Arap3 is dysregulated in a mouse model of hypotrichosis–lymphedema–telangiectasia and regulates lymphatic vascular development

Joëlle Kartopawiro1, Neil I. Bower1, Tara Karnezis3,4, Jan Kazenwadel2, Emmanuelle Lesieur1, Katarzyna Koltowska1, Jonathan Astin5, Philip Crosier5, Sonja Vermeren6, Marc G. Achen3,4, Steven A. Stacker3,4, Kelly A. Smith1, Natasha L. Harvey2,7, Mathias François1,* and Benjamin M. Hogan1,∗

1Division of Molecular Genetics and Development, Institute for Molecular Bioscience, The University of Queensland, St Lucia, QLD 4072, Australia, 2Division of Haematology, Centre for Cancer Biology, SA Pathology, Adelaide, Australia, 3Tumour Angiogenesis Program, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia, 4Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, VIC 3010, Australia, 5Department of Molecular Medicine and Pathology, School of Medical Sciences, The University of Auckland, Auckland, New Zealand, 6MRC Centre for Inflammation Research Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, UK and 7Discipline of Medicine, University of Adelaide, Adelaide, SA 5005, Australia

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Mutations in SOX18, VEGFC and Vascular Endothelial Growth Factor 3 underlie the hereditary lymphatic disorders hypotrichosis–lymphedema–telangiectasia (HLT), Milroy-like lymphedema and Milroy disease, respectively. Genes responsible for hereditary lymphedema are key regulators of lymphatic vascular development in the embryo. To identify novel modulators of lymphangiogenesis, we used a mouse model of HLT (Ragged Opossum) and performed gene expression profiling of aberrant dermal lymphatic vessels. Expression studies and functional analysis in zebrafish and mice revealed one candidate, ArfGAP with RhoGAP domain, Ankyrin repeat and PH domain 3 (ARAP3), which is down-regulated in HLT mouse lymphatic vessels and necessary for lymphatic vascular development in mice and zebrafish. We position this known regulator of cell behaviour during migration as a mediator of the cellular response to Vegfc signalling in lymphatic endothelial cells in vitro and in vivo. Our data refine common mechanisms that are likely to contribute during both development and the pathogenesis of lymphatic vascular disorders.

INTRODUCTION

Defects in the structure and function of lymphatic vessels can result in an accumulation of fluid in tissues and the interstitium of the body, leading to a condition known as lymphedema. Heritable lymphedema disorders of characterized aetiology include, among others, lymphedema-dystichiasis syndrome (LD, OMIM 153400), Hennekam syndrome (OMIM 235510), Milroy disease (MD, OMIM 153100), Milroy-like primary lymphedema (1) and hypotrichosis–lymphedema–telangiectasia (HLT, OMIM 607823) and occur due to impaired formation of a normal lymphatic vasculature in the developing embryo. The genes responsible for these syndromes include FOXC2 (LD) (2), CCBE1 (Hennekam syndrome) (3,4), VEGFR3 (MD) (5), VEGFC (Milroy-like primary lymphedema) (1) and SOX18 (HLT) (6,7).

In vertebrate embryos, lymphatic vessels arise during development from pre-existing veins by a process known as lymphangiogenesis. First, a subset of venous endothelial cells (VECs) in the dorsal walls of the cardinal veins is specified to the lymphatic lineage (7,8). These lymphatic endothelial cell (LEC) precursors emerge from the vein and, in mice, give rise to lymph sacs that are
thought to subsequently mature into deeper lymphatic vessels. The subcutaneous lymphatic vascular network is thought to form from a population of migratory superficial LECs, which derive at least in part from a structure known as the peripheral longitudinal lymphatic vessel (PLLV) [see 9,10,11,16].

Vascular endothelial growth factor receptor 3 (VEGFR3) and its ligand VEGF are key regulators of lymphatic vascular development. VEGFR3 is expressed in blood vessels and LEC precursors sprouting from veins and becomes enriched in LECs at ~11.5 dpc [12]. Vegf mRNA is expressed by mesenchymal cells adjacent to VEGFR3-expressing endothelial cells (ECs) and is required for LEC precursors to exit the veins [13–16]. Homozygous Vegf-deficient mice fail to assemble a lymphatic vasculature [15] and correspondingly, expression of a VEGFR3 ligand trap in skin blocks lymphangiogenesis [17], while ectopic expression of Vegf stimulates lymphangiogenesis [18].

LympA function is conserved in zebrafish models, where depletion of Vegf or inactivation of Vegfr3 kinase domain activity, results in a complete loss of all lymphatic vessels [19–21]. Finally, in humans, MD, otherwise known as hereditary lymphedema type I, and Milroy-like primary lymphedema result from mutations in VEGFR3 and VEGF, respectively, and are characterized by lymphedema of developmental origin [1,5].

