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Analysis of *talpid*^{3} and wild-type chicken embryos reveals roles for Hedgehog signalling in development of the limb bud vasculature

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Abstract

Chicken *talpid*^{3} mutant embryos have a wide range of Hedgehog-signalling related defects and it is now known that the *talpid*^{3} gene product encodes a novel protein essential for Hedgehog signalling which is required for both activator and repressor functions of Gli transcription factors (Davey, M.G., Paton, I.R., Yin, Y., Schmidt, M., Bangs, F.K., Morrice, D.R., Gordon-Smith, T., Buxton, P., Stamataki, D., Tanaka, M., Münsterberg, A.E., Briscoe, J., Tickle, C., Burt, D.W. (2006). The chicken *talpid*^{3} gene encodes a novel protein essential for Hedgehog signalling. Genes Dev 20 1365–77). Haemorrhaging, oedema and other severe vascular defects are a central aspect of the *talpid*^{3} phenotype (Ede, D.A. and Kelly, W.A (1964a). Developmental abnormalities in the head region of the *talpid*^{3} mutant fowl. J. Embryol. exp. Morp. 12:161–182) and, as Hedgehog (Hh) signalling has been implicated in every stage of development of the vascular system, the vascular defects seen in *talpid*^{3} are also likely to be attributable to abnormal Hedgehog signalling. Gene expression of members of the *VEGF* and *Angiopoietin* families of angiogenic growth factors has been linked to haemorrhaging and oedema and we find widespread expression of *VEGF-D*, *rigf* and *Ang2a* in the *talpid*^{3} limb. Furthermore, ectopic expression of these genes in *talpid*^{3} limbs points to regulation via Gli repression rather than activation. We monitored specification of vessel identity in *talpid*^{3} limb vasculature by examining expression of artery-specific genes, *Np1* and *EphrinB2*, and the vein-specific genes, *Np2a* and *Tie2*. We show that there are supernumerary subclavian arteries in *talpid*^{3} limb buds and abnormal expression of an artery-specific gene in the venous submarginal sinus, despite the direction of blood flow being normal. Furthermore, we show that Shh can induce *Np1* expression but has no effect on *Np2a*. Finally, we demonstrate that induction of *VEGF* and *Ang2a* expression by Shh in normal limb buds is accompanied by vascular remodelling. Thus Hedgehog signalling has a pivotal role in the cascade of angiogenic events in a growing embryonic organ which is similar to that proposed in tumours.

Keywords: *talpid*^{3}; Hedgehog; Limb; Vascular development; VEGF; Angiopoietin

Introduction

Homozygous *talpid*^{3} chicken embryos have severe vascular abnormalities including "cork screw" blood vessels in the extra-embryonic vasculature and subcutaneous haemorrhaging and oedema in the embryo (Ede and Kelly, 1964a) which results in lethality between 2.5 and 5 days of development on the current genetic background. Previous studies have also reported a hypervascularised vascular network in *talpid*^{3} limbs (Mohammed, 1986) although the direction of blood flow appears normal (this study). The limb bud is a well known site of Sonic Hedgehog (Shh) signalling which patterns the antero-posterior axis of the limb (review Tickle, 2003) and the loss of normal Hh signalling in *talpid*^{3} results in polydactylous limbs with many unpatterned digits (Davey et al., 2006; Lewis et al., 1999). Recently we showed that there is defective processing of Gli proteins, the transcriptional effectors of Hedgehog (Hh) signalling, in *talpid*^{3} embryos and that both activation and repression of Hh target genes fail (Davey et al., 2006). Here we examine *talpid*^{3} limb buds to gain insights into the role of Shh signalling in patterning the vasculature.

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Talpid^3 mutants can first be recognised morphologically at stage 20 HH by their broad limb buds. By stage 20 HH, wild-type chick wing buds have a clearly established single subclavian artery (Wilson, 1983; Jargiello and Caplan, 1983; Caplan, 1985; Drushel et al., 1985) which has become dominant from an earlier simple capillary plexus. This artery forms midway along the antero-posterior axis of the limb bud and supplies the distal vascular plexus of capillaries. This plexus then drains into the venous sub-apical marginal sinus running around the edge of the limb bud. Additional limb buds induced from the flank also have this antero-posterior vascular pattern suggesting that the limb mesenchyme regulates the location of the subclavian artery (Tamura et al., 2003).

Hedgehog signalling has been implicated in the processes of haematopoiesis, vasculogenesis and angiogenesis in early mouse and chick development (Dyer et al., 2001; Byrd et al., 2002; Vokes et al., 2004). Shh protein has been shown to regulate the expression of the angiogenic growth factors, *VEGF-A*, *Ang1* and *Ang2* and *bFGF* in mouse fibroblasts in vitro (Pola et al., 2001) and Shh can induce expression of members of the *VEGF* family of angiogenic growth factors in zebrafish embryos (Lawson et al., 2002) and in developing chick limb buds (Diaz-Trelles et al., 2002; Tamura et al., 2003). Perturbation of VEGF expression can result in leaky and haemorrhagic vessels (reviewed Yancopoulos et al., 2000). For example, in the chick wing, overexpression of *VEGF-A* results in haemorrhaging and oedema (Flamme et al., 1995). Here we examine the expression of angiogenic growth factors in *talpid*^{3} limbs in order to understand the basis of the haemorrhaging and oedema in *talpid*^{3} embryos.

One of the most intriguing roles for Shh signalling is an influence on arterial–venous blood vessel identity (reviewed Adams, 2003; Byrd and Grabel, 2004). Patterning of venous and arterial endothelial identity can be controlled by both haemodynamic forces and molecular mechanisms that act prior to establishment of the circulation (reviewed Adams, 2003). Addition of Shh protein to the adult rabbit cornea can induce formation of arterio-venous shunts (Pola et al., 2001) suggesting that normal arterial–venous specification is perturbed. In zebrafish, Hedgehog signalling has been shown to control formation of the dorsal aorta and endothelial arterial identity (Lawson et al., 2002). In the chick, fate mapping and grafting experiments between venous and arterial tissues have shown that the endothelial identity of the angioblasts in the limb remain plastic until late in development and that endothelial identity is patterned by the limb environment (Kardon et al., 2002, Othman-Hassan et al., 2001; Moyon et al., 2001a). As Shh signalling is abnormal but well characterised in *talpid*^{3} limb buds (Lewis et al., 1999; Davey et al., 2006), we examine arterial–venous identity in the developing mutant limb buds. Genes expressed specifically in the chick arterial circulation include *ephrinB2* (Moyon et al., 2001a; Othman-Hassan et al., 2001) and *neuropilin1* (*Np1*; Moyon et al., 2001a, Herzog et al., 2001), and in the chick venous circulation, *Neuropilin2a* (*Np2a*; Herzog et al., 2001) and *Tie2* (Moyon et al., 2001a,b).

Finally we complement our studies on *talpid*^{3} limb vasculature by examining the effects of applying Shh to normal limb buds on morphology and gene expression of the vasculature. Through these manipulations, we have gained insights into the way in which the vascular network remodels itself as the limb grows.

