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Lef1 plays a role in patterning the mesoderm and ectoderm in *Xenopus tropicalis*

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ABSTRACT Tcf/Lef HMG box transcription factors are nuclear effectors of the canonical Wnt signaling pathway, which function in cell fate specification. Lef1 is required for the development of tissues and organs that depend on epithelial mesenchymal interactions. Here, we report the effects of lef1 loss of function on early development in *X. tropicalis*. Depletion of lef1 affects gene expression already during gastrulation and results in abnormal differentiation of cells derived from ectoderm and mesoderm. At tail bud stages, the epidermis was devoid of ciliated cells and derivatives of the neural crest, e.g. melanocytes and cephalic ganglia were absent. In the Central Nervous System, nerve fibers were absent or underdeveloped. The development of the paraxial mesoderm was affected; intersomitic boundaries were not distinct and development of the hypaxial musculature was impaired. The development of the pronephros and pronephric ducts was disturbed. Most striking was the absence of blood flow in lef1 depleted embryos. Analysis of blood vessel marker genes demonstrated that lef1 is required for the development of the major blood vessels and the heart.

KEY WORDS: *lef1, Xenopus, tropicalis, knockdown, wnt*

Introduction

The Tcf/Lef family of HMG box transcription factors functions in the development of multiple tissues and organs during development as well as in the maintenance of stem cell compartments in adult tissues. The vertebrate Tcf/Lef family consists of four members: Tcf1 (TCF7), Lef1 (LEF1), Tcf3 (TCF7L1) and Tcf4 (TCF7L2) (reviewed in Arce *et al.,* 2006). The interaction of Tcf/Lef proteins with β-catenin is required for the formation of a transcriptional active protein complex to regulate target gene expression (references in Arce *et al.,* 2006). Tcf/Lef proteins also function as transcriptional repressors by binding to members of the Groucho/TLE family. Groucho related proteins mediate a repressor function for all Tcf/Lef proteins through interaction with histone-deacetylase-1 (references in Arce *et al.,* 2006). Lef1 is required for the proper development of hair follicles, mammary glands and teeth which all depend on epithelial mesenchymal interactions (van Genderen *et al.,* 1994). *Lef1* deficient mice lack the mesencephalic nucleus of the trigeminal nerve, which is the only neural crest-derived neuronal population affected (van Genderen *et al.,* 1994).

The endogenous expression pattern of *Lef1* in the mouse and *Xenopus* indicates additional functions also during early development (Oosterwegel *et al.,* 1993; Molenaar *et al.,* 1998; Galceran *et al.,* 1999). *Xenopus lef1* is expressed at high levels in the branchial arches and neural crest derived cells, in the developing heart, lateral plate mesoderm, the tail bud, fins and the mesencephalon (Molenaar *et al.,* 1998). In addition, *Lef1* and *Tcf1* double mutant mice showed additional developmental abnormalities compared to the single mutants and demonstrated redundant functions between Lef1 and Tcf1 during development (Galceran *et al.,* 1999).

In *Xenopus, lef1* expression starts at stage 9 (Molenaar *et al.,* 1998) just prior to mesoderm specification and successive patterning of the mesoderm during gastrulation. Mesoderm patterning is under the control of different signaling pathways including

Abbreviations used in this paper: APJ, angiotensin IIreceptor-like 1; Lef, lymphoid enhancer-binding factor; Msr, mesenchyme-associated serpentine receptor/angiotensin receptor related protein; Myf, myogenic factor; nr-3, nodal-related-3; Wnt, wingless-type MMTV integration site family.

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Wnt signaling. In *Xenopus*, wnt8 is required to pattern the ventro-lateral mesoderm (Christian and Moon, 1993; Hoppler *et al.,* 1996) and restricts the dorsal mesoderm to the prospective dorsal side (Hoppler and Moon, 1998). Recently, we showed in loss of function experiments that lef1 is downstream of wnt8 and is required for patterning the paraxial mesoderm by regulating the expression of *myod* (Roël *et al.,* 2002).