A number of key transcription factors regulate the early commitment and specification of ECs to the lymphatic lineage (reviewed in [22]). Sox18 is expressed in the embryonic blood vascular endothelium [23] and is required for the specification of LECs from VECs, directly activating Proxl transcription in the embryonic veins [7]. In zebrafish, sox18 acts redundantly in vascular development with sox7 to regulate the early differentiation of arteries and veins [24] and recent work indicates that sox18 and vegfc genetically interact during lymphangiogenesis [25]. HLT patients are characterized by lymphedema, cutaneous telangiectasias, other abnormal dilations of superficial vasculature in hair follicles [6,7,26]. Sox18 mutations documented in HLT include missense mutations in the transcription domain of Sox18 that introduce frame shifts, and generate dominant-negative forms of Sox18 [27]. Mouse phenotypes due to spontaneous mutations of the Ragged (Ra) allelic series closely resemble the phenotypes seen in HLT. While homozygous mutants are embryonic lethal and fail to initiate lymphangiogenesis, adult heterozygous mutants develop abnormal subcutaneous lymphatic vessels that are tortuous, thin and increased in both number and density [7]. The underlying cause of the Ra phenotypes, as in HLT, is mutations in Sox18 that result in dominant inhibitory Sox18 protein variants [7,26,28,29].

Although the developmental role of Sox18 is becoming more defined, the molecular mechanisms of vascular pathology in HLT and their relationship to other lymphatic vascular pathologies remain unexplored. Taking advantage of the utility of the Ragged Opossum (Raop) mouse model, we performed an expression-based screen to identify functionally relevant genes dysregulated in Raop/-/ mutant vessels. Using mouse, zebrafish and primary LEC models, we identified Arap3 as a novel regulator of lymphatic vascular development. Arap3 (ArfGAP with Rhogap domain, Ankyrin repeat and Ph domain 3) is a GTPase activating protein (GAP) for the small GTPases RhOA and ARF6. Arap3 is controlled by phosphoinositide 3-OH kinase (PI3K), which regulates both the catalytic activity and subcellular localization of Arap3 [30,31]. Upon activation of PI3K, Arap3 translocates to the plasma membrane, where its substrates RHOA-GTP and ARF6-GTP are localized. RAP-GTP, which also resides at the plasma membrane, is a further regulator of Arap3’s RHO GAP activity. Work with transfected and knock-down cells, as well as with primary Arap3−/− cells demonstrated that Arap3 is a regulator of cell adhesion and migration. In this context, the RHO GAP activity appears to be particularly relevant [32–35]. During vascular development, Arap3 has been shown to be essential in the formation of the blood vasculature [36]. How Arap3 function integrates into known signalling pathways, and whether Arap3 also regulates lymphatic vascular development or pathological processes in lymphatic endothelium remains unexplored.

Overall, our study finds that Arap3, acts downstream of Vegfc during lymphatic vascular development. Arap3 modulates LEC migratory behaviour and is essential for embryonic lymphatic development. Its down-regulation in the heterozygous Raop+/− model of HLT may contribute to vascular endothelial pathogenesis in that context. Delineating the molecular regulators of developmental and pathological lymphangiogenesis is a necessary precursor to the development of therapeutic strategies able to effectively target vascular pathologies.

RESULTS

Arap3 expression is reduced in Raop+/− subcutaneous lymphatic vessels

In order to identify novel modulators of pathological lymphangiogenesis, we took advantage of the Raop mouse model of HLT. Homozygous Sox18Raop−/− is embryonic lethal at 14.5 dpc with a failure of all lymphatic vascular development, precluding the use of these animals to sort adult LECs. We isolated LECs from adult ear skin of 30 wild-type and 30 heterozygous Sox18Raop+/− mutant mice using FACs analysis, based on their expression of PODoplanin and Pan-Endothelial Cell Adhesion Molecule 1 (PECAM-1) (PODOPLANIN+/−/PECAM-1+) (Fig. 1A, red box in graphs). A microarray analysis was performed using an Affymetrix platform and a list of genes was generated that was dysregulated in heterozygous Sox18Raop+/− mutant LECs compared with wild-type (data not shown). Classic LEC markers were found down-regulated in Sox18Raop+/− LECs, validating the specificity of the cell population isolated and indicating the defective nature of the mutant LECs (Fig. 1B). Given that these known LEC markers showed a 1–2-fold down-regulation, it was interesting to observe many genes that displayed changes in expression level of a much greater magnitude. Selecting genes with a fold change of ≥5, we based our further expression analysis on 289 genes that were putatively dysregulated in Sox18Raop+/− LECs (data not shown).