Materials and methods

Talpid^3 *embryo collection*

Eggs from the *talpid*^{3} flock, maintained at the Roslin Institute, Midlothian, were incubated at 38°C for 3 days and then windowed to assess phenotype (Ede and Kelly, 1964a) and stage (Hamburger and Hamilton, 1951). Embryos were injected with Indian ink to reveal the vascular pattern or fixed at appropriate stages for RNA in situ hybridisation or transmission electron microscopy.

Embryo manipulation

Fertilised chick eggs (Bovan's Gold Line) were incubated for 3 days at 38°C, windowed and incubated until stage 20 HH. Extraembryonic membranes lying over the right wing bud were torn using forceps and fine tungsten needles. A cut was made between the anterior apical ectodermal ridge and the underlying mesenchymal avascular zone in the right wing bud using fine tungsten needles and the apical ridge was stretched to make a loop. Control QT-6 cells and QT-6 cells expressing Shh which had been grown to confluence were scraped from the surface of the culture dishes and Nile Blue Sulphate was added to medium to aid visualisation. Sheets of cells were kept at 37°C in fresh medium and then cut into smaller fragments, pipetted onto the prepared chick wing buds and tucked under the apical ridge loop. Embryos were then reincubated. At specific time points, embryos were either fixed for in situ hybridisation or injected with Indian ink.

RNA in situ hybridisation

As per Nieto et al., 1996, the following templates and sample numbers were used: *Ang2a* (UMIST ChEST 603481051F1; ta $n=3$, wt $n=5$); *VEGF-D* (UMIST ChEST 603493810F1; ta $n=3$, wt $n=6$); *Tie2* (UMIST ChEST 603850655F11 ta $n=1$, wt $n=2$); *Rigf* (Tamura et al., 2003, ta $n=3$, wt $n=3$); *Neuropilin1* (forward primer 5′-TGGGCTCATTCCTGACTC-3′, reverse primer 5′-CCCTTTCCAATTTCTCCCTC; pGEM antisense Nco1/Sp6, ta $n=2$, wt $n=6$); *Neuropilin2a* (forward primer 5′-GCGTCCCTGTCTCTCACCTTC-3′, reverse primer 5′-AACCACACAGGTCTTCAGGG-3′, ta $n=2$, wt $n=6$). Post hybridisation embryos were embedded in gelatin, frozen and sectioned at 30 μm.

Indian ink labelling

Indian ink was diluted 1:1 with PBS, sonicated and filtered with a 45 μm syringe filter. A finely drawn glass capillary needle was attached to a 100 μl Hamilton syringe via Tygone tubing, back filled with mineral oil and then filled with Indian ink. The needle was inserted into an extraembryonic yolk sac artery, and ink was gently expelled from the needle. Once ink had reached all blood vessels, embryos were fixed in 5% Trichloroacetic acid (TCA) for several hours, dehydrated and cleared in methyl salicylate.

Transmission electron microscopy

Limbs of one wild-type and one *talpid*^{3} embryos were fixed after 5 days of incubation, *in ovo* then quickly transferred into fix upon dissection. Tissues were fixed overnight in 4% PFA, 2% gluteraldehyde in 0.1% cacodyate buffer. Limbs were post-fixed in 1% osmium tetroxide, washed in dH_2O and dehydrated through graded ethanols into propylene oxide and then infiltrated with Durcupan araldite resin through propylene oxide/Durcupan steps. Tissue in Durcupan was then placed in moulds and baked at 60°C overnight. Sections were either cut at 1–2 μm thick (Leica Ultramicrotome) and stained with

toluidine blue or cut at between 60–70 nm and post stained with osmium tetroxide. Ultrathin sections were viewed on a JEOL1200X.

Results

Vascular pattern and cellular morphology of blood vessels in talpid^3 and wild-type chick limb buds

We injected Indian ink into the vascular system of wild-type and *talpid^3* chick embryos at stages 20 HH and 25 HH (Figs. 1A–D) and confirmed the vascular patterns described by Mohammed (1986). At stage 20 HH, there is an extensive vasculature in *talpid^3* limb buds in the form of an unpatterned capillary plexus with no dominant subclavian artery (Fig. 1B) as is seen in wild-type wing buds (arrowhead, Fig. 1A) although the avascular zone (AVZ; arrow) is present distally under both wild-type and *talpid^3* limb ectoderm (Figs. 1A, B, C, D). In wild-type stage 25 HH limbs, the dominant subclavian artery has become more prominent (arrowhead, Fig. 1C) but, in *talpid^3* limbs, the vasculature has still not coalesced centrally (Fig. 1D). Instead several larger calibre vessels have formed over a wide central area in the proximal part of the limb bud (arrowheads, Fig. 1D). These may represent multiple 'subclavian arteries' (see later).

We compared the morphology of the vasculature of *talpid^3* and wild-type limb buds at the stage 25 HH in semi-thin toluidine blue stained sections (Figs. 2A, B). *Talpid^3* limb blood vessels are enlarged, as previously described by Ede and Kelly, 1964b (Fig. 2B) and more numerous. In thin sections of blood vessels of wild-type limb buds, endothelial cells were seen to have bulging nuclei (endothelial soma labelled 'E' in red, Fig. 2C) and long thin cellular projections (arrowheads, Fig. 2C) which interdigitate with projections of neighbouring endothelial cells (Fig. 2E) and are connected by electron dense areas (arrows, Fig. 2E). Endothelial cells in vessels in *talpid^3* limb buds have similar morphology (endothelial cell labelled 'E', arrows at cellular projections, Fig. 2D) except that overlapping cellular projections appeared thicker (arrows, Fig. 2D) and electron dense junctions between *talpid^3* endothelial cells appear less well defined (arrows, Fig. 2F).

Expression of members of the VEGF family talpid^3 and wild-type chick limb buds

Over expression of *VEGF-A*, a member of the VEGF family of vascular growth factors can lead to hypervascularisation, vessel fusion, haemorrhaging and oedema in the developing chick limb (Flamme et al., 1995), in other areas of the developing