Here, we report the effects of loss of function of lef1 during development in *Xenopus tropicalis*. We show that *lef1* is differentially expressed already at gastrula stages in the ectoderm and the mesoderm. Loss of function experiments demonstrate that lef1 is required for the proper expression of *nodal related 3, myod* and *msr/APJ* to pattern the dorsal and ventro-lateral mesoderm during gastrulation. Our results indicate that in *Xenopus* lef1 regulates the genetic program of somitogenesis through *myod* but not through *myf5*. Analysis of lef1 depleted embryos at the tail bud stage revealed impaired development of different tissues and organs derived from the ectoderm, the mesoderm and the endoderm**.** In particular, formation of major blood vessels and the pronephric ducts that are derived from the lateral plate mesoderm (Walmsley *et al.,* 2002) was affected. The different tissue components of the heart, i.e. endocardium and myocardium, did not develop properly.

Results

Xenopus lef1 *lacks exon VI*

Xenopus lef1 cDNA sequences reported so far (Molenaar *et al.,* 1998) do not contain the exon, annotated as VI in human *LEF1*, which is also represented in other Tcf/Lef members (Arce *et al.,* 2006). *LEF1* exon VI encodes an activation domain and exon VI isoforms may have different functions during development (Gradl *et al.,* 2002). Analysis of *Xenopus* genomic *lef1* sequences between corresponding exons V and VII revealed that *lef1* does not contain exon VI related sequences (data not shown). Also, sequence analysis of *lef1* of *Fugu rubripes*, using the genomic database of the pufferfish (DOE Joint Genome Institute), revealed the lack of exon VI related sequences. A more extensive phylogenetic analysis is required to determine whether this exon was acquired in mammalian genomes or lost in amphibians and fish. We have shown by northern-blot analysis that *lef1* produces only one mRNA during early *Xenopus* development (Molenaar *et al.,* 1998). In addition, screening of *Xenopus* cDNA libraries and 5'-RACE did not uncover evidence for alternative splicing of *lef1* (data not shown). Thus, our lef1 knockdown experiments in *Xenopus* concern the loss of function of a single lef1 isoform not containing the exon VI encoded activation domain present in mammals.

Lef1 is required for patterning the mesoderm in Xenopus tropicalis

To determine functions of lef1 during early development we analyzed the effects of knockdown with antisense morpholino oligonucleotides in *X. tropicalis* embryos. *X. tropicalis* has a diploid genome and is therefore a favorite organism for gene knockdown experiments (Nutt *et al.,* 2001). We determined the endogenous expression pattern of *lef1* in *X. tropicalis* with *X. tropicalis* specific probes (Fig. 1A and Roël *et al.,* unpublished results), which appeared to be very similar to the previously described expression pattern in *X. laevis* (Molenaar *et al.,* 1998). Expression of *lef1* starts at Mid-Blastula Transition, i.e. before gastrulation (Molenaar *et al.,* 1998). *Lef1* is expressed at high levels in lateral and ventral domains of the involuting mesoderm overlapping with the *wnt8* expression domain (Fig. 1B and Christian and Moon, 1993), and it is also expressed in the adjacent endoderm (Fig. 1A).

To block lef1 protein expression during early development, embryos were injected at the two-cell stage with 10 ng of *lef1* morpholino antisense oligonucleotide (morpholino) in each blastomere. In rescue experiments, embryos were co-injected with lef1 morpholino and human *LEF1* RNA, which is not sensitive to the morpholino.

