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A Myc-Slug (Snail2)/Twist regulatory circuit directs vascular development

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Myc-deficient mice fail to develop normal vascular networks and *Myc*-deficient embryonic stem cells fail to provoke a tumor angiogenic response when injected into immune compromised mice. However, the molecular underpinnings of these defects are poorly understood. To assess whether Myc indeed contributes to embryonic vasculogenesis we evaluated Myc function in *Xenopus laevis* embryogenesis. Here, we report that *Xc-Myc* is required for the normal assembly of endothelial cells into patent vessels during both angiogenesis and lymphangiogenesis. Accordingly, the specific knockdown of *Xc-Myc* provokes massive embryonic edema and hemorrhage. Conversely, Xc-Myc overexpression triggers the formation of ectopic vascular beds in embryos. Myc is required for normal expression of *Slug/Snail2* and *Twist*, and either XSlug/Snail2 or XTwist could compensate for defects manifest by *Xc-Myc* knockdown. Importantly, knockdown of *Xc-Myc*, *XSlug/Snail2* or *XTwist* within the lateral plate mesoderm, but not the neural crest, provoked embryonic edema and hemorrhage. Collectively, these findings support a model in which Myc, Twist and Slug/Snail2 function in a regulatory circuit within lateral plate mesoderm that directs normal vessel formation in both the vascular and lymphatic systems.

KEY WORDS: Myc, Slug/Snail2, Twist, Vasculogenesis, Lymphangiogenesis, *Xenopus*

INTRODUCTION

Myc, and its closest family members N-myc (Mycn) and L-Myc, function as basic helix-loop-helix/leucine zipper transcription factors that regulate diverse cellular responses and are commonly activated in cancer (Nesbit et al., 1999; Grandori et al., 2000). Under normal conditions, the transcription of Myc genes is mitogen dependent and is suppressed by growth inhibitory signaling pathways and by the tumor suppressors p53 and pRb (Grandori et al., 2000; Bernard and Eilers, 2006). The pervasive selection for Myc overexpression in cancer reflects its pivotal roles in regulating progression through the cell cycle (Roussel et al., 1991), increases in cell mass (Iritani and Eisenman, 1999) and the tumor angiogenic response (Pelengaris et al., 1999; Baudino et al., 2002; Knies-Bamforth et al., 2004).

Loss-of-function experiments have revealed a conserved role for Myc in regulating cell growth and division in both vertebrates and invertebrates. For example, in *Drosophila* mosaic *dmyc* (*dm* – FlyBase) mutants, the wing discs grow very poorly, are smaller in size and are out-competed by wild-type cells (Johnston et al., 1999). Furthermore, the conditional knockout of *Myc* in mice established its essential role in the G1- to S-phase transition of the cell cycle (de Alboran et al., 2001; Trumpp et al., 2001). However, there are also obvious consequences of *Myc* loss on developmental processes. For example, the conditional deletion of *Myc* in the hematopoietic compartment leads to an accumulation of hematopoietic stem cells that fail to migrate from the bone marrow (Wilson et al., 2004) and *Myc* loss in the mouse leads to stunted growth, neural tube closure defects and pericardial swelling, and to profound defects in embryonic vasculogenesis, angiogenesis and hematopoiesis (Davis et al., 1993; Trumpp et al., 2001; Baudino et al., 2002).

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The broad and devastating effects of *Myc* loss on mouse embryonic development suggested that many might be attributed to hematopoietic failure, and/or to defects of $Myc^{-/-}$ embryos in vasculogenesis (Baudino et al., 2002). Indeed, mouse embryos haploinsufficient for the angiogenic cytokine vascular endothelial growth factor (Vegf) (Carmeliet et al., 1996; Ferrara et al., 1996) or its receptors Flt1 (VegfR1) (Fong et al., 1995) or Flk1 (Kdr – Mouse Genome Informatics) (Shalaby et al., 1995) display phenotypes that are similar to those manifest by the Myc knockout. Furthermore, Myc is expressed in endothelial-like progenitors of the blood islands that are known to give rise to the primitive vascular and hematopoietic system, and Myc-deficient cells have defects in the expression of angiogenic regulators (Baudino et al., 2002).

Although the defects in vascular development in the mouse *Myc* knockout suggested this was a cause of lethality, the failure of these embryos could also be due to placental defects. *Xenopus laevis* is a tractable model system that lacks this concern and allows the analysis of pathways that control cell fate decisions during early embryogenesis. More importantly, the vascular system of the *Xenopus* embryo is easily visualized and is very similar to that of higher vertebrates (Levine et al., 2003; Ny et al., 2005). Furthermore, these embryos can survive without a circulatory system for an extended time, permitting mutants to be analyzed well into development (Mohun et al., 2000). Indeed, here we report that specific knockdown of *Xc-Myc* in post-gastrulation stages does not lead to defects in embryonic hematopoiesis or vasculogenesis per se, but rather leads to defects in the maturation and completion of vessel development in both the vascular and lymphatic systems. Importantly, these defects were rescued by the transcription factors Twist and/or Slug (also known as Snail2), indicating that a Myc-Twist/Slug circuit is required to direct and complete normal vessel development.

MATERIALS AND METHODS

Morpholino oligonucleotides, plasmids and mRNA

To knockdown Xc-Myc expression, we designed a morpholino oligonucleotide (Gene-Tools), Mo I (5′-CATCTTTCCTGCCATGCGAT-TGGTC) to target *Xc-Myc1* transcripts, which are expressed throughout

embryonic development (see Fig. S1 in the supplementary material). A standard, commercially available control morpholino (5′-CCTCTT-ACCTCAGTTACAATTTATA) and a scrambled *Xc-Myc* morpholino (5′-**C**GA**T**AATT**C**GCGTTAA**C**AGGCAT**G**T) with five mispairs (in bold) were used in this study. For rescue experiments, the *Xc-Myc* cDNA was used to generate a construct (Xc-Myc-Mut) in which the morpholino recognition region was mutated by site-directed mutagenesis using the Stratagene Quick Change Site-directed Kit. Mutations were confirmed by sequencing. Morpholino oligos designed to target *XSlug* (5′-TCTTGACCAGAA-AAGAGCGTGGCAT) and *XTwist* (5′-TGGACTCTTCCTGCATCA-TTTTCT) were obtained from Gene-Tools. *XSlug* and *XTwist* were cloned into the pGEMT-Easy vector. Capped mRNA from *Xc-Myc-Mut*, wild-type *Xc-Myc*, *XSlug* (*Snail2*), *XTwist* and *β-galactosidase* were synthesized using the mMessage mMachine kit (Ambion).