To identify the subset of genes most likely to be involved in vessel formation, we examined expression patterns of the top 100 dysregulated genes in zebrafish embryos during development. Sixteen genes were identified as having strong vascular expression (Fig. 1B, Supplementary Material, Fig. S1A). Subsequent loss-of-function screening by morpholino injection for seven of these selected genes (Supplementary Material, Fig. S1B), identified a robust requirement for one candidate
Figure 1. Arap3 expression is decreased in skin lymphatic vessels of 
RαOp−/− mutant mice. (A) Upper panels: FACS analysis of isolated LECs from ear 
skin tissue of wild-type (left panel) and 
mutant mice. (B) Heat map depicting dysregulated genes in 
pure LECs from RαOp−/− adult mice (right panel). The red box indicates LECs sorted for cell surface expression of PODOPLANIN and PECAM-1. Lower panels: skin lymphatic vessels stained for PODOPLANIN in RαOp−/− mice appeared abnormal and dysfunctional compared with wild-type as previously described (7). (B) Heat map depicting dysregulated genes in pure LECs from RαOp−/− mutant mice compared with wild-type (up-regulation = yellow, down-regulation = blue). Lymphatic markers, Prox1, Lyve1, Nrp2, Foxc2, Efnb2, Vegfc, Vegfr3 and Syk, were down-regulated in RαOp−/− vessels (left panel). Genes that were identified as expressed in zebrafish embryonic vasculature were misexpressed in RαOp−/− mutant vessels, including Arap3 (9.44-fold down-regulated, right panel). (C and D) Quantitative-PCR analysis of mRNA isolated from PECAM-positive ECs sorted from wild-type and RαOp−/− ear skin. Results were normalized against Prox1 and Vegfr3 (lymphatic markers) and validated the down-regulation of Arap3 gene expression in RαOp−/− mutant animals.

Arap3 expression is increased in the developing vasculature and LECs 
independent of Sox18 or Vegfc function

We examined the expression profile of arap3a during zebrafish 
embryonic development (Fig. 2Ai–iv). In zebrafish, two copies of 
arap3 exist, arap3a and arap3b. In situ hybridization (ISH) 
analysis showed that arap3a was enriched in somites at the 10 
somite stage (ss) (Fig. 2Ai). Furthermore, we observed that 
arap3a was enriched in neurones (Fig. 2Aii), intersegmental 
veins (ISVs), the dorsal aorta (DA) and posterior cardinal 
vein (PCV) at later embryonic stages (21 ss, 28 and 48 hpf, 
respectively) (Fig. 2Aiii–iv). In contrast, arap3b was ubiquitously 
expressed in the head with no expression observed in the vasculature (Supplementary Material, Fig. S2A–E).

In order to quantify arap3a expression specifically in developing 
zebrafish LECs, we took advantage of a recently described transgenic line expressing DsRed in embryonic veins and lymphatics Tg(lyve1:DsRed) (37) crossed to a transgenic line select-
ively expressing EGFP in blood vessels Tg(6.5kdrl:EGFP) (38). We used FACS to sort populations of LECs and VECs from whole embryo cell suspensions (data not shown, Supplementary Material, Methods). kdrl was enriched in VECs compared with LECs at all-time points as expected (Fig. 2B). In contrast, arap3a was enriched in both VECs and LECs at 3 and 5 dpf, but not at 7 dpf (Fig. 2C), time points that correspond to LEC migration and vessel formation in zebrafish.

To examine whether arap3a is regulated by sox18, analysis of 
arap3a expression was performed in sox18/sox7 double 
mutants at ~21 ss and revealed normal vascular expression com-
pared with uninjected controls (Fig. 2Ei–ii). In addition, normal 
expression of sox18 was observed at 24 hpf in arap3a morphants (Fig. 2Ev–vi). To determine whether the vegf or vegfc/vegfr3 
signalling pathways regulate arap3a expression, we looked at the expression of arap3a in morphant embryos depleted of 
plc-gamma and mutant for vegfr3 (21) at ~20 ss. We found no 
changes in arap3a transcript expression (Fig. 2Eiii and iv).

In mouse embryos, Arap3 expression was examined at 
11.5 dpc by ISH. LEC precursors actively sprouting from the 
dorsal-lateral walls of the cardinal veins in response to 
Vegfc (8) were found to express Arap3, confirmed by co-staining 
with PROX1 (Fig. 2Di–ii). Analysis of Arap3 expression in 
RαOp−/− mutant mouse embryos showed normal expression in 
various endothelial beds such as veins, arteries or endocardium 
that normally express Sox18 at that stage (11.5 dpc) (Fig. 2F, 
black arrowheads). In addition, HUVECs treated with VEGFC 
with or without SOX18RαOp−/− protein showed no significant 
changes in ARAP3 transcripts levels (Supplementary Material, 
Fig. S3). Together, these data indicate that while it is down-
regulated in RαOp−/− lymphatic vessels, Arap3 transcription is 
not directly regulated by Sox18 or Vegfc.

