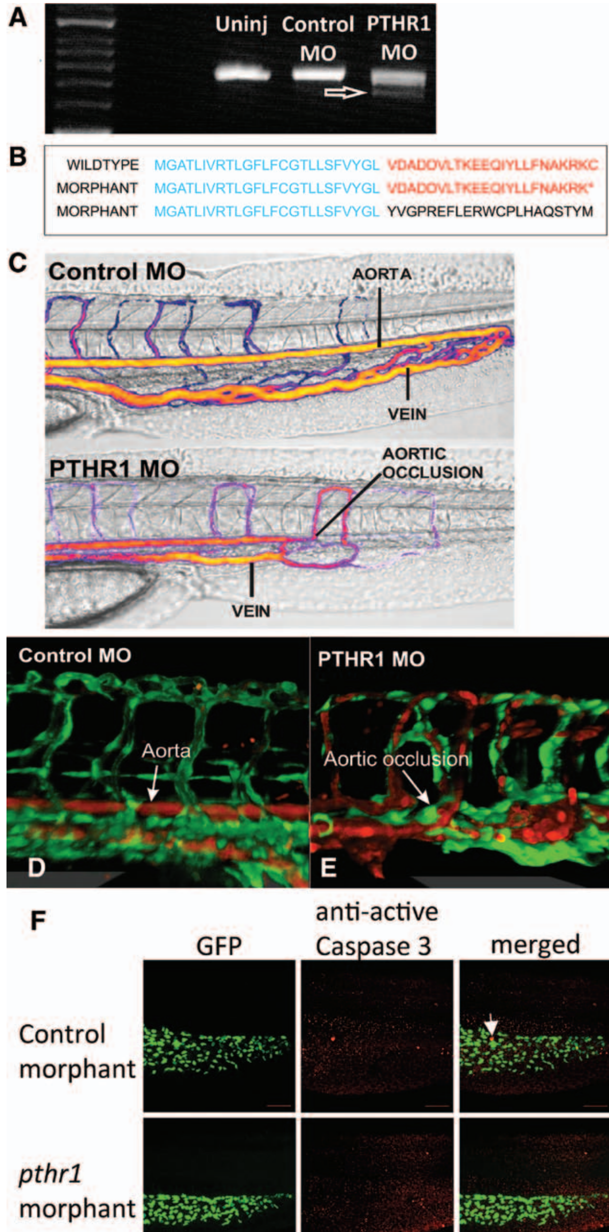

Figure 2. *pthr1* knockdown induces aberrant mRNA splicing and localized aortic occlusion attributable to loss of endothelium. **A**, rt-PCR for *pthr1* mRNA in uninjected embryos (Uninj), control morphants, and *pthr1* splice-blocking morphants, showing reduction in normally spliced *pthr1* mRNA and aberrantly spliced transcripts in *pthr1* morphants (arrow). **B**, Predicted protein sequences of wild-type and 2 representative morphant (aberrantly spliced) *pthr1* transcripts. Exon 1 in blue, exon 2 in red. *Premature stop codon. Black indicates shifted open reading frame. **C**, Digital motion angiograms of representative 2 dpf control and *pthr1* morphant embryos demonstrating aortic occlusion in *pthr1* morphant. Head is to the left. **D**, *Fli1:green fluorescent protein (GFP)/GATA1:dsRED* control morphant showing blood flow in aorta and cardinal vein. **E**, *pthr1* morphant showing blunt-ended occlusion of the aorta with loss of endothelium for 2 somites and diversion of blood flow into intersegmental vessels. **F**, Active Caspase 3 immunostaining in 24 hpf *Flk1:GFP-NLS* transgenic embryos, showing no difference in endothelial apoptosis between control and *pthr1* morphants (1 apoptotic endothelial cell is arrowed in the control morphant). MO indicates morpholinos.