Embryology and microinjections

Xenopus laevis embryos were generated and staged according to standard procedures (Sive et al., 2000). Knockdown experiments were performed by injecting embryos at the one-cell stage with 40 ng of *Xc-Myc* morpholino (Mo). Control morpholinos were injected at 40-80 ng per embryo. Following injections, embryos were visually inspected at the indicated stages of development until they reached stage 45, and were scored for the presence of edema. Embryos were stained with benzidine (Hemmati-Brivanlou and Thomsen, 1995) to visualize effects on Xc-Myc knockdown on blood development and the vascular system. For rescue experiments, 500 pg of *Xc-Myc-Mut*, *XSlug* or *XTwist* mRNA were mixed with 40 ng Mo and injected at the one-cell stage. Injections of wild-type *Xc-Myc* or *β-galactosidase* mRNA with Mo were used as controls. Embryos used for in situ hybridization were injected at the two-cell stage, in one blastomere, with half the dose of Mo (20 ng) or RNA (250 pg) mixed with mRNA encoding *β-galactosidase* to mark the injected side. At least three different batches of embryos obtained from different frogs were used for each set of experiments.

Lineage analysis was initially carried out at the 32-cell stage, yet here the morpholino dose proved toxic or failed to produce any phenotype at the lower doses tested. Therefore, lineage analysis was done at the 16-cell stage and provoked lineage-specific phenotypes. Following the fate maps of Moody (Moody, 1987), blastomeres of either the D2.1, V1.2 or V2.1 lineage were injected with a 1 nl mixture of rhodamine-dextran and either *Xc-Myc* morpholino (800 nM), or a combination of *XSlug* (266 nM) and *XTwist* (534 nM) morpholinos. The embryos were cultured overnight and sorted at stage 13 based on the presence of the rhodamine signal. Embryos which showed positive labeling were followed until stage 45 and scored for developmental defects including hemorrhage and edema. Lineage analyses were from different batches of embryos obtained from four different frogs.

Western blot analyses, whole-mount in situ hybridization and histology

Whole embryo extracts were prepared by Freon extraction and western blots were performed using a Myc antibody (Santa Cruz) that recognized Xc-Myc protein. In situ hybridization and β-galactosidase staining were performed according to standard methods (Sive et al., 2000). Digoxigenin-UTP-labeled antisense RNA probes were generated against *Xc-Myc*, *XTwist*, *XSlug* and *X-msr* using MegaScript Kit (Ambion). All results were collected from two to four different batches of embryos obtained from different frogs.

For histology, staged embryos were fixed in MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde] overnight at 4°C, dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 μm) were prepared and were stained with Hematoxylin and Eosin.

RESULTS

Xc-Myc is expressed in the primitive mesoderm and ventral blood islands that give rise to the vasculature

As a consequence of genome duplication during evolution (Bisbee et al., 1977), *Xenopus* harbors two copies of *Xc-Myc* (*Xc-Myc1* and *Xc-Myc2*), both of which are maternally expressed. However, only

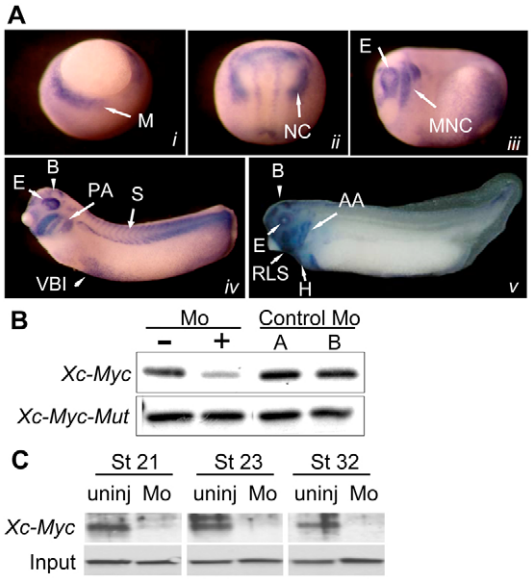

Fig. 1. *Xc-Myc* expression and morpholino knockdown. (**A**) In situ hybridization of *Xc-Myc* expression during development. (i) Gastrula: expression in involuting mesoderm (M) that flanks the yolk plug. (ii) Neurula: expression in anterior region and in the lateral edges of neural crest (NC). (iii) Early tailbud: expression in the developing eye (E) and migratory neural crest cells (MNC). (iv) Tailbud (~stages 27/28): expression in eyes (E), brain (B), somites (S), pharyngeal arches (PA) and ventral blood islands (VBI). (v) Stage 35/36: expression is predominant in head and heart region (eyes, E; brain, B; heart, H; rostral lymph sac, RLS; aortic arches, AA). (**B**) The Xc-Myc morpholino blocks translation of Xc-Myc. In vitro translation of transcripts for wild-type (Xc-Myc) or a morpholino-resistant mutant (Xc-Myc-Mut) in the absence (–) or presence (+) of *Xc-Myc* morpholino (Mo) is shown. Control morpholinos were non-specific (A) or a scrambled Xc-Myc (B) morpholino. (**C**) Knockdown of Xc-Myc protein expression in vivo at different stages. Western blot of Xc-Myc expression in embryos injected with *Xc-Myc* morpholino (Mo); uninjected (uninj); loading control (input).

Xc-Myc1 is zygotically expressed following the mid-blastula transition (Vriz et al., 1989) (see Fig. S1 in the supplementary material). In the mouse embryo, Myc is expressed in endothelial-like (PECAM-1$^+$) cells that line the blood islands of the yolk sac (Baudino et al., 2002), which give rise to both the embryonic vasculature and primitive hematopoietic cells. In *Xenopus*, the precursors of blood and vasculature arise in an analogous region coined the ventral blood island (Turpen, 1998). To define precisely the spatial and temporal patterns of *Xc-Myc* expression in *Xenopus* embryos, we performed whole-mount in situ hybridization (Fig. 1A). The probe was specific for *Xc-Myc* and does not recognize *XN-Myc*. During gastrulation, *Xc-Myc* is expressed in the area flanking the yolk plug corresponding to the involuting mesoderm (Fig. 1A, part i). Furthermore, during the neurula stages, *Xc-Myc* is expressed in the anterior, posterior and lateral edges of the embryo, as previously described (Bellmeyer et al., 2003). The latter region harbors pre-migratory and migratory neural crest precursors (Fig. 1A, parts ii,iii). At the tailbud stage, *Xc-Myc* expression was evident in the developing brain, eyes, somites, pharyngeal arches and, importantly, in the region corresponding to the ventral blood islands (VBI, Fig. 1A, part iv). By stage 35/36, *Xc-Myc* expression expanded into the region where the heart and outflow tract are formed and is strongly expressed in the rostral lymph sac (RLS, Fig.

