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

Claudia O. Rodrigues1,2, Steve T. Nerlick3, Elsie L. White1, John L. Cleveland1,4,* and Mary Lou King3,4*

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 knockout 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 XTwit could compensate for defects manifest by Xc-Myc knockout. Importantly, knockdown of Xc-Myc, XSLug/Snail2 or XTwit 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., 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 transition of the cell cycle (de Alborn 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).

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′-CATTTTCTGCTGCATGCGAT-TGGTC) to target Xc-Myc 1 transcripts, which are expressed throughout
embryonic development (see Fig. S1 in the supplementary material). A standard, commercially available control morpholino (5'-CCTCTT-ACCTCAGTTACAAATTTA) and a scrambled Xc-Myc morpholino (5'-CGATAATTCGCTTAAACGGCATG) with five mismatches (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'-CTTGGACCCAGA- AAGGCCGTGGCAT) and Xtwist (5'-TGGACTTCTTCTGCATCA- 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**

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), blastomers 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-mur 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 MgSO4, 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 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 coincident with 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 Xen-Myc. During gastrulation, Xc-Myc is expressed in the area flanking the yolk plug corresponding to the involving 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.
Myc was assessed by western blot analysis. In vivo morpholino. The specificity and extent of the knockdown of Xc-scrambled embryos were injected with a standard control morpholino, a Xc-Myc protein were drastically reduced in embryos injected with the at earlier stages (stage 10.5-11) was not detected (data not shown). Through the tailbud stage (stage 32, Fig. 1C). Reduction of Xc-Myc embryos, and this was sustained from the neural tube (stages 19-21) and cardinal vein.

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).

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 lenti) 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
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 throughout the body proper in Xc-Myc morpholino-injected embryos (Fig. 3B). 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, although 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).
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 X Slug or XT twist expression (see Fig. S3 in the supplementary material). Therefore, either X Slug or XT twist can function downstream of Myc.

To test the potential functions of X Slug or XT twist 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 X-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, XT twist and X Slug were acting through to affect vessel development, we injected their corresponding morpholinos together with a lineage

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

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 X Slug or XT twist expression (see Fig. S3 in the supplementary material). Therefore, either X Slug or XT twist can function downstream of Myc.

To test the potential functions of X Slug or XT twist 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, XT twist and X Slug were acting through to affect vessel development, we injected their corresponding morpholinos together with a lineage

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
tracer into blastomeres of the 16-cell stage embryo whose descendants mostly contribute to either neural crest or lateral plate mesoderm. Both \( \text{XTwist} \) (Hopwood et al., 1989) and \( \text{XSlug} \) (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, descendants 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 descendants 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 Slug 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.

**DISCUSSION**

Myo 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 \( \text{Erg} \), \( \text{Scl} \) and \( \text{Prox1} \), transcription factors that regulate vascular and lymphatic development (Mead et al., 1998; Wigle and Oliver, 1999; Wigle et al., 2002; Tahtakran and Sellock, 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 development.
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).

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-Jennet, 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).
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 Slugg 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 Myc-Slug/Twist 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.

We thank Dr Robert N. Eisenman for providing the Xc-Myc cDNA, Dr Paul Krieg for the X-msr and ERG cDNA, Dr Amar Singh for advice, and Dr Paul Mead for providing the β-galactosidase expression construct and for invaluable guidance. This work was supported by NIDDK R01 grant DK44158 (U.L.C.), by NIGMS R01 grant GM33932 (M.L.K.), by the Cancer Center (CORE) support grant CA21765, and by the American Lebanese Syrian Associated Charities (ALSAC) of St Jude Children’s Research Hospital.

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

References