Arap3a depletion in zebrafish leads to reduced thoracic 
duct formation

We targeted transcripts encoding both arap3 paralogues (arap3a 
and arap3b) with morpholino oligomers (MO) and looked at po-
tential blood vascular defects at 48 hpf (data not shown) as well
as the formation of the thoracic duct (TD) at 5 dpf (Fig. 3). We used both a translation blocking (ATG) and a splice blocking (Spl) morpholino to target *arap3a* and validated the splice blocking morpholino by Q-PCR (Supplementary Material, Fig. S4). Uninjected control embryos displayed normal development of the blood vasculature at 48 hpf and lymphatic vessel development at 5 dpf (Fig. 3A–A′, arrowheads). *arap3a*-depleted embryos displayed significantly reduced or absent TD development (Fig. 3B–B′, asterisks), but overall grossly normal blood vasculature. Quantification of TD extent showed that the observed loss of TD in *arap3a* morphants was highly significant (Fig. 3E). In *arap3b* morphants, quantification of PLs and TD formation showed no difference compared with uninjected control embryos (Supplementary Material, Fig. S2F–G). Since *arap3b* showed no restricted developmental expression and morphants showed normal blood and lymphatic vessels, no further functional studies were undertaken for this gene.

**Figure 2.** *Arap3* is expressed in the developing vasculature of mouse and zebrafish independent of Sox18 or VegfC function. (A) *arap3a* in situ expression profile during zebrafish embryonic development, displaying vascular expression. (i) At 10 ss, *arap3a* was enriched in somites (arrow), (ii) at 21 ss in neurones (black arrowheads), the DA and PCV. At later embryonic stages, (iii) 28 hpf and (iv) 48 hpf, enriched expression of *arap3a* was observed in ISVs (open arrowheads). (B and C) FAC sorted LECs (L) from whole Tg(−6.5kdrl:EGFP; lyve1:DbRed) zebrafish embryo extracts (3, 5 and 7 dpf) show reduced expression of kdrl (B) and robust expression of *arap3a* (C) compared with veins (V). (D) Enriched *Arap3* mRNA expression in mouse LEC precursors migrating from the dorso-lateral wall of the cardinal vein in sections (11.5 dpc embryos, white arrowheads) (i), with PROX1 co-localization (immunofluorescence blue staining) (ii). (E) Depletion of Plcγ and Sox18/7 or mutation of vegfr3 does not affect *arap3a* expression in developing zebrafish (Panels i–iv). Similarly, depletion of Arap3a does not affect sox18 expression in zebrafish embryos (Panels v and vi). (F)ISH revealed that *Arap3* transcript is normally expressed by arterial, venous and heart ECs in *RaOp2−/−* homozygous mouse embryos (black arrowheads). Error bars indicate mean ± SEM. DA, dorsal aorta; PCV, posterior cardinal vein; L, lymphatic; V, vein; CV, cardinal vein.

*arap3a* morphants have reduced numbers of parachordal lymphangioblasts but normal intersegmental veins

Following the observation of TD loss in *arap3a*-depleted zebrafish embryos, we examined whether this was due to a defect in the formation of precursor LECs (parachordal lymphangioblasts (PLs)), which bud from the cardinal vein at 32 hpf and are found at the horizontal myoseptum (HM) at ~54 hpf (4,39). Using the Tg(fli1a:EGFP;−6.5kdrl:mCherry) transgenic line, we compared uninjected controls with embryos injected with *arap3a* ATG or splice blocking morpholinos (Fig. 3C, D and F). In uninjected embryos, a population of PLs was observed at the HM at 58 hpf (PLs scored across 10 body segments) (Fig. 3C). In contrast, *arap3a* morphants showed significantly fewer PLs at the HM (Fig. 3D–F).

Zebrafish mutants in the Vegfc/Vegfr3 pathway all show a block in venous sprouting (secondary angiogenesis), failing to
give rise to both venous intersegmental vessels (vISVs) and PLs (20,40). To address whether this was the case in arap3a morphants, we assessed the number of intersegmental arteries (aISVs) compared with vISVs that had formed at 3 dpf using the Tg(fli1a:EGFP; -0.8flt1:tdTomato) transgenic line (Fig. 3G, Supplementary Material, Fig. S5A). We first scored PL formation, confirming that arap3a morphants showed reduced PLs. However, at 3 dpf the same morphants displayed normal aISVs and vISVs (Supplementary Material, Fig. S5A). We also looked at markers of arterial and venous identity in arap3a morphants. Normal expression of arterial markers hey2 and efnb2a, and venous markers dab2 and coupTFII, was observed (Supplementary Material, Fig. S5B). Taken together, these data suggest that the loss of TD formation observed in arap3a zebrafish morphants is due to a specific defect in PL formation. In the absence of a specific marker strain to examine LEC specification in zebrafish (41), defects in either migration or specification of LECs may explain this phenotype.