1A, part v). Therefore, in addition to its known expression and role in neural crest development, *Xc-Myc* is also expressed in primitive mesoderm, in the VBI that gives rise to the vascular system, and in the rostral lymph sac, a crucial component of the lymphatic system.

Xc-Myc loss provokes massive edema and marked defects in organogenesis

Given its early expression in primitive mesoderm, we assessed the potential role of Xc-Myc in vascular and blood development by knocking down Xc-Myc expression in one-cell stage embryos using morpholino oligonucleotides that were designed to block specifically translation of *Xc-Myc* mRNA (Fig. 1B). One-cell stage embryos were injected with a standard control morpholino, a scrambled *Xc-Myc* control morpholino or with the *Xc-Myc*-specific morpholino. The specificity and extent of the knockdown of Xc-Myc was assessed by western blot analysis. In vivo, the levels of Xc-Myc protein were drastically reduced in embryos injected with the *Xc-Myc*-specific morpholino compared with uninjected control embryos, and this was sustained from the neural tube (stages 19-21) through the tailbud stage (stage 32, Fig. 1C). Reduction of Xc-Myc at earlier stages (stage 10.5-11) was not detected (data not shown).

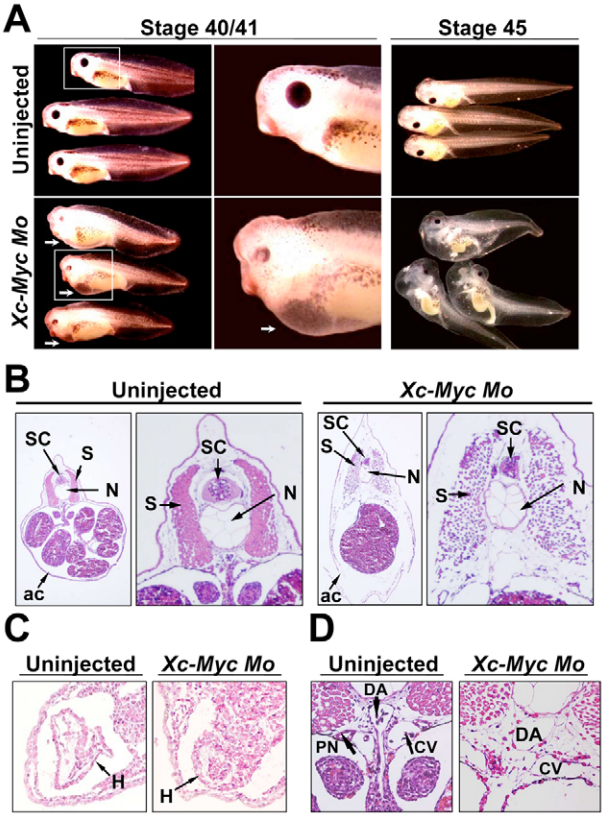

Fig. 2. Knockdown of Xc-Myc provokes edema and compromises organogenesis. (**A**) Embryos injected with *Xc-Myc* morpholino (*Xc-Myc Mo*) at the one-cell stage developed edema in the head and heart region (white arrows). By stage 45 (*right panels*) massive edema and associated defects in organogenesis were obvious in Xc-Myc knockdown tadpoles (*Xc-Myc Mo*). (**B-D**) Histological analysis (stage 37) of Xc-Myc knockdown embryos revealed profound developmental defects: somites (S), spinal cord (SC), notochord (N), abdominal cavity (ac), heart (H), pronephros (PN), dorsal aorta (DA) and cardinal vein (CV). (C) The heart is shown. (D) The region containing the dorsal aorta and cardinal vein.

By contrast, Xc-Myc levels were unaffected by either of the two control morpholino oligonucleotides, as determined by in vitro translation (Fig. 1B). A successful phenotypic rescue of the Xc-Myc knockdown is presented in Fig. 5.

Knockdown of Xc-Myc did not lead to obvious external developmental abnormalities through stage 28 (see Fig. S2 in the supplementary material), although changes in gene expression were evident at this stage (see below, Fig. 4B). As expected (Bellmeyer et al., 2003), *Xc-Myc* morpholino-injected embryos showed craniofacial defects by stage 41 (Fig. 2A, Fig. 3A). However, in addition, we also observed obvious signs of ventral edema in Xc-Myc-knockdown embryos, and by stage 45 this edema was profound (Fig. 2A, white arrows). Cross-sections of embryos at stage 37 revealed marked defects in the development of the digestive tract that were associated with edema (Fig. 2B), as well as defects in the development of the somites and spinal cord, which were atrophied or missing in Xc-Myc-knockdown embryos (Fig. 2B). Furthermore, at stage 37 the hearts of Xc-Myc knockdown embryos lacked normal chambers (Fig. 2C) and large blood vessels such as the dorsal aorta and posterior cardinal vein appeared extremely thin (Fig. 2D, Fig. 3C). Therefore, knockdown of Xc-Myc in *Xenopus* leads to marked edema and associated catastrophic effects on organogenesis.

Xc-Myc knockdown impairs endothelial cell assembly into patent tubules

We reasoned that many of the abnormalities observed during organogenesis and the massive edema in Xc-Myc-knockdown embryos might reflect a defective vasculature and/or lymphatic system. We ruled out one explanation for the source of edema, incomplete closure of the neural tube, as we found this step to be normal in Xc-Myc-knockdown embryos (data not shown). At the stages of embryonic development affected, blood vessels are composed exclusively of endothelial cells and pericytes, which are derived from mesoderm and neural crest progenitors, respectively (Etchevers et al., 2001; Cox et al., 2006). To determine whether vascular endothelial cells were correctly specified and/or assembled in Xc-Myc-depleted embryos, we assessed the effects of Xc-Myc knockdown on the expression of the embryonic endothelial lineage marker *Xenopus mesenchyme-associated serpentine receptor* [*X-msr* (Devic et al., 1996)].