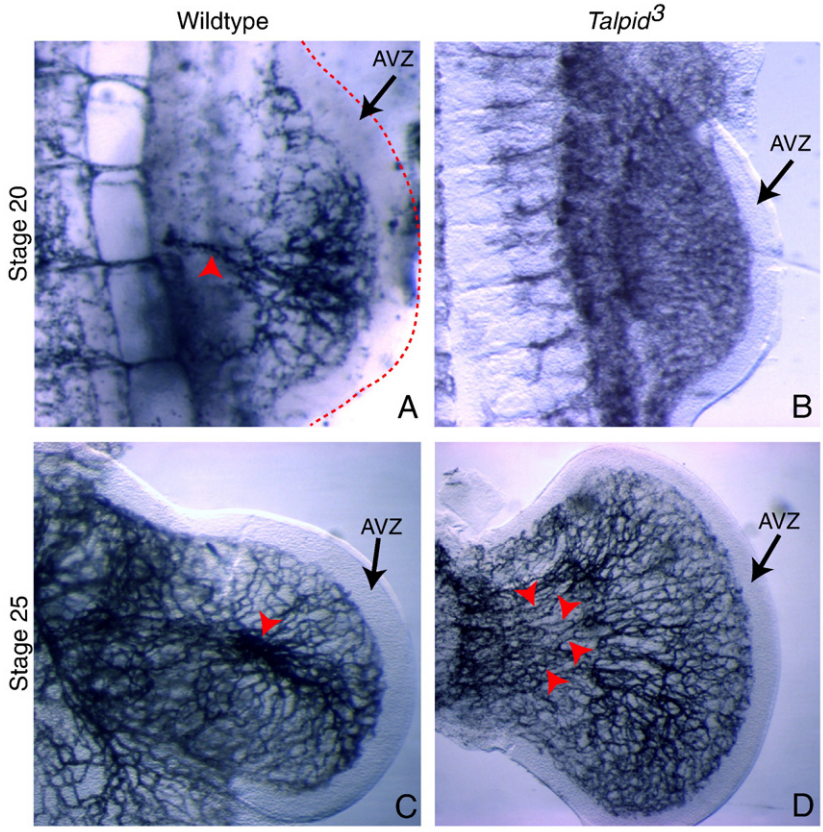

Fig. 1. Vasculature of wild-type and *talpid^3* limb buds. (A–D) Vasculature of the limb as shown by injection of Indian ink. Panels A and B are shown at the same magnification. Panels C and D are shown at the same magnification. (A) Vasculature of wild-type stage 20 HH limb bud outlined by red dashed line, red arrowhead indicates SA, arrow indicates AVZ. (B) Vasculature of stage 20 HH *talpid^3* limb bud showing normal AVZ (arrow). (C) Vasculature of stage 25 HH wild-type limb bud, red arrowhead indicates SA, arrow indicates AVZ. (D) Vasculature of stage 25 HH *talpid^3* embryo, red arrowheads indicates multiple SAs, arrow indicates normal AVZ. Abbreviations: SA, subclavian artery; AVZ, avascular zone.

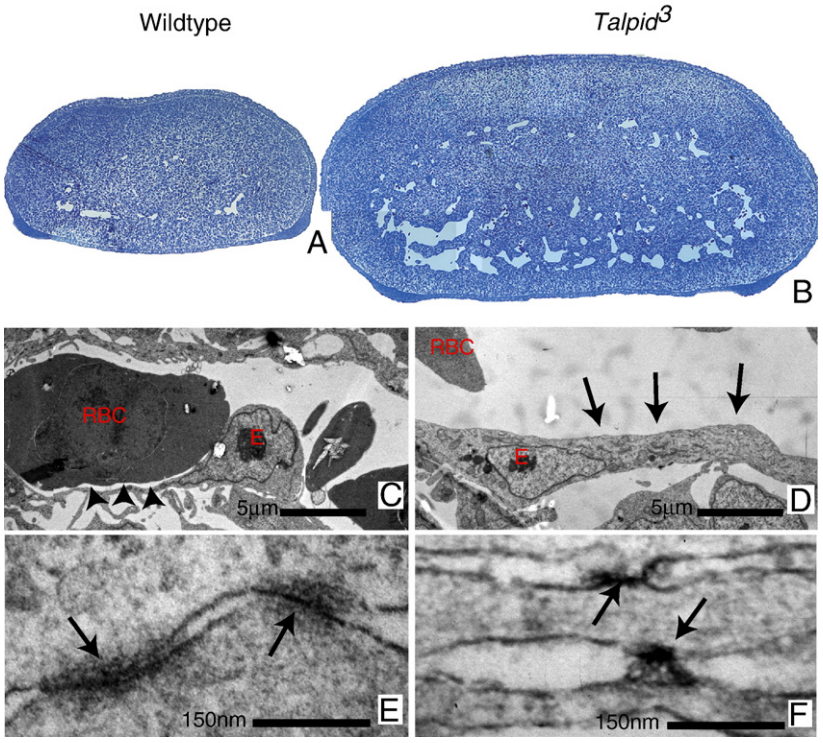

Fig. 2. Histology of wild-type and *talpid*^{3} limb blood vessels. (A and B) Semi-thin sections of stage 25 HH wild-type (A) and *talpid*^{3} limb buds (B) stained with toluidine blue (panels A and B are at the same magnification). (C–F) Ultra-thin sections stained with osmium tetroxide. (C) Capillary in a stage 25 HH day wild-type limb. Arrowheads indicate long thin cellular projection of endothelial cell (E). (D) Capillary in a stage 25 HH *talpid*^{3} limb. Arrows indicate long cellular projection of endothelial cell (E) which appears much thicker than cellular projections seen in wild-type endothelial cells. (E) Wild-type blood vessel showing junctions between endothelial cell projections (arrows). (F) *Talpid*^{3} blood vessel; junctions between endothelial cell projections (arrows) much less defined. Abbreviations: RBS, red blood cell; E, endothelial cell.

embryo (Drake and Little, 1995) and in human adult pathologies (reviewed Yancopoulos et al., 2000). We therefore examined expression of members of the VEGF family to see if this might account for the *talpid*^{3} limb hypervascularisation and susceptibility to haemorrhaging and oedema.

No changes were seen in the expression of *VEGF-A* in *talpid*^{3} buds (not shown) but both *VEGF-D* and *rigf* (other members of the VEGF family) showed changes in expression. In the wild-type limb, *VEGF-D* has discrete areas of expression during development, with strong expression in particular localised to the middle and posterior mesenchyme between stages 17 and 24 HH (Diaz-Trelles et al., 2002; Fig. 3A). At stage 25 HH in wild-type embryos, *VEGF-D* expression is seen in both wing and leg in a central stripe, which corresponds to the developing brachial and femoral arteries, respectively (arrow, Fig. 3C). Distally, *VEG-D* expression is associated with the capillary plexus throughout the posterior 2/3 of the autopod (Diaz-Trelles et al., 2002; Fig. 3C) except in regions where digits are developing which are known to be avascular (asterisk, Fig. 3C). In contrast to the posteriorly restricted expression seen in wild-type limb buds, in *talpid*^{3} limb buds *VEGF-D* is expressed strongly throughout the antero-posterior axis of stage 22 HH limb buds (Fig. 3B). By stage 25 HH, *VEGF-D* expression is still widespread in *talpid*^{3} limb buds with strong expression anteriorly, although domains of weaker expression have developed in both wing and leg at central (wing) (arrow, Fig. 3D) or central-posterior (leg) locations likely corresponding with chondrogenic condensations.

Rigf, another member of the VEGF family, is expressed strongly in proximal and posterior mesenchyme and is mostly absent from the anterior mesenchyme of wild-type chick limb buds (Tamura et al., 2003; Fig. 3E). In contrast, in *talpid*^{3} embryos at the same stage, *rigf* is not only expressed throughout the limb but is most highly expressed anteriorly (Fig. 3F). Thus, *VEGF-D* and *rigf*, members of the VEGF family, which can be induced in the anterior margin of wild-type limb buds in response to Shh (Diaz-Trelles et al., 2002; Tamura et al., 2003), are both ectopically expressed and expressed at higher levels in *talpid*^{3} limb buds.