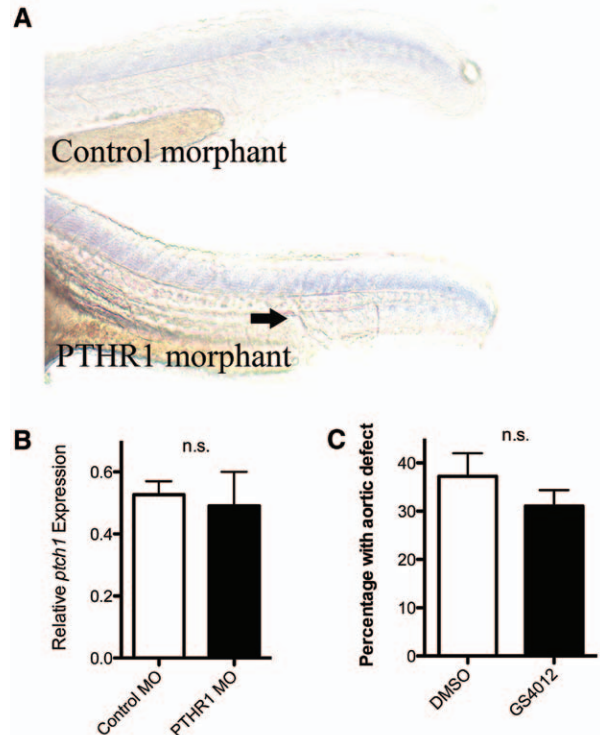

Figure 3. The aortic defect induced by *pthr1* knockdown is not associated with alteration in *ptch1* expression, and cannot be rescued by vascular endothelial growth factor induction. **A**, In situ hybridization for ptch1 in 24 hpf control and *pthr1* morphants. *Ptch1* expression is seen in the neural tube, with no defect at the site of aortic occlusion (arrowed). **B**, Quantitative rt-PCR for *ptch1* expression in control and parathyroid hormone receptor 1 (PTHR1) morphants. **C**, Incubation of *pthr1* morphants in 25 umol/L GS4012 did not significantly reduce the percentage with an aortic defect. MO indicates morpholinos.

responsible for Notch signaling. When we used this system to upregulate Notch signaling in 12 hpf *pthr1* morphants, we did not observe alterations in general embryonic morphology at 24 hpf, but we were able to significantly reduce the proportion of embryos with defective aortae at 2 dpf (Figure 5). Heatshocking wild-type *pthr1* morphants (that do not carry the temperature inducible transgene) had no effect on the percentage of embryos with aortic occlusions (data not shown), indicating that the morphant phenotype is not simply heat sensitive. These results indicate that the aortic defect induced by *pthr1* knockdown is indeed attributable to abnormal Notch signaling.

Finally, we attempted to determine which ligand is responsible for *pthr1* signaling. Zebrafish have 3 *pthr1* ligands; *pth1*, *pth2,* and PTH-related peptide (*pthrp*).[20] Previous work has demonstrated that *pth1* and *pth2* are not expressed in the vasculature of the developing zebrafish.[20] During bone development, hedgehog signaling has been shown to induce *pthrp* expression in developing chondrocytes.[13] We therefore asked whether a similar mechanism regulates vascular development.

When we performed in situ hybridization to examine *pthrp* expression, we observed a similar ubiquitous staining pattern to that of *pthr1* expression, with no vascular (or hypochordal) staining to account for the *pthr1* morphant phenotype (Figure 6A). We then knocked down *pthrp* by morpholino antisense and found that this induced an identical aortic occlusion at the same location as PTH1R knockdown (Figure 6B), with a similar penetrance (Figure 6C). When we examined the effect of *pthrp* knockdown on Notch signaling in the *Tg(CSL:Venus)qmc61* transgenic, this induced defective

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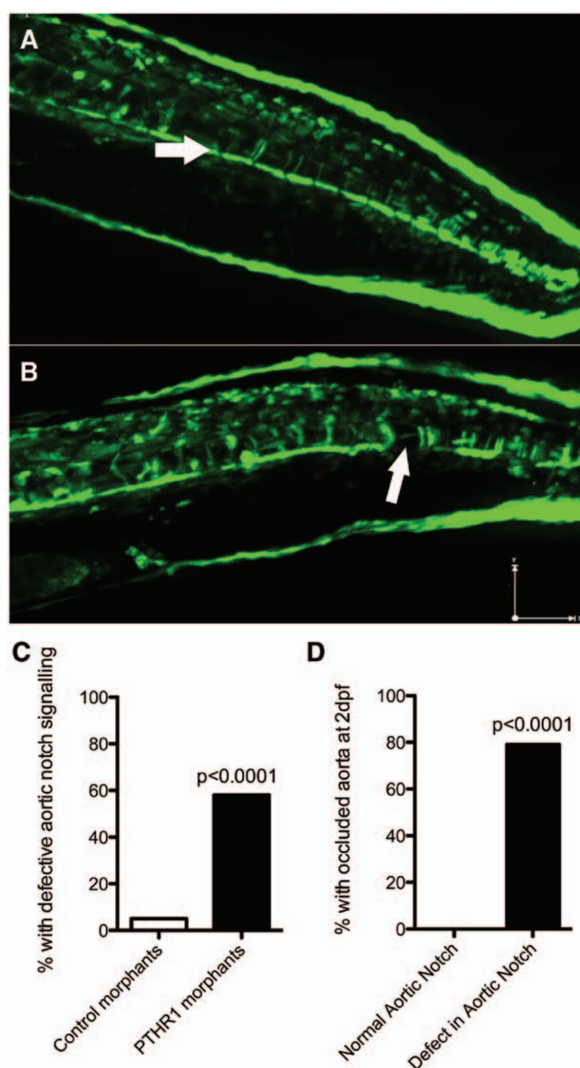