Whole-mount in situ hybridization showed a reduced and more diffuse staining pattern for *X-msr* in Xc-Myc knockdown stage 37 embryos compared with uninjected embryos. This was especially evident in the blood vessels of the head region, including the aortic arches and the anterior cardinal vein, as well as in the vitelline vein network (Fig. 3A,B). The vasculature surrounding the lens (tunica vasculosa lentis) and the developing retina (choroid) was also incomplete and/or collapsed (Fig. 3A,B). One of the most telling defects was in the rostral lymph sac (RLS), which appeared completely missing. Normally this distinct structure, found just anterior to the heart region, strongly expresses *X-msr* (Fig. 3B) (Ny et al., 2005). This sac is composed of endothelial cells derived from a common endothelial precursor that also gives rise to vascular progenitors originating in the lateral plate mesoderm (Ny et al., 2005). In Xc-Myc-depleted embryos *X-msr* staining is essentially absent in this region (Fig. 3B, arrowhead). Therefore, the Myc knockdown phenotype also includes defects in lymphatic vessel development.

In most cases, the endothelial cells detected by *X-msr* expression in Xc-Myc knockdown embryos appeared to align in a roughly normal vascular pattern, yet their staining was much weaker and was

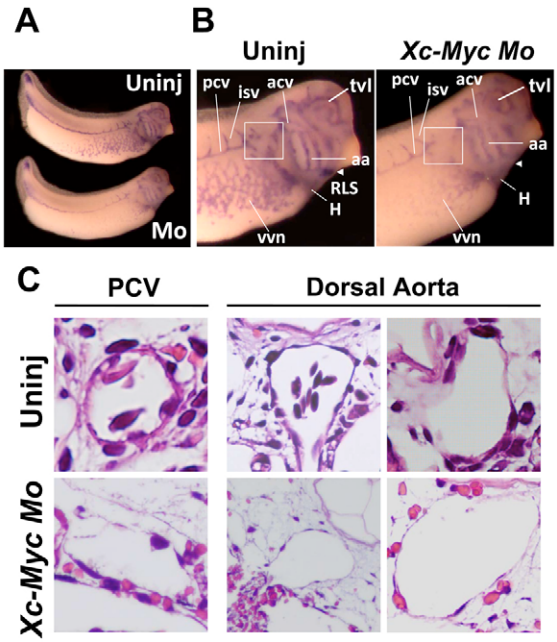

Fig. 3. Xc-Myc knockdown impairs endothelial cell development. (**A**,**B**) Whole mount in situ hybridization (stage 37) for endothelial lineage marker *X-msr*; (B) enlargement of the embryos shown in A. *X-msr* staining is reduced and diffuse in Xc-Myc knockdown embryos throughout, and the vitelline vein network (vvn), aortic arches (aa), tunica vasculosa lentis (tvl), anterior cardinal vein (acv) and rostral lymph sac (RLS) are reduced or missing. H, heart; pcv, posterior cardinal vein; isv, intersegmental vessels. (**C**) Histology of posterior cardinal vein (pcv) and dorsal aorta at stage 37. Uninj, uninjected control; *Xc-Myc Mo*, *Xc-Myc* morpholino-injected embryos. The vessels found in Xc-Myc morpholino-injected embryos have remarkably thin walls and empty lumens. Analyses based on five or six embryos from two different experiments.

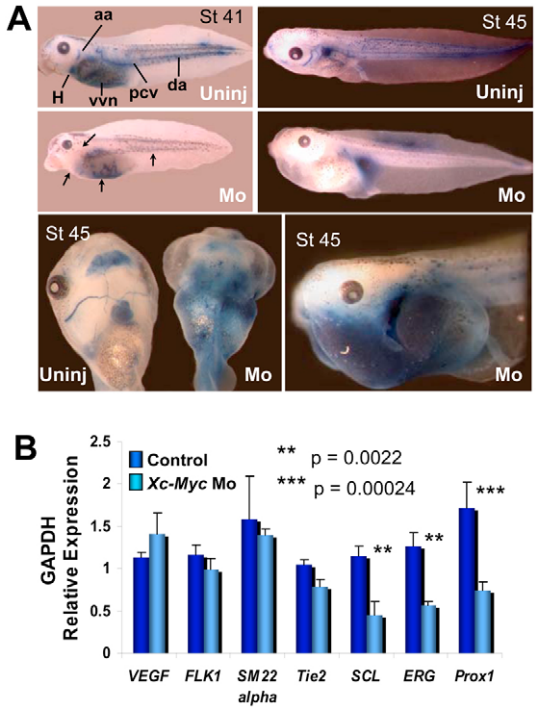

Fig. 4. Edema provoked by Xc-Myc knockdown is associated with massive hemorrhage and impaired expression of *X-ERG*, *Scl* and *Prox1*. (**A**) The vascular networks of uninjected (Uninj) and *Xc-Myc* morpholino (Mo)-injected embryos were visualized by staining blood cells with benzidine. Hemorrhagic areas are present throughout body proper of Mo-injected embryos. H, heart; aa, aortic arches; vvn, vitelline vein network; pcv, posterior cardinal vein; da, dorsal aorta. (**B**) The effects of Xc-Myc knockdown on the expression of genes involved in vascular development was assessed by qRT-PCR in control (dark blue bars) and *Xc-Myc* morpholino-injected (light blue bars) embryos at stage 28. Expression was normalized to *Gapdh*.

often diffuse (Fig. 3A,B). Indeed, this was confirmed by detailed histological analyses of the posterior cardinal vein and the dorsal aorta, which revealed that endothelial linings of these major blood vessels were very thin and appeared to be composed of fewer endothelial cells (Fig. 3C).

Xc-Myc is essential for normal vascular development in *Xenopus*

To assess the status and function of the hematopoietic and vascular system in Xc-Myc-depleted embryos, we initially stained erythrocytes at stage 45 with benzidine, which stains red blood cells and thus allows visualization of the entire circulatory system (Hemmati-Brivanlou and Thomsen, 1995). Benzidine staining revealed that tadpoles injected with control morpholinos had a well-developed vasculature, including the aortic arches emanating from the heart (Fig. 4A). In Xc-Myc-knockdown tadpoles, blood was also evident; therefore, there are no obvious effects of Xc-Myc loss on erythrocyte development. However, the blood was pooled and localized in hemorrhagic areas in different regions of the body proper in Xc-Myc-depleted embryos, most often in the abdominal cavity (Fig. 4A). Moreover, in most of the Xc-Myc-knockdown embryos, the aortic arches and peripheral vessels in the tail were not stained by benzidine, indicating that blood had leaked from the vasculature. Indeed, quantification of these phenotypes demonstrated that ~85% of *Xc-Myc* morpholino-injected stage 45 embryos had profound edema (Fig. 5A, n=339) and that nearly all of these (n=316) had associated defects in vessel development (Fig. 5B, part ii; Fig. 5C), clearly linking these two events. By contrast, fewer than 5% of uninjected embryos (n=617) or embryos injected with a scrambled control *Xc-Myc* morpholino (n=190) showed edema and vascular defects (Fig. 5A,B, part i; Fig. 5C).