Analysis of gastrula stage embryos showed that lef1 depletion

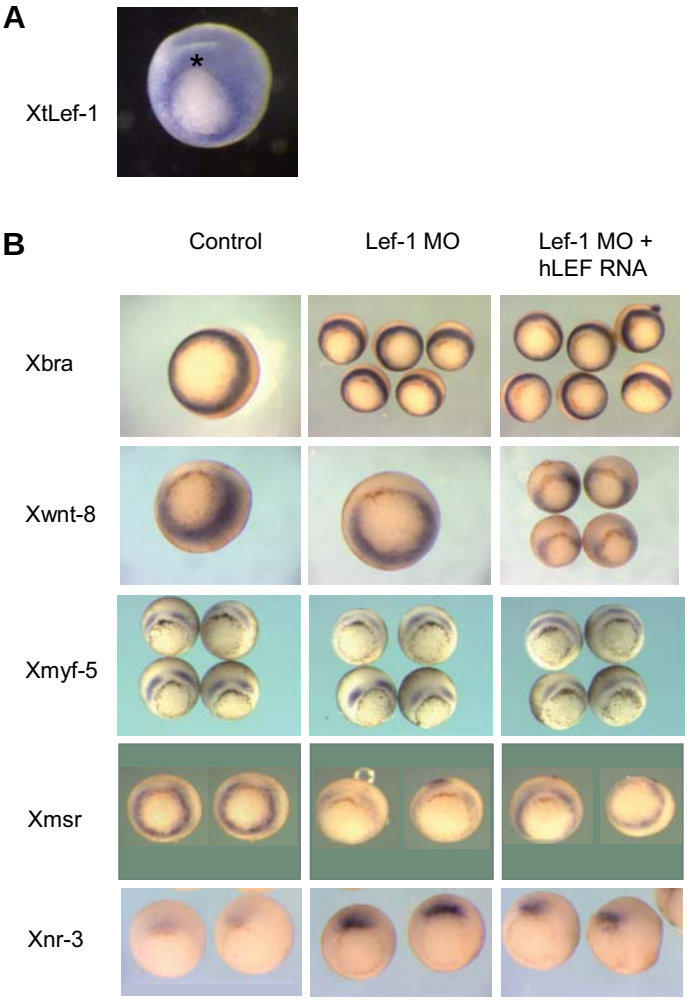

Fig. 1. Lef1 depletion affects expression of genes in the ventral and dorsal mesoderm. In situ *hybridization for* X. tropicalis lef1 *RNA at stage 10.5* **(A)** *shows high levels of expression in the lateral and ventral marginal zone, low expression in the ectoderm and in the dorsal marginal zone (dorsal side up). Expression of marker genes in gastrula stage embryos, stage 10.5* **(B)**, *dorsal side up.* Lef1 *depletion does not alter the expression of* brachyury, wnt8 *or* myf5. Msr/APJ *expression is down-regulated by* lef1 *depletion whereas* nodal related 3 (nr-3) *expression is upregulated. Co-injection of human* LEF1 *RNA rescues the expression of* msr/APJ *and partially downregulates* nr-3 *expresssion.*

did not affect the expression of the pan-mesodermal marker gene *brachyury* (Fig. 1B), indicating that lef1 is not required for mesoderm specification. Both *Myod* and *Myf5* were found to be Wnt targets in mouse paraxial mesoderm explants (Tajbakhsh *et al.,* 1998) and, in *Xenopus*, *myod* and *myf5* are expressed within the *lef1* expression domain. Promoter analysis showed that *myf5* expression might be regulated by β-catenin/tcf in *Xenopus* (Yang *et al.,* 2002; Shi *et al.,* 2002). However, the expression of *myf5* at mid-gastrula stage was not affected by lef1 depletion (Fig. 1B) in contrast to that of *myod* (Roël *et al.,* 2002). These results demonstrate that, in *Xenopus*, lef1 is required for activation of only part of the myogenic genetic program to pattern the presomitic mesoderm during gastrulation.

The expression domain of the endothelial marker *msr* (Devic *et al.,* 1996) in the gastrula embryo overlaps with the expression domains of *lef1* and *wnt8* (Christian and Moon, 1993) and contains high levels of nuclear β-catenin (Schohl and Fagotto, 2002). Therefore, *msr* may be regulated by Wnt/β-catenin/lef1. Indeed, lef1 depletion resulted in down regulation of *msr* expression in the marginal zone in all of the embryos analysed by *in situ* hybridisation (n=14) (Fig. 1B). *Wnt8* expression was not affected by lef1 depletion (Fig. 1B) indicating that these effects of lef1 depletion were not due to an indirect effect via wnt8. Co-expression of mRNA encoding human *LEF1* restored the expression of *msr/APJ* in all of the embryos analysed (n=12) (Fig. 1B). These results demonstrate that lef1 is required for normal expression of *msr/APJ* in the marginal zone and suggest that *msr/APJ* may be a new Wnt response gene downstream of lef1 signaling.

Since *lef1* is also expressed in the dorsal marginal zone (Fig. 1A) we asked whether lef1 also functions in patterning the organizer of the *Xenopus* embryo. Therefore, we analyzed the expression of *nodal related 3,* which is a direct target of maternal β-catenin/tcf3 (McKendry *et al.,* 1997; Houston *et al.,* 2002). To our surprise, expression of *nodal related 3* was elevated at the endogenous site with an ectopic extension towards the animal pole (Fig. 1B), as after depletion of maternal tcf3 (Houston *et al.,* 2002). These results show that lef1 is required for the proper expression of *nodal related 3* in the dorsal marginal zone.