Expression of members of the Angiopoietin family talpid^3 *and wild-type chick limb buds*

A second family of angiogenic growth factors, the angiopoietins, has also been shown to be induced by Hedgehog signalling in fibroblasts in vitro (Pola et al., 2001). Furthermore, a member of this family, the potent vascular growth factor *Ang2a* and one of its receptors, *Tie2* (reviewed Gale and Yancopoulos, 1999) has been described as being restricted in expression to mesenchyme around arterial endothelium (*Ang2*) and to venous endothelium (*Tie2*; Moyon et al., 2001a,b) of axial blood vessels in chick embryos. As overexpression of

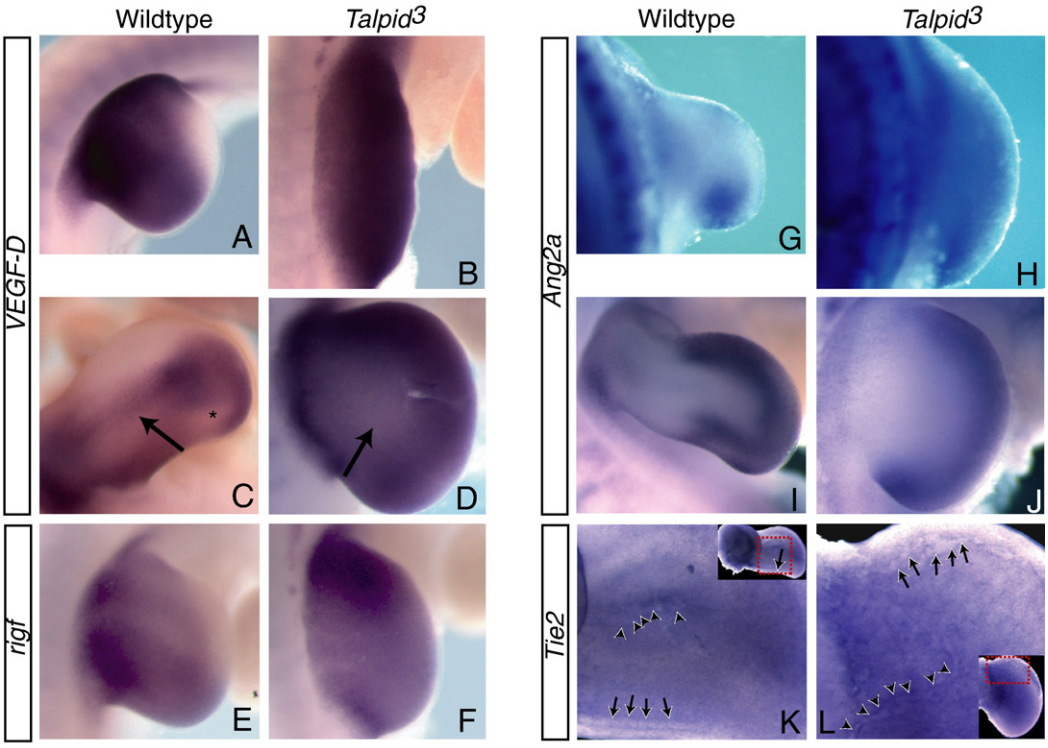

Fig. 3. Expression of *VEGF-D* and *rigf* in wild-type and *talpid*^{3} limbs. (A–D) *VEGF-D* expression. (A) Wild-type stage 22 HH wing bud, note lack of expression of *VEGF-D* anteriorly. (B) *Talpid*^{3} stage 22 HH wing bud, strong *VEGF-D* expression throughout the antero-posterior axis. (C) Wild-type stage 25 HH wing bud, note strong expression of *VEGF-D* in a central stripe (arrow) which correlates with the SA and absence of expression from prospective digit 4 (asterisk). (D) *Talpid*^{3} stage 25 HH wing bud; strong expression throughout the wing bud except for a central weaker domain correlating with the fused chondrogenic condensation (arrow). (E, F) *Rigf* expression. (E) Wild-type stage 22 HH wing bud, note lack of *rigf* of expression anteriorly. (F) *Talpid*^{3} stage 22 HH wing bud, note strong expression of *rigf* anteriorly. (G–J) *Angiopoietin2a* expression. (G) Wild-type stage 20 HH wing bud, note posterior expression. (H) *Talpid*^{3} stage 20 HH wing bud, expression throughout the antero-posterior axis. (I) Stage 25 HH wild-type wing buds; note distal *Ang2a* expression. (J) Stage 25 HH *talpid*^{3} wing buds; symmetrical *Ang2a* expression distally. (K, L) *Tie2* expression. (L) Wild-type wing buds stage 25 HH; *Tie2* expression in both arteries (arrowheads) and veins (arrows). Insert picture shows area magnified (red outlined box). (L) *Talpid*^{3} wing buds stage 25 HH; expression in both arteries (arrowheads) and veins (arrows). Insert picture shows area magnified (red outlined box). Abbreviation: SA, subclavian artery.

VEGF-A can lead to the differentiation of arterial endothelium (Mukouyama et al., 2002; Lawson et al., 2002) and the loss of venous endothelial identity (Lawson et al., 2002) in zebrafish, we used clones from the UMIST chick EST database (Boardman et al., 2002) to examine expression of *Ang2a* and *Tie2*, to observe whether abnormal Hedgehog signalling in the *talpid*^{3} limb alters expression of this family of growth factors and whether there are changes in arterial–venous identity of the blood vessels in the *talpid*^{3} limb.

In stage 20 HH wild-type chick embryos, *Ang2a* is expressed strongly in posterior limb mesenchyme (Fig. 3G). In contrast, in *talpid*^{3} limbs, expression of *Ang2a* is found throughout the antero-posterior axis, lacking any posterior restriction (Fig. 3H). At stage 25 HH, *Ang2a* expression in wild-type limbs is concentrated in a distal arc around the edge of the developing autopod particularly in the avascular zone with higher levels of expression anteriorly (Fig. 3I). In stage 25 *talpid*^{3} limb buds, *Ang2a* is also expressed distally but expression is fairly even across the antero-posterior axis, with higher expression posteriorly (arrow, Fig. 3J). Unlike previous reports of expression of *Tie2* in the body of the chick embryo, *Tie2* appears to be expressed ubiquitously in all blood vessels in wild-type limbs (arrow posterior marginal sinus, arrowhead subclavian artery, Fig. 3K) and expression was not altered in *talpid*^{3} chick embryos (arrows indicate marginal sinus, arrowheads indicating multiple *talpid*^{3} subclavian arteries Fig. 3L). Thus, abnormal Hedgehog signalling in *talpid*^{3} limbs alters dramatically arterial restricted expression of *Ang2a* expression but no change was observed in *Tie2*.