Defects in vascular development in Xc-Myc-knockdown embryos were observed well before edema became obvious at stages 40/41. For example, athough blood could be observed throughout the circulatory system of control embryos, it was largely absent in the peripheral circulatory system of *Xc-Myc* morpholino-injected stage 37 embryos (Fig. 3B).

To confirm that the developmental defects provoked by the *Xc-Myc* morpholino were not due to off-target effects, embryos were co-injected with the *Xc-Myc* morpholino and mRNA encoding a wobble mutant of Xc-Myc (Xc-Myc-Mut) designed to block its recognition by the *Xc-Myc* morpholino. Indeed, in vitro translation of Xc-Myc-Mut protein was unaffected by the *Xc-Myc* morpholino (Fig. 1B). Importantly, all of the defects that were manifest in Xc-Myc knockdown embryos, including edema (n=327) and vascular defects (n=240), were rescued by co-injection of mRNA encoding the mutant of Xc-Myc (Fig. 5A,B, part iii; Fig. 5C).

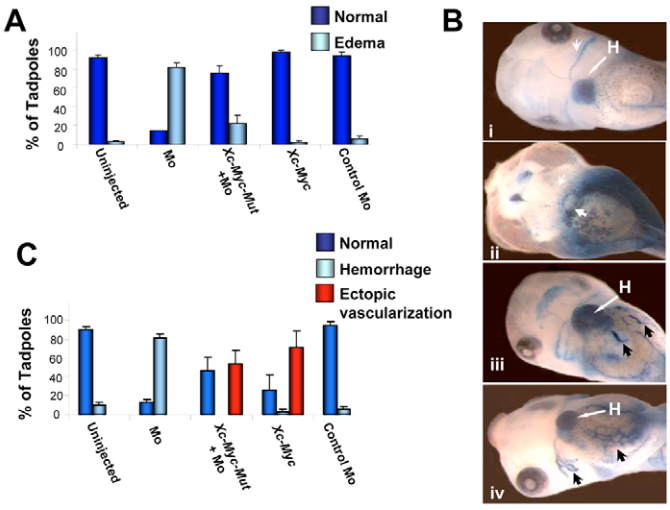

Fig. 5. Edema provoked by Xc-Myc knockdown is intrinsic and specific and Xc-Myc overexpression induces ectopic vascular beds. (**A**) The percentage of *Xc-Myc* morpholino-injected embryos that developed edema (light blue bars) or appeared normal (dark blue bars) is shown. Co-injection of *Xc-Myc-Mut* rescued the edema phenotype. (**B**) Representative images of stage 45 control (i) or those injected with *Xc-Myc* morpholino (ii), with both *Xc-Myc-Mut* mRNA and *Xc-Myc* morpholino (iii) [note rescue of phenotype and hypervascularization (black arrows)], or with *Xc-Myc* RNA (iv), showing hypervascularization and ectopic vascular beds (black arrows). (**C**) The percentage of *Xc-Myc* morpholino-injected embryos with hemorrhage. Co-injection of *Xc-Myc-Mut* mRNA rescued the phenotype. Injection of either wild-type *Xc-Myc* or mutant *Xc-Myc-Mut* mRNA induced hypervascularization (red bars).

In addition to ameliorating the edema and vascular phenotypes of Xc-Myc knockdown embryos, overexpression of Xc-Myc-Mut often led (in 55% of injected embryos) to abnormal patterns of blood distribution, which were characterized by ectopic vascular beds and hypervascularization (Fig. 5B, part iii, black arrows; Fig. 5C, red bars, n=240). Furthermore, 70% of embryos injected with wild-type *Xc-Myc* mRNA alone displayed an ectopic formation of blood vessels (Fig. 5B, part iv, black arrows; Fig. 5C, red bars, n=146), consistent with findings in mice that have demonstrated Myc overexpression can provoke angiogenesis (Pelengaris et al., 1999; Knies-Bamforth et al., 2004). Therefore, physiological thresholds of Myc are essential for normal formation of patent vessels during vasculogenesis.

Xc-Myc is required for the normal expression of *Egr*, *Scl* and *Prox1*

To assess whether the defects in vessel development of the vascular and lymphatic systems caused by Myc knockdown were associated with specific deficits in the expression of key regulators of vasculogenesis or lymphangiogenesis, we analyzed the expression of *Vegf* and its receptor *Flk1*, which are important for the initial steps of differentiation of angioblasts into endothelial cells. We also examined expression of the angiopoietin receptor Tie2, which regulates the recruitment and migration of smooth muscle cells and pericytes that cover newly forming blood vessels (Rossant and Howard, 2002; Carmeliet, 2003; Cleaver and Melton, 2003; Cleaver, 2004). In addition, we assessed the expression of *X-erg*, an Ets family transcription factor that is expressed in vascular endothelial cells, in the neural crest-derived mesenchymal cells of pharyngeal arches, and in the endocardium. *X-erg* is thought to direct cell migration during vascular development (Remy and Baltzinger, 2000; Tahtakran and Selleck, 2003). Although *Vegf*, *Flk1* and *Tie2* expression changed little in Xc-Myc knockdown embryos, *X-erg* expression was reduced by ~50% (Fig. 4B).

Additional transcriptional regulators of vasculogenesis and lymphangiogenesis include the transcription factors Scl, which plays critical roles in yolk sac erythropoiesis and in angiogenic remodeling of the yolk sac capillaries into complex vitelline vessels (Mead et al., 1998), and Prox1, a master regulator of lymphangiogenesis (Wigle and Oliver, 1999; Wigle et al., 2002). Notably, knockdown of Xc-Myc led to marked reductions in the expression of both *Scl* and *Prox1* (Fig. 4B). Like *X-erg*, reduced levels of Scl and Prox1 would be predicted to contribute to the edematous phenotype of Xc-Myc knockdown embryos, which displayed marked defects in the ventral vascular plexus and rostral lymph sac.