Figure 4. *pthr1* knockdown induces localized defects in hypochordal Notch signaling. **A**, Trunk and tail (head to the left) of 24 hpf *Tg*(*CSL:Venus*)*qmc61* transgenic showing Notch signaling in aorta (thick **arrow**). Scale bar, 100 um. **B**, *pthr1* morphant transgenic showing localized defect in hypochord Notch signaling (thin **arrow**). **C**, Percentage of embryos with defective Notch signaling at 24 hpf. Groups of 20 to 40 control and *pthr1* morphant embryos were observed blinded for defective Notch signaling. Statistical analysis was by χ^2 analysis. **D**, Percentage of *pthr1* morphant embryos with aortic occlusion at 48 hpf. No embryos with normal hypochord Notch signaling at 24 hpf had aortic occlusion at 48 hpf. Statistical analysis was by χ^2 analysis.

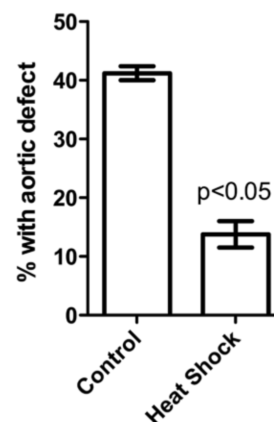

Figure 5. Upregulation of Notch signaling rescues the aortic defect in parathyroid hormone receptor 1 (PTHR1) morphants. *Heatshock:Gal4;UAS:NICD* transgenics were injected with PTHR1 morpholino antisense. At 16 hpf, embryos were either heatshocked to upregulate Notch signaling or sham (underwent the same manipulation at room temperature). Embryos were then observed for aortic defects at 2 dpf. Statistical analysis was by χ^2 analysis. No effect of heatshocking alone was seen in PTHR1 morphants in a wild-type background (ie, the morphant phenotype is not generally heat sensitive).

hypochordal Notch signaling similar to that seen in *pthr1* morphants (Figure 6D). These data suggest that *pthrp* is the ligand responsible for induction of Notch signaling by *pthr1*.

Discussion

We have shown that despite apparently ubiquitous expression during the early stages of embryonic development, morpholino antisense knockdown of either *pthr1* or *pthrp* induces a localized occlusion in the aorta in developing zebrafish embryos. Coupled with the fact that humans with homozygous mutations in PTHR1 have been described to suffer aortic coarctation in association with the other severe skeletal manifestations of Blomstrand chondrodysplasia, these data strongly suggest that PTHR1 is required for correct aortic formation in both humans and zebrafish. The fact that defective aortic formation can be observed in *pthr1* morphant zebrafish before skeletal development and without other abnormalities suggests *pthr1* contributes directly to aortic formation, rather than inducing aortic defects as an effect of other malformations.

The reason for the localized nature of the defect remains unclear. Mutations in *hey2* (*gridlock*) cause a similarly localized (although more proximal) aortic occlusion, although *hey2* is expressed throughout the aorta.[33] It is clear therefore that during aortic development, some locations are more sensitive to genetic perturbation. We found no evidence to suggest the affected region expresses different levels of *pthr1* or *pthrp*, which might have accounted for our findings. Although it is possible that persistence of maternal mRNA might protect aortic development in its early stages, we saw the same effect with a splice-blocking morpholino, making this less likely. We frequently saw restoration of distal aortic flow via collateral vessels, confirming distal aortic lumenization. Nevertheless, we cannot completely exclude aortic abnormalities distal to the occlusion.