Figure 3. arap3a is required for TD formation and parachordal lymphangioblast migration in zebrafish. (A, A’) Uninjected control embryos Tg(fli1a:EGFP; -6.5kdrl:mCherry) display normal development of blood and lymphatic vessels (white arrowheads indicate the TD). (B, B’) arap3a morphants (splice MO) display reduced TD formation (asterisks) but grossly normal blood vasculature. (C) Uninjected Tg(fli1a:EGFP; -6.5kdrl:mCherry) control embryos display PLs (white arrowheads) at the HM at 58 hpf. (D) arap3a splice morphants (asterisks) display reduced PL formation. (E) Quantification of TD formation in uninjected controls versus arap3a ATG and splice morphants (Spl) at 5 dpf. TD extent/ per somite was 96% in control embryos (n = 59), ATG morphants 33% (n = 43) and splice morphants 14% (n = 52). Error bars indicate mean ± SEM. (F) PL quantification in morphants shows significantly reduced numbers of PLs compared with uninjected embryos, scored across 10 somite segments (ANOVA statistical analysis, P < 0.0001). (G). In morphant embryos, scores reveal normal formation of aISV’s (P = 0.94) and vISV’s (P = 0.65) but reduced PL formation (p < 0.0001, n = 23 embryos) in the Tg(fli1a:EGFP; -0.8flt1:tdTomato) strain. Error bars indicate mean ± SEM. DA, dorsal aorta; PCV, posterior cardinal vein; UIC, uninjected control; MO, morpholino oligomer. DLAV, dorsal longitudinal anastomotic vessel; ISV, intersegmental vessel.

Arap3 is required cell-autonomously for development of the dermal lymphatic vasculature in mice

Previous studies in mouse showed an important role for Arap3 in angiogenesis (36), but lymphatic vessels were not examined.
Arap3−/− mice showed severe blood vascular defects and were embryonic lethal by 11 dpc. Moreover, blood endothelial loss of Arap3 function (Arap3fl/fl–Tie2Cre+) triggered a phenotype reminiscent of that observed in Arap3-null mice, indicating an EC-autonomous function (36).

To specifically disrupt Arap3 function at the initial stage of lymphangiogenesis, we took advantage of an inducible Cre line, Sox18CreERT2/+ (42), that enables temporal induction of Cre activity in ECs, including in the precursor LEC compartment. The efficacy of Cre-mediated excision using this line was validated by crossing with Rosa26R Cre reporter mice (43) (Supplementary Material, Fig. S6A). To assess whether conditional deletion of the Arap3 gene in LECs would cause lymphatic defects, we generated Sox18CreERT2+/-:Arap3fl/fl homozygous mice. Tamoxifen was administered intraperitoneally to pregnant females on three consecutive days (11.5, 12.5 and 13.5 dpc) in order to induce efficient excision of exon 6–8 of Arap3 (Supplementary Material, Fig. S6B–D) (36). The gross morphology of Arap3 mutants at 14.5 dpc was normal compared with wild-type embryos (Supplementary Material, Fig. S6E). In mice, dermal lymphatic vessels grow from each flank of the embryo towards the dorsal midline, subsequently forming a complete subcutaneous lymphatic vascular network (44). The dermal lymphatic vasculature was examined in both wild-type embryos and homozygous Arap3 mutants at 14.5 dpc by immunofluorescent immunostaining using antibodies directed against the blood vascular endothelial marker ENDO-MUCIN, together with the lymphatic selective markers NEUROPILIN2 and PROX1 (Fig.4A–F). Wild-type mouse embryos showed remodelled blood vasculature (Fig. 4A), as well as lymphatic vessels developing progressively towards the midline of the skin (Fig. 4C). Homozygous Sox18CreERT2+/-:Arap3fl/fl mutants exhibited a dermal vascular network that appeared improperly remodelled towards the midline of the skin where large loops and gaps between vessel branches were observed (Fig. 4B, Supplementary Material, Fig. S6G). In addition, homozygous Arap3 mutants displayed severely abnormal lymphatic vessels (Fig. 4D, Supplementary Material, Fig. S6H). Distance to midline of the lymphatic vascular network was increased in mutants compared with wild-type (Fig. 4G). The vessels that had formed were reduced in overall length, were increased in width (Fig. 4H) and appeared bulbous in morphology (Fig. 4E–F, Supplementary Material, Fig. S6H). These data suggest a role of Arap3 in the sprouting or migration of LECs that drives the outgrowth of lymphatic vessels during mouse embryonic development.