A Myc-to-Slug/Twist pathway directs vasculogenesis and lymphangiogenesis

Knockdown of Myc in *Xenopus* results in the loss of neural crest derivatives, such as craniofacial structures, cartilage and fins, and compromises the expression of the *Slug* and *Twist* transcription factors in the neural crest. Indeed, *Slug* and *Twist* are also important for cell fate determination and migration of the neural crest and function downstream of Xc-Myc (LaBonne and Bronner-Fraser, 2000; Bellmeyer et al., 2003). However, *Slug* and *Twist* are also expressed in the lateral plate mesoderm, which generates endothelial precursor cells (Hopwood et al., 1989; Mayor et al., 1995). This suggested that Slug and/or Twist might also function downstream of Xc-Myc to coordinate normal blood and lymph vessel development. As expected, injection of *Xc-Myc* morpholino into one cell of a two-cell embryo disrupted the expression of *Twist* and *Slug* on the injected side, and these defects were rescued by the co-injection with the mRNA encoding the wobble mutant of Xc-Myc (see Fig. S3 in the supplementary material). Furthermore, co-injection of *Xc-Myc* morpholino and *Slug* or *Twist* mRNA into one cell of two-cell embryos rescued defects in endogenous *XSlug* or *XTwist* expression (see Fig. S3 in the supplementary material). Therefore, either *XSlug* or *XTwist* can function downstream of Myc.

To test the potential functions of XSlug or XTwist downstream of Myc in vessel development, one-cell embryos were co-injected with *Xc-Myc* morpholino and *Slug* or *Twist* mRNAs, and assessed at stage 45 by staining with benzidine. Notably, many embryos injected with Xc-*Myc* morpholino together with *Slug* (n=181) or *Twist* (n=242) mRNA did not develop edema or hemorrhage, a hallmark of those injected with the Xc-*Myc* morpholino alone (Fig. 6A,B, n=316). Rescue of this phenotype was specific for *Slug* or *Twist* mRNA, as embryos co-injected with the Xc-*Myc* morpholino and β-*galactosidase* mRNA (n=169) displayed profound defects in vascular development (Fig. 6A). Indeed, *Slug* or *Twist* mRNA/Xc-*Myc* morpholino co-injected embryos (n=327) were phenotypically similar to normal embryos and to those rescued by co-injection of the wobble Xc-*Myc-Mut* mRNA (n=108, Fig. 6A,B). However, *Slug*- or *Twist*-injected embryos lacked the hyper-vascularization phenotype that characterized Xc-Myc overexpression (Fig. 5B,C). Therefore, enforced expression of either *Slug* or *Twist* can specifically rescue the defects in vessel development provoked by Xc-Myc knockdown.

To investigate more directly what cell lineage Xc-Myc, XTwist and XSlug were acting through to affect vessel development, we injected their corresponding morpholinos together with a lineage

tracer into blastomeres of the 16-cell stage embryo whose descendants mostly contribute to either neural crest or lateral plate mesoderm. Both XTwist (Hopwood et al., 1989) and XSlug (Mayor et al., 1995) are expressed in lateral plate mesoderm. The V1.2 blastomeres are a major contributor to both trunk and head neural crest, whereas D2.1 mostly to head neural crest and lateral plate mesoderm. V2.1 contributes mostly to lateral plate mesoderm and nothing to head neural crest (Fig. 7A,B) (Moody, 1987). Specifically, descendents of the D2.1 lineage give rise to embryonic blood, endocardium and endothelial cells that comprise the ventral aorta and vitelline veins, whereas V2.1 descendents give rise to blood precursor cells and endothelial cells of major blood vessels (Walmsley et al., 2002). Notably, Xc-Myc or XSlug/XTwist knockdown within the neural crest lineage (V1.2 or D1.2) did not result in edema (0%, *n*=41; 0%, *n*=29) and very few (3%, *n*=41; 7%, *n*=29) developed hemorrhagic spots (compare Fig. 7B,C, V1.2, controls with Fig. 4A). In sharp contrast, Xc-Myc morpholino injections into D2.1 (lateral plate mesoderm) resulted in 44% edematous and 54% hemorrhagic embryos (*n*=57, Fig. 7B). Similar results were obtained for the XSlug/XTwist MO D2.1 injections (33%, 33%, *n*=18) with most embryos displaying both phenotypes (Fig. 7B,C). Interestingly, knockdown of Xc-Myc or XSlug/XTwist in the posterior lateral plate mesoderm (V2.1) was sufficient to cause edema in the head (33%, *n*=15) and edematous or hemorrhagic areas throughout the embryo (66%, *n*=15; and 75%, *n*=12). These findings are inconsistent with a cell-autonomous effect of Xc-myc, XSlug or XTwist in the neural crest lineage for vascular development. Furthermore, a lack of benzidine staining of the brachial arches, heart and outflow tract was also observed in the posterior lateral plate mesoderm targeted knockdowns (Fig. 7B,C). Collectively, these findings show that Xc-Myc and XSlug/XTwist operate in a regulatory pathway outside of the neural crest lineage, but within the lateral plate mesoderm, to affect both the endothelial and blood lineages.

Interestingly, although *Xc-Myc* expression precedes *Slug*, morpholino-induced knockdown of Slug also affected *Xc-Myc* expression (Fig. 6C, parts i-iii, *n*=11). Indeed, almost 90% of *Slug* morpholino-injected embryos showed effects on *Xc-Myc* expression in the somites, eye region and neural crest (Fig. 6C). Furthermore, although neural crest cells expressing *Xc-Myc* were affected by *Slug* knockdown, the vascular defects evident following Xc-Myc knockdown were never observed (Fig. 6C, part iv,v). Therefore, although Slug is sufficient to rescue the effects of knockdown of Myc on vessel development, loss of *XSlug* alone is not sufficient to disrupt vascular integrity, but also required loss of *XTwist*. These results indicate a level of functional redundancy downstream of Myc in this pathway.