We speculate that rather than affecting a specific part of the aorta, *pthr1* knockdown exerts its effects at a specific time during embryonic development, at which point that portion of the aorta is developing and hence most sensitive. Human aortic coarctation can occur at various sites, including preductally and postductally in the aortic arch but also more distally, including the abdominal aorta, including in association with Alagille syndrome.[34] Although the most obvious phenotype in our studies and in human coarctation is the localized occlusion, it is entirely possible that there are more subtle defects affecting arterial formation generally; this would be supported by the 5-fold increased risk of cerebral aneurysms in patients with aortic coarctation.[35]

Although we have shown that *pthr1* is expressed in isolated endothelial cells, the ubiquitous expression of both the receptor and *pthrp* make nonendothelial cell autonomous explanations possible. Again, we can only speculate on these potential

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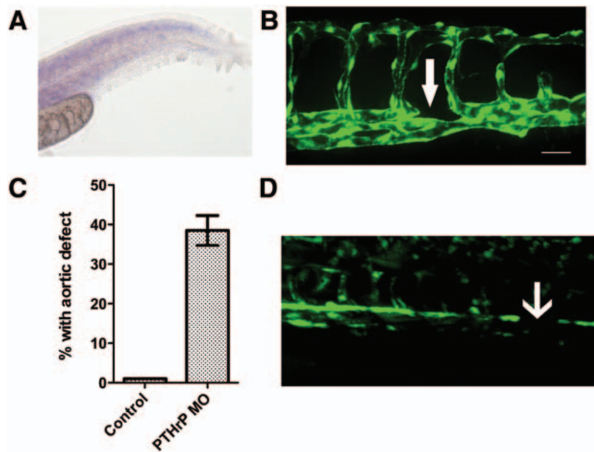

Figure 6. *pthrp* knockdown induces an identical aortic defect to *pthr1* knockdown associated with defective Notch signaling. **A**, In situ hybridization for *pthrp* of 24 hpf zebrafish embryo showing ubiquitous trunk expression. **B**, When *pthrp* was knocked down in Fli1:GFP embryos by morpholino antisense, this induced an aortic occlusion attributable to lack of endothelium (**arrow**) identical in appearance and site to that seen in *pthr1* morphants (Figure 2). Scale bar represents 100 um. **C**, Penetrance of abnormal aortic phenotype in *pthrp* morphants compared with control morphants. **D**, *pthrp* knockdown induces a localized defect in hypochordal Notch signaling (**arrow**) identical to that seen in *pthr1* morphants. MO indicates morpholinos.

explanations, and our ability to explore these mechanisms via endothelial specific gene knockdown has been thus far unsuccessful. The aorta at the stages detailed possesses very few, if any, pericytes, which appear around 72 hpf.[36] Nevertheless, it is possible that reductions in Notch ligand expression in structures surrounding the developing aorta could explain our findings.

We attempted to place PTHR1 within the Shh-VEGF-Notch pathway. Both *pthr1*and *pthrp* knockdown induced a localized defect in hypochordal Notch signaling at the site of the future aortic occlusion. Global upregulation of Notch signaling significantly rescued these aortic occlusions, suggesting that *pthr1*/*pthrp* signaling controls Notch expression to orchestrate correct aortic formation, at least at the site of the observed reduction in Notch signaling and subsequent aortic defect. *Pthr1* is unlikely to lie upstream hedgehog in this pathway, as a reduction in *ptch1* expression would be expected if hedgehog signaling is perturbed by *pthr1* knockdown. Equally, the failure to rescue the phenotype by small molecule induction of VEGF (a manipulation which completely rescues the *gridlock* mutant) suggests *pthr1* does not lie upstream VEGF to drive aortic formation.

Many questions remain about the contribution of *pthr1* to aortic formation, particularly whether this is a cell autonomous effect, and the mechanism of dowregulation of Notch signaling. The reason for the localized nature of the aortic defect is unclear, given the ubiquitous nature of *pthr1* and *pthrp* expression. We are currently attempting to dissect these mechanisms. Nevertheless, our study is the first to prove a role for PTHR1 in aortic formation and to link PTHR1 with Notch signaling during vascular development.