To assess whether initial sprouting of LECs from the cardinal vein was affected upon Arap3 deletion, we characterized homozygous Arap3 mutants at 11.5 dpc after Tamoxifen treatment at 8.5, 9.5 and 10.5 dpc (Fig. 4J–M). Mutant embryos were grossly normal (Supplementary Material, Fig. S6F) and displayed normal expression of PROX1 in the cardinal vein (Fig. 4K and M). Further, Arap3 mutants had initiated sLEC sprouting from the PLLV at this stage (Fig. 4J – L′). Together these observations suggest that Arap3 is not required for specification or the earliest sprouting events but the progression of subcutaneous lymphangiogenesis between 11.5 and 14.5 dpc, which involves extensive migration, proliferation and remodelling of the network.

**ARAP3 is required for VEGFC-induced LEC migration in vitro**

To further characterize the cellular role of ARAP3, we performed RNAi knock-down of ARAP3 in cultured primary human LECs and looked at the response of cells in scratch, as well as transwell migration assays following VEGFC stimulation. A knock-down efficiency of ~80% was achieved 24 and 48 h following ARAP3 siRNA treatment of hLECs, as measured by Q-PCR (Fig. 5A).

The response of cells to VEGFC, during collective cell migration (measured by scratch assays) and chemotactic, single cell migration (measured in transwell assays) was significantly reduced following ARAP3 knock-down in hLECs (Fig. 5B–C). To investigate whether this effect was a result of defective cell polarity, junctional integrity or defective cell morphology we performed immunostaining with Phalloidin (Filamentous actin marker), VE-CADHERIN (cell–cell adhesion marker) and GM130 antibodies (Golgi marker) on hLECs post-scratch assay. No striking differences were observed (Fig. 5D–G). We also measured VEGFC-mediated proliferation in control siRNA and ARAP3 siRNA-treated human LECs. VEGFC-mediated proliferation was unaltered by ARAP3 knock-down (Supplementary Material, Fig. S7).

To further examine the behaviour of LECs upon ARAP3 depletion, a three-dimensional (3D) spheroid model was used (Fig. 5H–J). We observed a significant reduction in spheroid sprout numbers forming in response to VEGFC in ARAP3 siRNA-treated cells compared with controls (Fig. 5J). This assay demonstrated a role in LEC sprouting in three dimensions that is consistent with migration assays and increased distance to the midline of forming subcutaneous vessels in the mutant mice compared with wild-type controls.

**Arap3a deletion partially suppresses vegf-mediated venous angiogenesis in zebrafish**

We next examined in vivo whether arap3a may act as a downstream component of the Vegfc/Vegfr3 signalling pathway by performing Vegfc gain-of-function epistasis studies.
Using a heatshock-inducible vegfc zebrafish transgenic line Tg(hsp70l:Gal4;4XUAS:vegfc) crossed onto the Tg(fli1a:nl-sEGFP) background, we systematically over-expressed vegfc at selected time points during development (e.g. 28 hpf and again at 48 hpf). Previous studies have shown that increased vegfc in the embryo leads to hyperbranching ISVs that form largely between the HM and dorsal longitudinal anastomosing vessel (DLAV) (1,21,45,46) as well as an increase in PLs migrating to the HM (Fig.6A and C).

At 3 dpf, EC nuclei per body segment were scored in non-heatshocked and heatshocked transgenic embryos in the presence and absence of arap3a morpholino (Fig. 6A and C). Transgenic control embryos and arap3a non-heatshocked transgenic morphants displayed no ectopic EC nuclei between the DLAV and HM (Fig. 6A, B and E). arap3a non-heatshocked transgenic morphants showed the previously described loss of PLs at the HM. Heatshocked transgenic embryos (Fig. 6C) displayed an increased number of EC nuclei in somites between the DLAV and the HM (Fig.6C and E, blue bracket), as expected. We also observed large numbers of ectopic EC nuclei at the HM in heatshocked transgenic embryos that were present across nearly all somite segments (Fig.6C and F, PL orange bracket). Furthermore, we observed an increased number of nuclei within the PCV of heatshocked transgenic embryos compared with controls (Fig.6C and G, PCV brown bracket).

Intriguingly, arap3a heatshocked transgenic morphants (Fig. 6D) displayed a similar number of ectopic EC nuclei at the HM in heatshocked transgenic embryos that were present across nearly all somite segments (Fig. 6C and F, PL orange bracket). However, arap3a heatshocked transgenic morphants displayed significantly reduced numbers of ectopic PLs at the HM (Fig. 6D and F, PL orange bracket). Finally, an increase in