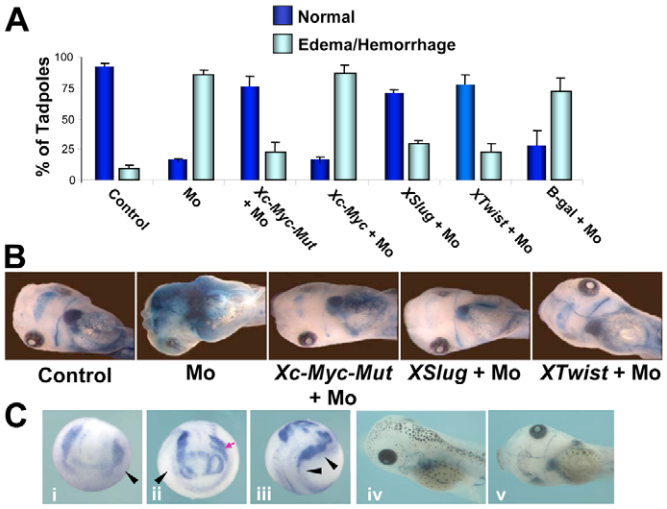

Fig. 6. Slug or Twist rescue the vascular defects provoked by Xc-Myc knockdown. (**A**) The percentage of embryos that appeared normal (dark-blue bars) or showed edema associated with hemorrhage (light-blue bars) after injection of *Xc-Myc* morpholino alone or mixed with *Xc-Myc-Mut*, wild-type *Xc-Myc*, *Slug*, *Twist* or *β-galactosidase* mRNAs. (**B**) Representative images of the results in A. showing the rescue of the Xc-Myc knockdown phenotype by Slug or Twist. (**C**) One blastomere of a two-cell stage embryo was injected with *Slug* morpholino. In situ hybridization at the neural tube stage using a probe specific for *Xc-Myc* showed that knockdown of Slug indeed affects *Xc-Myc* expression in the region corresponding to the neural crest (i-iii). On the injected side (black arrowheads) an accumulation of Xc-Myc-expressing cells is evident, while on the uninjected side cells expressing Xc-Myc have started to migrate (red arrow). A deviation in the body axis is also evident (ii,iii). Despite affecting *Xc-Myc* expression during neurulation, knockdown of *Slug* in two-cell embryos is not sufficient to cause the edema and hemorrhagic phenotype (iv,v), as observed upon Xc-Myc knockdown using *Xc-Myc* morpholino.

DISCUSSION

Myc oncoproteins are required for cell growth and cell cycle traverse in most (but not all) tissue types. Accordingly, here we have shown that Xc-Myc is also required for normal vessel development. The pathway by which Xc-Myc regulates this process is, however, complex, where Xc-Myc functions are specifically required for endothelial cells to assemble into patent vessels during both vasculogenesis and lymphangiogenesis. Here, Xc-Myc is required for normal expression of *Erg*, *Scl* and *Prox1*, transcription factors that regulate vascular and lymphatic development (Mead et al., 1998; Wigle and Oliver, 1999; Wigle et al., 2002; Tahtakran and Selleck, 2003). Furthermore, Slug and Twist, transcription factors that function downstream of Myc in neural crest development (Bellmeyer et al., 2003), are shown here to also function downstream of Myc in directing normal vessel development within the lateral plate lineage, a site where both genes are also expressed (Mayor et al., 1995; Hopwood et al., 1989). Given the observed effects of knockdown of Slug on the expression of *Xc-Myc*, the ability of Slug or Twist to rescue the phenotypes manifest following Xc-Myc knockdown, and the ability of XSlug/XTwist morpholinos to cause vascular defects similar to Xc-Myc knockdowns, these findings support a model whereby Myc, Twist, and Slug function in a regulatory circuit that also directs the maturation of vessels of the vascular and lymphatic systems (Fig. 8).

Myc is required for normal vessel development in *Xenopus*

In the mouse, Myc appears required for primitive erythropoiesis and vasculogenesis (Baudino et al., 2002). In our studies, Xc-Myc was not fully depleted until the neural tube stage (stage 21), leaving open the possibility for an earlier role for Xc-Myc in the specification of endothelial progenitors. Maternal Xc-Myc is present at high levels in the unfertilized egg and could provide a source of this protein to the early embryo (Vriz et al., 1989). However, loss of Xc-Myc by stage 21 provokes massive hemorrhage and edema, which are indicative of vascular defects. Xc-Myc-depleted embryos displayed very thin vessels that were generally devoid of blood cells, which apparently had leaked out and pooled in the embryo body proper. These phenotypes were intrinsic to Myc. As progenitors for blood

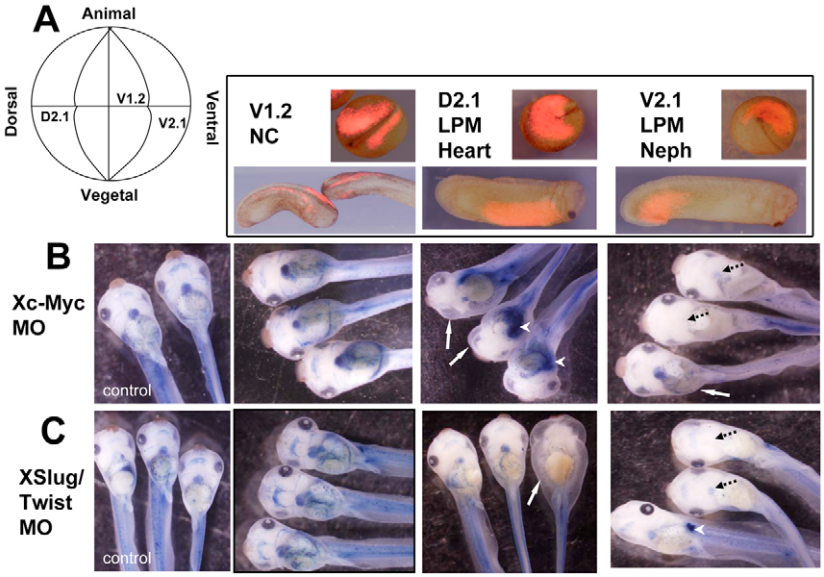

Fig. 7. Xc-Myc or XSlug/XTwist targeted knockdown in lateral plate mesoderm but not the neural crest lineage, provokes edema and hemorrhage. (**A**) Map showing the blastomeres targeted for knockdown of *Xc-Myc or XSlug/XTwist* in predominantly neural crest (V1.2) or lateral plate precursors (D2.1 or V2.1). Correct targeting of the morpholinos is shown in the next three images taken at neurula and tailbud when neural crest cells start migrating. NC, neural crest; LPM, lateral plate mesoderm; Neph, nephrotome. (**B**,**C**) Embryos were injected with a mixture of either *Xc-Myc* morpholino (B) or XSlug/XTwist morpholino (C), and were dextran fluorescently labeled with rhodamine. At stage 43, embryos were stained with benzidine to reveal the location of blood. Knockdown of *Xc-myc* or *XSlug/XTwist* within the lateral plate mesoderm (3rd and 4th column), but not the neural crest (2nd column), provoked edema (arrows) and hemorrhagic (arrowheads) phenotypes. Levels of blood in the V2.1 injected embryos (4th column) are significantly lower (dashed arrow).

and endothelial cells first appear just posterior to the cement gland and in the lateral plate mesoderm at the neurula stages (Walmsley et al., 2002; Cleaver, 2004), we infer that these progenitors, and the lymphangioblasts and endothelial cells that arise from them (Ny et al., 2005), require Xc-Myc to mature and form patent fully functional vessels. Conversely, we also infer that the hypervascularization phenotype observed in embryos injected with *Xc-Myc* mRNA may be due to increases in numbers of angioblasts and/or their progeny, which results in the production of excess vessels, a phenotype that is akin to those observed following ectopic expression of Vegf in *Xenopus* (Cleaver, 2004).