Artero-venous identity in talpid^3 *vasculature*

Widespread expression of *Ang2a* in the *talpid*^{3} limb suggests that there may be abnormal induction of arterial identity as is seen in zebrafish when *Shh* or *VEGF* is overexpressed (Lawson et al., 2002). To further investigate the arterial–venous identity of the blood vessels in the *talpid*^{3} limb, we examined expression of the genes expressed either in arteries, *Neuropilin1* (*Np1*) and *ephrinB2* (Herzog et al., 2001; Othman-Hassan et al., 2001; Moyon et al., 2001a; Wang et al., 1998; Adams et al., 1999) or in veins *Neuropilin2a* (*Np2a*). *Np2a* expression is associated with venous endothelium of early chick embryos; Moyon et al., 2001a; Herzog et al., 2001).

In sections of wild-type chick limb buds, *Np1* is expressed strongly in central mesenchyme around the subclavian artery and in the endothelium of the subclavian artery (SA, Fig. 4A).

Although there is weak *Np1* expression in mesenchyme surrounding the venous marginal sinus, expression was not seen in the vein endothelium (MS, Fig. 4C). In contrast, in sections of *talpid*^{3} limbs, *Np1* is expressed in mesenchyme and endothelium of all the large centrally located vessels, identifying them as arteries (SA, Fig. 4B). It is also expressed in the venous endothelium of the marginal sinus (MS, Figs. 4B, D). Thus, in *talpid*^{3} limb buds, there appear to be many subclavian arteries coupled with ectopic expression of the arterial marker, *Np1* in the endothelia of the venous marginal sinus.

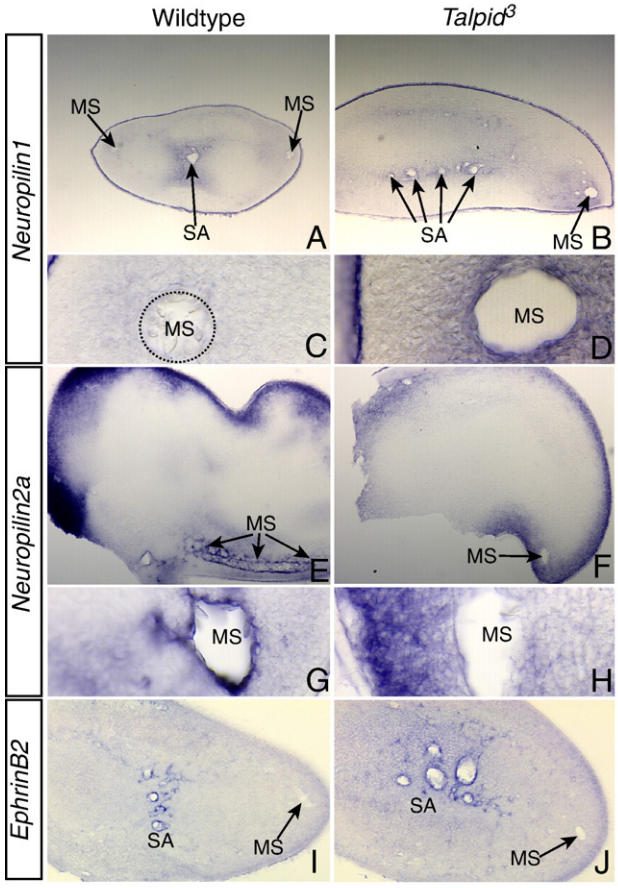

Fig. 4. Arterial–venous identity in wild-type and *talpid*^{3} limb bud vessels. Sections of limb buds after processing for RNA in situ hybridisation. (A–D) *Neuropilin1* expression (*Np1*, arterial). (A) Stage 25 HH wild-type wing bud; strong expression of *Np1* in central mesenchyme around SA but not around MS. (B) Stage 25 HH *talpid*^{3} wing bud; strong expression of *Np1* in central mesenchyme around SA and in mesenchyme around MS. (C) Higher magnification of the MS in stage 25 HH wild-type limb; no detectable expression of *Np1*. (D) Higher magnification of the MS in stage 25 HH *talpid*^{3} limb; expression of *Np1* in mesenchyme around the MS. (E–H) *Neuropilin2a* expression (*Np2a,* venous). (E) Stage 25 HH wild-type wing bud; strong expression of *Np2a* in endothelium of MS. (F) Stage 25 HH *talpid*^{3} wing bud; strong expression of *Np2a* in mesenchyme of the limb but not in endothelium of MS. (G) Higher magnification of the MS in stage 25 HH wild-type limb; *Np2a* expression in endothelium of the MS. (H) Higher magnification of the MS in stage 25 HH *talpid*^{3} limb; expression of *Np1* in mesenchyme around MS but not in endothelium of the MS. (I, J) Expression of *ephrinB2* (arterial). (I) Stage 25 HH wild-type wing bud strong expression of *ephrinB2* in endothelium of SA and not of the MS. (J) Stage 25 HH *talpid*^{3} wing bud; strong expression of *ephrinB2* in endothelium of the SA but not of the MS. Abbreviations: MS, marginal sinus (venous); SA, subclavian artery; A, B, E, F, I, J at same magnification; C, D, G, H at same magnification.

We then examined *Np2a* expression to see if expansion of *Np1* expression into the venous part of the *talpid*^{3} limb vasculature leads to alteration in the expression of *Np2a*, which is normally associated with veins. In sections of limb buds of stage 25 HH wild-type embryos, *Np2a* is expressed in the venous endothelium of the marginal sinus (MS, Figs. 5E, G). However, in stage 25 HH *talpid*^{3} limb sections, there is no detectable expression of *Np2a* in endothelium of the marginal sinus (MS, Figs. 4F, H); although expression can still be seen in other places in the limb mesenchyme. Thus although *Np2a* is expressed in the mesenchyme of the *talpid*^{3} limb, endothelial cells do not appear to express *Np2a*.

We also examined *Np1* and *Np2a* expression in the main axial blood vessels of the body and found that at stage 20 HH the *talpid*^{3} expression of *Np1* was normally limited to the axial mesenchyme and endothelium of the dorsal aorta, with no expression in endothelium of posterior cardinal veins (not shown) and that normal expression of *Np2a* was also retained in the posterior cardinal veins with no expression in the aorta (not shown).

As the marginal sinus of *talpid*^{3} limbs had lost expression *Np2a*, and gained expression of *Np1*, we observed the direction of blood flow in the posterior marginal sinus in *talpid*^{3} wings between stages 20 HH and 24 HH, because the direction of flow also patterns arterial–venous endothelial identity (le Noble et al., 2004). We observed that the direction of blood flow was normal (returning to the body i.e. venous; $n>10$). We then examined expression of *ephrinB2,* another gene normally associated with arteries. In wild-type chick embryos at stage 25 HH, *ephrinB2* is expressed in the endothelium of the centrally located subclavian artery and in arterial vessels radiating from it (SA, Fig. 4I), but not in the endothelium of the marginal sinus (MS, Fig. 4I). A normal pattern of expression is seen in *talpid*^{3} limb buds; *ephrinB2* is expressed in endothelia of subclavian arteries (SA) and the smaller arteries which emerge from them but not in the marginal sinus (MS; Fig. 4J). Therefore in *talpid*^{3} limbs, not all arterially expressed genes are misexpressed in the venous marginal sinus.