Figure 5. ARAP3 knock-down inhibits LEC migration in vitro. (A) Real-time PCR analysis validating ARAP3 mRNA knock-down in LECs at 24 and 48 h. (B) Quantitative analysis of individual cell migration in standard transwell migration assays. ARAP3 siRNA-treated HMVEC-dLyAd display significantly reduced migration in response to VEGFC compared with control siRNA-treated cells. Error bars indicate mean ± SD. P-values were calculated using Student’s paired t-test. ***P < 0.001, n = 12. n.s, non-significant. (C) Quantitative analysis of collective distance migrated by HMVEC-dLyAd in standard scratch assays. Migration of ARAP3 siRNA-treated HMVEC-dLyAd in response to VEGFC is significantly reduced compared with control siRNA-treated cells. Error bars represent mean ± SEM. P-values were calculated using Student’s paired t-test. **P < 0.005, n = 3. (D–G) Representative images of control and ARAP3 siRNA-treated LEC in response to VEGFC. Immunofluorescence for the golgi marker GM130, the vascular marker VE-CADHERIN and the cytoskeleton marker Phalloidin reveals that cell shape and cell polarity is not affected by ARAP3 knock-down. (H and I) ARAP3 siRNA-treated spheroids show reduced sprout formation in response to VEGFC. (J) Quantitative analysis of spheroid sprout number in control and ARAP3 siRNA-treated LEC spheroids in response to VEGFC. Data represent three independent experiments; 20–30 spheroids were counted per treatment per experiment. Error bars indicate mean ± SEM. For statistical analysis, Student’s t-test was performed (*P < 0.01).
cells within the PCV upon vegfc induction was also abrogated by arap3a depletion (Fig. 6D, and G).

These data show distinct requirements for arap3a during vegfc-driven cellular responses at the level of the HM-DLAV, HM and PCV. Specifically, these results suggest that arap3a functions as a modulator of vegfc-dependent cell sprouting in selective contexts.

DISCUSSION

In the present study, Arap3 was identified as dysregulated in LECs in a mouse model of HLT. During development, zebrafish arap3a is highly restricted in the developing vasculature, including sprouting LECs, but is not transcriptionally controlled by Sox18 or Vegfc. Zebrafish studies revealed that Arap3a depletion leads to significantly reduced numbers of PLs sprouting from the PCV to the HM, subsequently causing a significant loss of TD extent at 5 dpf. In contrast, the overall blood vasculature appeared normal in arap3a zebrafish morphants. This was a surprising observation since a previous report documented severe blood vascular defects in Arap3-null mice, which die prematurely (36) at mid-gestation. These two distinct observations resemble Vegfr3 phenotypes in mice and zebrafish (12,21); Vegfr3 null mice show severe early blood vascular defects (12) whereas vegfr3 zebrafish morphants and mutants show prominent lymphatic vascular defects and limited blood vascular defects (21).

Given these observations, it seemed plausible that arap3a...
might act downstream of the VEGFC/VEGFR3 pathway. Our studies used cultured LECs as a model system, together with zebrafish loss-of-function and epistasis approaches, provide evidence that ARAP3 mediates (at least partially) the cellular response to VEGFC/VEGFR3 signalling during LEC migration.

The Arap3 knockout mouse phenotype has been previously reported in the context of blood but not lymphatic vascular development. Here, to determine whether Arap3 also plays a role in lymphatic vascular development in the mouse, we generated a conditional EC Arap3 knockout model. This strategy demonstrated that Arap3 is necessary for normal lymphangiogenesis, at least during the formation of the dermal lymphatic vasculature but not required for specification of precursor LECs. The sprouting and outgrowth of the superficial LECs in mice that give rise to this vascular bed is dependent upon VEGFC/VEGFR3 signaling (16), consistent with our findings.

At a cellular level, ARAP3 has been shown to inhibit the formation of lamellipodia in vitro in PAE and HEK293 cells (32,47). The proposed mechanism for ARAP3 function is to act as a negative regulator of RHOA, a downstream effector essential to determine cell shape and to allow normal cell migration (30,31). Interestingly, in cultured primary LEC scratch assays, no dramatic changes were observed in cell morphology upon Arap3 depletion. In contrast, single cell migration of LECs in response to VEGFC in transwell migration assays as well as sprouting of LECs in 3D spherical assays was substantially compromised. This may indicate a role for ARAP3 in a restricted range of cell behaviours but particularly in sprouting and migration. This idea is supported by the defect in migration of LECs towards the midline in the mouse model as well as the specificity of epistasis phenotypes seen in vivo in zebrafish. In the fish model, Arap3a depletion rescued the number of ECs at the HM of Tg(hsp70:Gal4;4XUAS:vegfc) animals, but not the number of ECs in hyperbranched ISVs, suggesting contextual specificity in cellular behaviours requiring Arap3. Recent reports (48) show that over-expression of vegfc by mRNA injection in the common cardinal veins of zebrafish embryos, induce EC proliferation. Interestingly, the increased number of nuclei observed in the PCV of uninjected Tg(hsp70:Gal4;4XUAS:vegfc) controls was rescued upon arap3a depletion, perhaps suggesting a role for Arap3 in EC proliferation. However, this is not supported by direct cultured LEC data (Supplementary Material, Fig. S7) and it is hard to intuitively link Arap3 function with cellular proliferation. Further studies are needed to understand this phenotype.