We were unable to rescue the phenotype by mRNA injection, but are confident that the morphant phenotype is specific to *pthr1* knockdown rather than off target effects. This is based on the observations that 2 nonoverlapping (start and splice) MOs induce the same phenotype; knockdown of the ligand induces the same phenotype (which we have never observed with multiple other MOs); and that the phenotype can be rescued by upregulation of Notch signaling. We have been unsuccessful in identifying stable mutants of *pthr1* or *pthrp* by TILLING, although the use of zinc finger nucleases[37] may now make this possible. Nevertheless, the data obtained using MO seem sufficiently robust to support our conclusions. Indeed, the partial nature of the gene knockdown achieved with MO may be a better model of the effect of hypomorphic or haploinsufficient human mutations, such as those responsible for Alagille syndrome[38] than the total gene deletion achieved using murine knockouts. It is noteworthy that the *Hey2* mutation responsible for the *gridlock* mutant is hypomorphic and in fact reducing *Hey2* expression still further leads to widespread failure of aortic formation without a localized occlusion.[25] Although the *gridlock* mutant was originally described as a model of human aortic coarctation,[23] the mouse *Hey2* knockout does not display a coarctation, nor has any human case of aortic coarctation been associated with defects in *Hey2*. Given the incidence of CoA in Blomstrand chondrodysplasia, the *pthr1* morphant has arguably a better claim to being a model of CoA. However, although coarctation simply means occlusion, we do not contend the abnormality seen in *pthr1* morphants is the same as in human CoA. We do however consider it likely that some conserved function of PTHR1 underlies both the CoA in Blomstrand chondrodysplasia and the defects seen in our study, and that this is likely to mechanistically be linked to the Notch pathway.

Previous work has shown that *pthr1* signaling induces Notch signaling in other organ systems, such as the osteoblast[39] and periodontal ligament,[40] but ours is the first report of a similar function in the vasculature. Because Alagille syndrome is also associated with defective Notch signaling, we speculate that the Notch pathway may represent a final common pathway of aortic development that could be affected even in non-Mendelian or sporadic CoA. Supporting this is the finding that Notch mutations can be found in a significant proportion of adults with bicuspid aortic valve,[41,42] a common congenital abnormality that frequently coexists with CoA. We consider that this pathway is therefore an excellent candidate for examination during future attempts to delineate the causative mechanisms of congenital defects in aortic formation.

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Disclosures

None.

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Significance

We show that parathyroid hormone receptor 1 is required for aortic formation via a Notch-dependent mechanism. This provides insight into the aortic coarctation seen in Blomstrand chondrodysplasia (caused by parathyroid hormone receptor 1 mutations) and underlines the importance of the Notch pathway for aortic formation. Our study also highlights the usability of zebrafish for reverse genetic studies examining effects on vascular formation.

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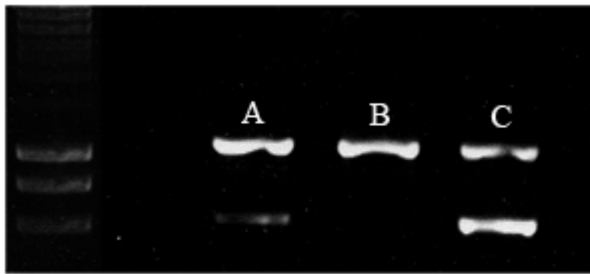
Supplemental Movie I. Light microscopy of 2dpf control morphant embryo. Arrow indicates aorta.
Supplemental Movie II. Digital subtraction movie of 2dpf control morphant embryo.
Supplemental Movie III. Light microscopy of 2dpf PTHR1 morphant embryo. Thick arrow indicates aorta, thin arrow indicates site of occlusion.
Supplemental Movie IV. Digital subtraction movie of 2dpf PTHR1 morphant embryo.



Supplemental Figure I. PTHR1 morphants without aortic abnormalities do not demonstrate aberrant PTHR1 transcripts.
PTHR1 morphants at 48hpf were separated into groups with either normal or abnormal aortic formation. Total RNA was extracted and rt-PCR for PTHR1 performed. A: unsorted PTHR1 morphants. B: PTHR1 morphants with normal aortae. C: PTHR1 morphants with abnormal aortae. Aberrently spliced mRNA can only be detected in those PTHR1 morphants with abnormal aortic formation, indicating that the morpholino has not been effective in those embryos with normal aortae.

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Materials and Methods

All studies conformed to the institutions ethical requirements and were performed under UK Home Office license 40/3434.