The morphological effect of Shh on chick limb vasculature

To examine morphological effects of Shh on normal limb vasculature, we grafted Shh-expressing QT-6 cells into wild-type chick limb buds and control QT-6 cells and visualised the vasculature pattern using injected Indian ink. The vasculature of contralateral unoperated limbs is shown for comparison.

At 6 h, 8 h and 12 h after *Shh*-expressing QT-6 cells had been grafted anteriorly under the apical ridge of stage 20 HH chick limb buds, a marked reduction of the capillary bed in the mesenchyme under the grafted cells was observed (6 h–6/7, 8 h–4/7, 12 h–4/5; Figs. 5B, D, F) and the distal marginal sinus was lost (arrow, Figs. 5B, F). By 16 h after grafting Shh expressing cells, 4/6 limbs had a dip distally in the capillary bed but the marginal sinus had now become reestablished (arrow at dip, Fig. 5H). At 6 h, 8 h, 12 h and 16 h in 6/10 limbs grafted

with control QT-6 cells, no change in the vasculature was detected (9/14; Figs. 5A, C, E); in 4 remaining limbs an indentation was observed but the marginal sinus was intact and in 4 other limbs the vascular plexus was intact and capillary growth was noted around the avascular graft (arrow, Fig. 5G). These observations taken together show that Shh added to a limb can induce a transient anti-angiogenic response followed by subsequent regrowth and remodelling of the capillary bed.

Effects of Shh on Np1, Np2a and Ang2a expression in wild-type limb buds

Np1, *Np2a* and *Ang2a* expression in wild-type limb buds has a posterior bias suggesting that expression of these genes may be regulated by Shh produced in the polarising region (*Np1* and *Ang2a* have posterior restriction at stage 20 HH, *Np2a* at stage 23–25; *Np1* and *Np2a* not shown, *Ang2a*, Fig. 3H). Furthermore, we have found that expression of *Np1*, *Ang2a* and *Np2a* is abnormal in *talpid*^{3} embryos which have a defect in Hh signalling. To examine the effect of ectopic Shh on the expression of these genes, we grafted Shh-expressing QT-6 cells and control QT-6 cells into the limb and examined expression of *Np1*, *Np2a* and *Ang2a* after 8 h and 16 h. With control QT-6 cells, no change in expression was seen in either *Np1* or *Np2a* expression after 8 or 16 h (7/7) (asterisk marks graft, Figs. 6A, C, E, G). In contrast, with Shh-expressing QT-6 cells at 8 and 16 h, a dramatic induction of *Np1* (4/4; asterisk marks graft, Figs. 6B, arrow D) and *Ang2a* (3/3; asterisk marks graft, Figs. 6J, L) expression was seen localised in the anterior limb bud around the graft. The effect on *Np2a* was negligible (5/5; asterisk marks graft, Figs. 6F, H). Shh may therefore control the specification of arterial identity through induction of *Ang2a* and *Np1*.

Discussion

The formation of a properly patterned limb crucially depends on the correct molecular clues specifying digit number and identity. In addition, the formation of a suitably patterned vascular network to supply the growing limb is also important. Shh signalling is known to be pivotal in the antero-posterior patterning of the limb skeleton (review Tickle, 2003) and the data we present here show that it also plays a role in controlling vascularisation of the limb including specification of arteries and veins.

Hh signalling is essential for the antero-posterior patterning of the limb vasculature

We have recently shown that *talpid*^{3} limbs have abnormal Hedgehog signalling and that both Gli activator and repressor function are lost (Davey et al., 2006). In *talpid*^{3} limbs, genes which are normally repressed anteriorly by Gli3R are ectopically expressed across the antero-posterior axis of the limb. Thus the ectopic expression of *VEGF-D*, *rigf* and *Ang2a* in

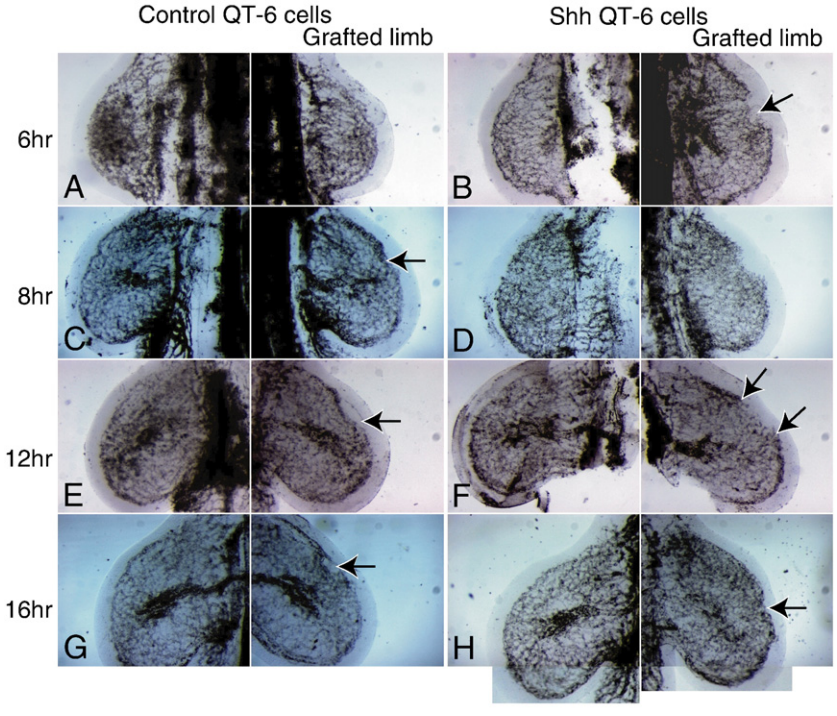

Fig. 5. Antiangiogenesis and revascularisation of the limb after treatment with Shh. Blood vessels shown by injection with Indian ink, in each panel grafted limb is on the right, unoperated limb is on the left for comparison. (A, C, E, G) Limbs implanted with control QT-6 cells. (B, D, F, H) Limbs implanted with Shh expressing QT-6 cells. (A) 6 h after QT-6 cells implanted. (B) 6 h after Shh QT-6 cells implanted; arrow indicates regression of vasculature. (C) 8 h after QT-6 cells implanted; arrow indicates dent in marginal sinus. (D) 8 h after Shh QT-6 cells implanted. (E) 12 h after QT-6 cells implanted; arrow indicates capillary growth in AVZ. (F) 12 h with Shh QT-6 cells implanted; marginal sinus is lost between arrows. (G) 16 h after QT-6 cells implanted; arrow indicates vascular growth around implanted cells. (H) 16 h after Shh QT-6 cells implanted; arrow indicates reformed marginal sinus; the vascular plexus has grown distally in the anterior limb bud.