Importantly, developmental deficiencies lead to lymphatic defects and lymphedema in HLT, Milroy-like lymphedema and MD due to mutations in Sox18 and VEGFC/VEGFR3. In the HLT model of Sox18 deficiency (Rao–/+), lymphatic vessels form but are tortuous, thin and increased in number in the skin (7). Little is known about the mechanisms that lead to these vascular phenotypes. We found that in this context, Arap3 is dysregulated. Given that ARAP3 is necessary for normal lymphatic vessel formation during embryogenesis, its down-regulation may play a significant role in the pathogenesis of HLT lymphatic defects. The full delineation of the molecular and cellular mechanisms at play during both developmental and pathological lymphangiogenesis will be essential to begin to tackle a host of diseases involving this important vascular network.

MATERIALS AND METHODS

Zebrasfish and mouse lines

Zebrasfish were kept under standard conditions as previously described (49). The Tg(Cy3ve.1:DsRed), Tg(-6.5kdrl:EGFP), Tg(fftIa:EGFP), Tg(-6.5kdrl:mCherry), Tg(-0.8fli1:ItdTomato), Tg(fftIa:nlsEGFP) and Tg(hsp70:Gal4)1.5kca4 lines were described previously (43,37,38,50–52). The Tg(4xUAS:vegfc) line was generated using the full length zebrafish vegfc cDNA cloned using the Gateway system (21,53,54).

Pregnant wild-type CD1 mice were sacrificed and embryos dissected at the required stages for ISH. Arap3fl/fl mice were generated as previously described (36). The Sox18CreERT2/+ line was obtained from A McMahon (USC, USA) (42). All animal work conformed to ethical guidelines and was approved by the animal ethics committees at the University of Queensland.

ISH and immunostaining

For zebrafish ISH, a 300–700 bp fragment of the gene of interest was amplified from wild-type zebrafish cDNA. Sequences for primers designed for mRNA probe synthesis are shown in Supplementary Material, Table S1. Reverse primers were designed for mRNA probe synthesis in the PAE and HEK293 cells (32,47).

For induction of Cre activity in Sox18CreERT2/+Arap3fl/fl mice, timed matings were established and the day of plug counted as 0.5 dpc. Tamoxifen was administered to pregnant females (10 mg/ml dissolved in sunflower oil, 40 mg/kg per mouse) by intraperitoneal injection once per day for 3 consecutive days prior to the time point at which embryos were harvested. Embryonic tissue was genotyped as previously described (Supplementary Material, Supplementary Table S5 for oligo sequences) (36).

Antibodies

The following dilutions were used for antibodies: rabbit polyclonal anti-PROX1 (Angiobio) 1:500; goat polyclonal anti-NEUROPLIN2 (R&D Systems) 1:400; rat monoclonal anti-ENDOMUCIN (Santa Cruz Biotechnology, Inc.) 1:200. Secondary antibodies anti-goat IgG Alexa488, anti-rabbit IgG Alexa594 and anti-rat IgG Alexa 647 (Molecular Probes) used at 1:200.

RNA and morpholino oligomer injections

MOs (Supplementary Material, Table S2) were dissolved in water and injected at 1 nl per embryo. Oligo sequences designed for validating the splice blocking morpholino by Q-PCR are given in Supplementary Material, Table S3. Oligo sequences for genotyping heatshock transgenic fish are given in Supplementary Material, Table S4.

Zebrafish quantitative-PCR

Procedures were performed in order to comply with the Minimum Information for Publication of Quantitative Real-Time PCR experiments guidelines (58). Full details including...
isolation of cell populations for cDNA synthesis are given in Supplementary Material, Methods. Primers were designed using Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to have Tm of 60°C, and where possible, to cross an exon–exon junction to avoid amplification of genomic DNA.

Imaging

Zebrafish whole mount in situ embryos were imaged using an Olympus SZX-12 Stereo microscope and flat mounts were imaged using an Olympus BX-51 Upright Brightfield/Darkfield microscope. Confocal imaging was performed on live zebrafish embryos mounted laterally in low-melting agarose (1%, Sigma-Aldrich) using a Zeiss LSM 510 META confocal microscope at the indicated stages. Nuclei counts in heatshock transgenic zebrafish were performed by using the ImageJ 1.44o software and Bitplane IMARIS 7.2.1 software for rendering. Mouse embryos were imaged by using Olympus SZX-12 Stereo microscope and whole mount immunofluorescence staining of mouse skin tissues were imaged using a Zeiss LSM 510 META confocal microscope. Quantitation of mouse vessel parameters was performed by using the ImageJ 1.44o software.

Analysis of statistical significance

To determine the significance of genetic interaction data, analysis of variance (ANOVA) or Student’s t-tests were performed using the Graphpad Prism software (Prism version 6 for Mac OS X). P < 0.05 was considered statistically significant.

Further methods can be found in Supplementary Material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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