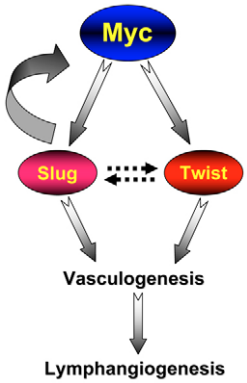

Fig. 8. A Myc-Slug/Twist regulatory circuit directs vasculogenesis and lymphangiogenesis. Xc-Myc is required for the normal expression of the *XSlug* and *XTwist* transcription factors, either of which are sufficient to rescue the defects in vascular and lymphatic development provoked by knockdown of Xc-Myc. Knockdown of XSlug also disrupts normal patterns of *Xc-Myc* expression, indicating a positive-feedback loop. Knockdown of both *XSlug* and *XTwist* in lateral plate mesoderm, but not neural crest, results in the vascular defects observed after knockdown of Xc-Myc, indicating both genes operate in the same pathway and downstream of Myc. Either ectopic Slug or Twist rescue defects in endogenous *XSlug* or *XTwist* expression induced by Xc-Myc knockdown, indicating that they also regulate each the expression of one another.

In the *Myc* knockout mouse embryo there appear to be defects in the expression of regulators necessary for initiation of the vasculogenesis program, in particular in the expression of *Vegf* and its receptors *Flk1* and *Flt1* (Baudino et al., 2002). By contrast, in *Xenopus*, in situ hybridization and histological analyses revealed that many of the early steps in vasculogenesis occurred normally in Xc-Myc knockdown embryos, including the specification of endothelial cell precursors (angioblasts), their migration and coalescence into continuous strands of endothelial cells at correct locations in the embryo, and proper expression of *Vegf* and *Flk1*. Thus, given that Xc-Myc is expressed in the lateral plate mesoderm and VBI where angioblasts form, these developmental steps are either independent of Xc-Myc, or occur before effective MO Xc-Myc knockdown at stage 21.

Xc-Myc is required in endothelial cells for vessel maturation

Xc-Myc may regulate *Xenopus* vascular development at different levels. First, the numbers of endothelial cells may be reduced in Xc-Myc knockdown embryos, suggesting at least a partial role for Xc-Myc in the early expansion of this cell lineage. Interestingly, Bellmeyer et al. (Bellmeyer et al., 2003) ruled out a role for Xc-Myc in proliferation within the neural crest lineage. Although we were unable to detect obvious differences in cell proliferation after knockdown of Xc-Myc using histone markers for proliferation (data not shown), Xc-Myc has been reported to regulate cell proliferation in *Xenopus* (Etard et al., 2005). Second, as endothelial cells assemble into tubes later in development, they may fail to form normal intercellular junctions following Xc-Myc knockdown, resulting in inadequate sealing. Third, smooth muscle cells or pericytes may fail to recruit to endothelial tubes, which would also result in leaky vessels. The latter hypothesis was appealing given the involvement of Myc in neural crest specification in *Xenopus* (Bellmeyer et al., 2003) and the contribution of neural crest cells to smooth muscle and pericytes in the anterior regions of the cardiovascular system (La Bonne and Bronner-Fraser, 1999; Huang and Saint-Jeannet, 2004). However, our data support endothelial cells as the crucial target as we observed leaky vessels devoid of red blood cells by stage 37, a time prior to the recruitment of smooth muscle cells and pericytes to vessels (Warkman et al., 2005; Cox et

al., 2006). In addition, Xc-Myc-depleted embryos lacked a distinct rostral lymph sac (Fig. 3B), a structure comprised only of endothelial cells that are held together by desmosomal-like structures (Ny et al., 2005). Collectively, these observations are therefore most consistent with Xc-Myc-depleted embryos being defective in endothelial numbers and cell-cell adhesion, rather than in mural cell recruitment. Together with our lineage studies, these results further support a role for Xc-Myc in vascular development independent of the neural crest. However, as neural crest cells migrate over the mesoderm, we cannot rule out that perturbing mesodermal lineages does not also affect neural crest migration, although we can rule out a cell-autonomous effect of c-Myc on the neural crest lineage in vascular development.

The Myc-Slug/Twist regulatory circuit

Slug and Twist perform important developmental roles as mediators of the epithelial-to-mesenchymal transition by regulating the expression of cell-adhesion molecules such as V-cadherin (Bolos et al., 2003; Marin and Nieto, 2004), which are also regulated indirectly by Myc through Slug (Wilson et al., 2004). More recently, Twist and Slug have been suggested to play crucial roles in cancer and tumor angiogenesis, processes that are also regulated by Myc (Pelengaris et al., 1999; Baudino et al., 2002; Valsesia-Wittmann et al., 2004; Elloul et al., 2005; Yang et al., 2006). Slug and Twist have well described roles in neural crest development in *Xenopus* but have never been associated with vascular development in this organism (LaBonne and Bronner-Fraser, 1999; LaBonne and Bronner-Fraser, 2000; Huang and Saint-Jeannet, 2004). Notably, Xc-Myc knockdown disrupted the normal expression of *Slug* and *Twist* (data not shown), and ectopic Twist or Slug were able to rescue the vascular defects of Xc-Myc knockdown embryos. Moreover, Slug knockdown affected the expression and/or migration of *Xc-Myc*-expressing cells, indicating a feedback loop in this regulatory circuit (Fig. 8). However, knockdown of XSlug alone did not elicit vascular defects, but an XSlug/XTwist dual knockdown did reveal redundancy in this pathway (Fig. 7B,C). Collectively, these findings support a model whereby Myc, Twist and Slug also function in a regulatory circuit that directs vasculogenesis and lymphangiogenesis outside the neural crest lineage (Fig. 8).

We propose that endothelial cell-cell interactions are directed by Xc-Myc. Also consistent with this model, we found that Xc-Myc depletion compromised the expression of *Scl*, which directly induces the expression of vascular endothelial (VE)-cadherin (Deleuze et al., 2007). Notably, VE-cadherin is an essential adhesion factor at intercellular junctions important in the patency of blood and lymph vessels (Ny et al., 2005). Therefore, Myc, together with Slug and/or Twist, may regulate the assembly of blood vessels by directing the expression of these crucial adhesion receptors. Future studies will explore these models of Myc function.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/1903/DC1

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