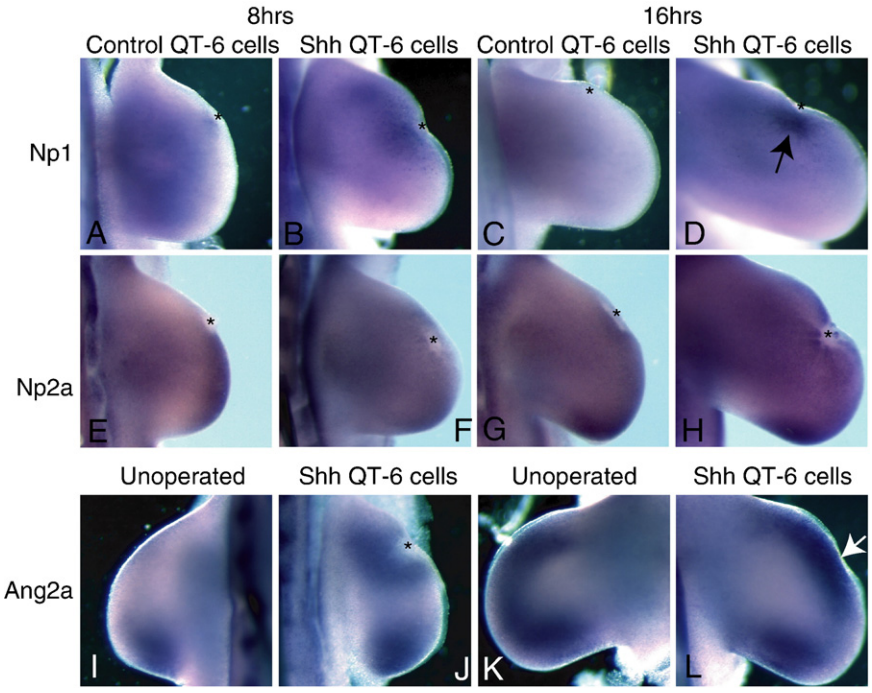

Fig. 6. Expression of *Np1*, *Np2a* and *Ang2a*, in wild-type limbs in response to Shh. Asterisk indicates grafted cells, all figures at same magnification. (A–D) *Np1* expression. (E–H) Np2a expression. (I–L) *Ang2a* expression. *Np1* expression 8 h after QT-6 cells (A) or Shh QT-6 cells implanted (B). *Np1* expression 16 h after limbs grafted with QT-6 cells(C) or Shh QT-6 cells (D) arrow indicates region of increased expression. (E–H) *Np2a* expression. *Np2a* expression 8 h after QT-6 cells implanted (E) and Shh QT-6 cells implanted (F). *Np2a* expression 16 h after QT-6 cells implanted (G) and Shh QT-6 cells implanted (H). (I–L) *Ang2a* expression. *Ang2a* expression in limb 8 h after implanted with Shh QT-6 cells (J), in unoperated limb (I). *Ang2a* expression 8 h after Shh QT-6 cells implanted (L), arrow indicates region of increased expression; (K) unoperated limb.

talpid^3 suggests that expression of these genes is normally restricted to the posterior region of the limb bud because of Gli repression anteriorly.

The loss of polarised gene expression in *talpid*^{3} limb buds results in an increased number of unpatterned digits (Lewis et al., 1999). Here we have shown that the antero-posterior vascular pattern is also lost in *talpid*^{3} limbs. In the *talpid*^{3} limb, no central dominant central subclavian artery forms and instead several large calibre vessels form. This correlates with ectopic expression of the vascular growth factors, *VEGF-D*, *rigf* and *Ang2a*, across the antero-posterior axis of the limb. In particular, *rigf* is expressed in the mesenchyme around the subclavian artery and is thought to position it (Tamura et al., 2003). The large calibre vessels that form centrally in *talpid*^{3} limb buds express the artery-specific genes *EprhinB2* and *Np1*. Therefore the widespread expression of *rigf* in the *talpid*^{3} limb appears to be correlated with multiple subclavian arteries arising at several different antero-posterior levels. Therefore Shh signalling is essential for the antero-posterior patterning of the vasculature of the limb and determining position of the subclavian artery.

Hh signalling regulates vessel stablility through vascular growth factors

The *talpid*^{3} limb is hypervascularised, with a dense vascular network characterised by large calibre blood vessels. As well as the haemorrhaging and oedema suffered by *talpid*^{3} embryos, these defects are very similar to those seen in chick wings in which *VEGF-A* has been over expressed (Flamme et al., 1995; Yin and Pacifici, 2001). We have shown that other members of the VEGF family, *VEGF-D* and *rigf* are ectopically expressed in the *talpid*^{3} limb and hypothesise that the ectopic expression of these genes may cause the *talpid*^{3} vascular defects. *VEGF-A* is a vascular permeability factor (reviewed Yancopoulos et al., 2000) which is important for the maturity of tight junctions (Wang et al., 1998). Hedgehog signalling has also been shown to be important in the formation of tight junctions between Schwann cells (Parmantier et al., 1999) and perturbation of Desert hedgehog leads to abnormally permeable blood vessels in peripheral nerves (Sharghi-Namini et al., 2006). Thus the abnormalities in junctions between *talpid*^{3} endothelial cells may due to a combination of loss of normal Hh signalling and over expression of *VEGF*. Furthermore, when *VEGF-A* and *Ang2* are expressed together, as we see in *talpid*^{3}, blood vessels can become unstable (Visconti et al., 2002) further adding to the possibility of haemorrhaging and oedema.

Hh signalling contributes to vessel identity in the limb

Our analysis of vessel identity in *talpid*^{3} limb buds shows a loss of expression of a venously expressed gene, *Np2a*, coupled with ectopic expression of an arterially expressed gene, *Np1*, in the venous endothelium of the marginal sinus. Expression of *Ang2a* which is also associated with arterial identity, was also expressed throughout the *talpid*^{3} limb. We have shown that

application of Shh protein can lead to ectopic expression of *Np1* and *Ang2a* expression in the anterior limb, but does not effect expression of *Np2a*. Therefore, we conclude that the loss of *Np2a* expression in the *talpid*^{3} marginal sinus is not due directly to defective Hh signalling but to the induction of partial arterial identity in the marginal sinus cells of the *talpid*^{3} limb. However, as *ephrinB2*, which is normally expressed in the subclavian artery, is not expressed ectopically in the *talpid*^{3} marginal sinus, the defect in the Shh pathway is not sufficient to entirely transform venous identity. Direction of blood flow has also been shown to be an important mechanism in specification of venous or arterial identity (le Noble et al., 2004). Here we show that the direction of blood flow is normal in the *talpid*^{3} limb and therefore blood flow may inhibit the expression of *ephrinB2* in the marginal sinus.