Zebrafish husbandry and transgenic lines

Adult zebrafish were housed in groups of 40 mixed males and females at 28°C on a 12hr light/dark cycle. To obtain embryos, adults were either pairmated or clutches from the entire tank were obtained by placement of a marble tank. We used the following transgenic lines; *Fli1:GFP* expressing GFP in endothelial cytoplasm[1], *Flk1:GFP-NLS* in endothelial nuclei[2], *GATA1:dsRED* expressing dsRED in erythrocytes[3], *Tg(CSL:Venus)qmc61* expressing the YFP derivative Venus driven by concatemerised CSL binding sites. CSL is a transcription factor that mediates upregulation of Notch responsive genes; this transgenic therefore expresses Venus at sites of Notch signalling, *hsp70:Gal4/UAS:NICD*, which upregulates the Notch intracellular domain (and hence Notch signalling) in response to heatshock[4, 5].

Isolation of zebrafish endothelial cells

Endothelial cells were isolated by FACS from dissociated *Flk1:GFP-NLS* embryos as described previously[6].

Morpholino antisense knockdown

We used the following morpholino antisense oligonucleotides (MO) (Gene-Tools); *pthr1* translation blocking: ATCCCGACGCAAGTTCACGCAAATG, *pthr1* splice blocking: GAAAACCTTTGGAACTTACCAGACG, PTHrP splice blocking: AAAGCCACAGACACTTACATTCGGC, Control: CCTCTTACCTCAGTTACAATTTATA

Each MO was injected into one-cell embryos (0.5nl of 0.83mmol). Vascular development was assessed in groups of 30-40 embryos by stereomicroscopy, spinning-disk confocal microscopy, and digital motion analysis as previously published[3]. All experiments were performed at least twice (in triplicate for assessment of aortic defects).

In situ hybridisation and rt-PCR

pthr1 and *ptch1* expression was assessed by rt-PCR and *in situ* hybridisation either alone or with co-immunostaining as described previously[7]. The following primers were used for PCR; PTHR1 Forward: CAG CAC ATT TGC GTG AAC TT Reverse: GAA ACA GGT GCA TGT GGA TG, PTHrP Forward: CCA GCA GTC AGT TGG TCA GA Reverse: AAG GTC AGC AGC ACC TTG AT. Quantitative rt-PCR for *ptch1* was performed as previously described[3, 7].

Active caspase 3 staining

Control and *pthr1* MO-injected *flk1:EGFP-NLS* embryos were fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Following washes in PBS containing 0.1% Tween 20 (PBST) and in 1% dimethylsulfoxide in PBST (PDT), embryos were permeabilised in 0.3% Triton X-100 in PDT, at room temperature for 20 min. After washes in PDT, the embryos were incubated in blocking solution (5% fetal calf serum and 2 mg/ml bovine serum albumin in PDT) at room temperature for 1 hour. The embryos were then incubated at 4ºC overnight with rabbit anti-human/mouse active caspase 3 antibody (R&D Systems), diluted 1:200 in blocking solution. Following washes in PDT, the embryos were incubated in blocking solution at room temperature for 1 hour before incubation for 2 hours at room temperature in Alexa Fluor 546 goat anti-rabbit secondary antibody (Life Technologies), diluted 1:500 in blocking solution. The embryos were then extensively washed in PBST and mounted in Vectashield mounting medium for fluorescence (Vector Laboratories). Imaging was performed using an Olympus FV1000 laser scanning confocal microscope with a 40x (numerical aperture (NA) 1.0) oil immersion objective.

The effect of small molecule VEGF induction on *pthr1* morphants

Groups of 30-40 *pthr1* morphants were incubated in various doses of the VEGF inducer GS4012 (Calbiochem) or DMSO from immediately post morpholino injection (2 cell stage) to 2dpf as previously described[8]. The percentage of each group with abnormal aortic development was observed

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blinded by stereomicroscopy. Each group was considered as a single replicate and repeated at least three times.

The effect of Notch induction on *pthr1* or *pthrp* morphants

hsp70:Gal4/UAS:NICD transgenic or wildtype embryos were injected with the *pthr1* or *pthrp* morpholino. At 12hpf, groups of 30-40 morphants were placed in thermal cycler tubes in a heatblock. After initial incubation at 22°C, embryos were heated for 10 minutes at 40°C to activate the heatshock responsive transgene. The percentage of each group with abnormal aortic development was observed blinded by stereomicroscopy. Each group was considered as a single replicate and repeated at least twice.

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