Hh signalling can control a cascade of angiogenic events

We have shown that applying Shh to the wild-type limb can affect the local morphology of the vasculature. Soon after Shh application, an anti-angiogenic event occurs which results in loss of the distal marginal sinus and regression of the capillary bed proximal to the graft, followed by re-growth of the capillary plexus and finally re-establishment of the marginal sinus. Since the limb vasculature lacks true pericytes before stage 35 HH (Drushel et al., 1985), these events occur independently of pericytes. Previous work by others has shown that application of Shh can induce localised expression of *VEGF-D* and *rigf* in the chick limb (Diaz-Trelles et al., 2002, Tamura et al., 2003) and we have shown that Shh can also induce localised expression of *Ang2a*. It has been shown in gliomas and adult

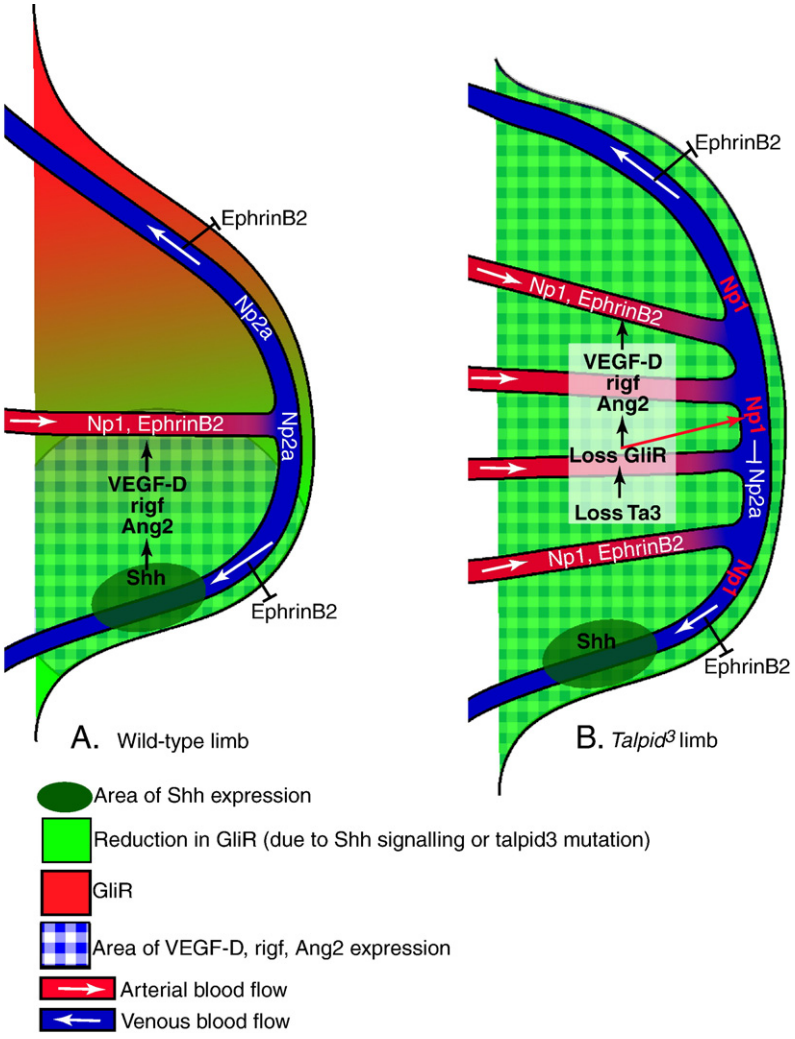

Fig. 7. Model of action of Hedgehog signalling in limb vasculature development. (A) Model of Shh signalling on the development of the vasculature in wild-type limb. Shh produced posteriorly (dark green), inhibits formation of the GliR (orange) thus GliR is reduced posteriorly (light green). Reduction of GliR allows expression of *VEGF-D*, *rigf* and *Ang2* (hatched area). The central location of the subclavian artery is specified at a particular position by the relative levels of GliR and GliA. A combination of blood flow and Shh dependent gene expression induces arterial identity (*Np1*, *ephrinB2*) in the subclavian artery. Arterial identity is suppressed in the marginal sinus by blood flow. (B) Model of the consequences of loss of functional Gli transcription factors on the development of the vasculature in the *talpid*^{3} limb. Loss of functional GliR throughout the limb (light green) allows unpolarised expression of Shh dependent genes throughout the limb bud (hatched area, *VEGF-D*, *rigf*, *Ang2a*). As a result, no central subclavian artery is specified, and multiple subclavian arteries form. Partial arterial identity is seen in the marginal sinus (*Np1*) although blood flow still inhibits complete conversion to arterial identity (*ephrinB2* is not expressed in the marginal sinus).

tissue that induction of *Ang2* in the absence of *VEGF* can cause blood vessels to become unstable and regress causing tissue to become hypoxic. Subsequent induction of *VEGF* (due to the hypoxia) combined with *Ang2* expression, then causes the blood vessels to undergo a new phase of angiogenesis, a similar pattern of events to that which we see in the chick limb (Holash et al., 1999). Furthermore, in the mouse heart, the combined expression of *VEGF* and *Ang2* leads to arterial gene expression (Visconti et al., 2002) which we also observe in the chick wing due to the localised induction of *Np1* by a graft of Shh expressing cells. Our work thus demonstrates that the same mechanism of blood vessel remodelling that occurs during tumourgenesis and adult angiogenesis also occurs in the embryo and indicates that Shh is a key modulator of gene expression in new angiogenic events.

Taken together, we propose the following model for the role of Shh in controlling vascular pattern of the limb bud. Shh diffuses from the polarising region (dark green Fig. 7A) into posterior mesenchyme, preventing processing of Gli3 into the Gli3 Repressor (green = inhibited Gli processing vs. orange = GliR gradient Fig. 7A; te Welscher et al., 2002). Lack of Gli3 repressor allows the combined expression of *VEGF-D*, *rigf* and *Ang2a* in the posterior half of the limb bud (hatched area, Fig. 7A) which has been shown to lead to vascular remodelling and induction of arterial identity. This posteriorly localised signalling may allow a high rate of vascular remodelling to occur in order to supply the posterior limb tissue which is expanding the greatest (Vargesson et al., 1997) with an appropriate blood supply. Posteriorly restricted signalling could also allow the correct induction of boundary which positions the subclavian artery as suggested by Tamura et al., 2003. The combined expression of *VEGF-D*, *rigf* and *Ang2a* together with blood flow may induce arterial identity in the subclavian artery (*ephrinB2, Np1*, Fig. 7A). It is unclear how the posteriorly located marginal sinus maintains a venous identity being so close to the polarising region but possibly this is due to the direction of blood flow inhibiting expression of arterially restricted genes such as *ephrinB2* (le Noble et al., 2004; Fig. 7A).

In the *talpid*^{3} limb, loss of functional Gli Repressor (green gradient throughout limb model, Fig. 7B) allows widespread, unpolarised expression of *VEGF*, *rigf* and *Ang2a*. This allows the formation of multiple subclavian arteries in the *talpid*^{3} limb and the greater blood supply throughout the bud may be a factor in its rapid growth. Venous identity of the marginal sinus is compromised either as a result of widespread expression of a combination of *VEGF*, *rigf* and *Ang2a* or due to an unknown function of the Gli Repressor. Consequently *Np1* expression is induced in the venous endothelium precipitating a loss of *Np2a* expression (Fig. 7B). We would also have to hypothesise that the normal direction of blood flow maintains repression of *ephrinB2* in venous endothelium (Fig. 7B). Thus taken together, our analysis of the vasculature of the *talpid*^{3} limb buds suggests that both intercellular signalling and haemodynamic forces act to establish artero-venous vascular identity but that molecular clues maybe more important in arterial identity while haemodynamic forces may play a larger role in venous identity.

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