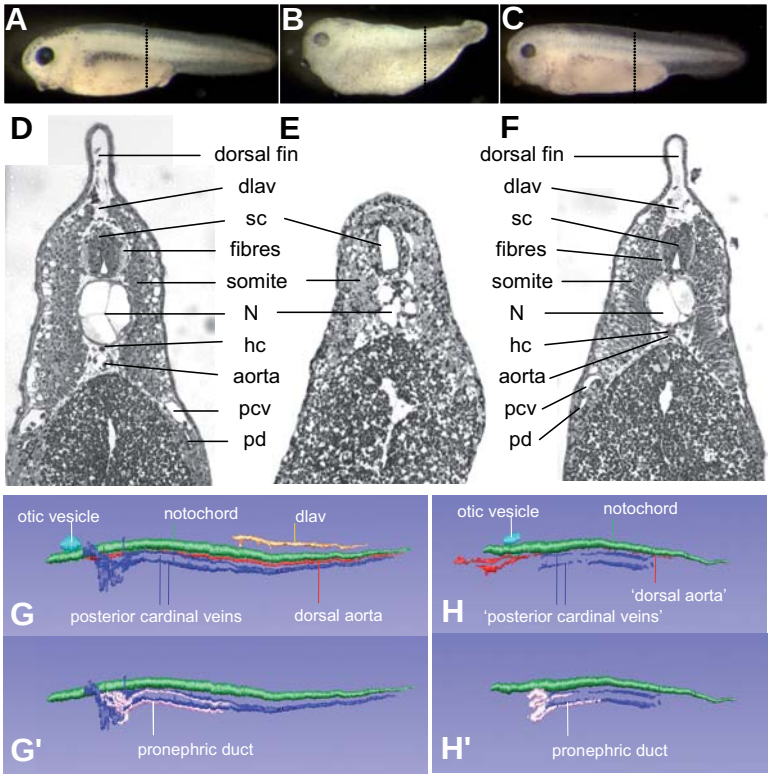

Fig. 2. Lef1 depletion results in impaired development of different tissues. *Non-injected control* X. tropicalis *embryo, stage 38* **(A)**. Lef1 *morpholino oligo injected embryos* **(B)** *are short and lack a tail with a dorsal and ventral fin. The eyes are small and pigmentation is absent. Co-injection of mRNA encoding human* LEF1 *rescues the* lef1 *MO effects* **(C)**. *Broken lines in (A-C) indicate level of sections shown in (D-F). Transverse section of non-injected control embryo* **(D)**. Lef1 *MO injected embryos* **(E)** *lack the dorsal longitudinal anastomosing vessel (dlav), the posterior cardinal veins (pcv), the pronephric ducts (pd), the hypochord (hc) and the dorsal aorta. The somites and notochord (N) are poorly differentiated. The spinal cord (sc) lacks nerve fibers and the notochord (N) is smaller in diameter. Embryo co-injected with* LEF1 *RNA* **(F)** *shows rescue of the structures affected by* lef1 *depletion. 3D reconstructions of transverse sections* **(G-H')** *of embryos shown in (A,B). Noninjected control embryo* **(G,G')** *and embryo injected with* lef1 *MO* **(H,H')**. *Reconstructions were aligned relative to the otic vesicle.*

Phenotypic analysis of lef1 depleted embryos at later stages

We studied the effects of lef1 depletion on later development in more detail. During tail bud stages, growth of 74% (n=178/241) of the lef1 depleted embryos was strongly retarded, resulting in embryos with a very short tail (Fig. 2B). Lef1 depleted embryos lacked both dorsal and ventral fins and pigmentation of neural crest derived melanophores. The head and eyes were smaller and the eyes were less pigmented compared to those of non-injected controls (Fig. 2A,B). Moreover, lef1 depleted embryos did not show a blood flow and did not respond to mechanical stimuli.

Histological analysis and 3D-reconstructions of representative lef1 depleted embryos show that many of the major organs are present at stage 38, including brain and spinal cord, notochord, somites and gut, demonstrating that general patterning along the anterior-posterior and dorsal-ventral body axes was unaffected (Fig. 2B,E,H,H') compared to noninjected embryos (Fig. 2G,G'). However, multiple tissues and organs were abnormal as will be described below. Co-injection of human *LEF1* mRNA resulted in 82% (n=219/267) embryos with normal head, body, tail and fins (Fig. 2F). Rescued embryos also showed wild type melanophore patterns, reacted to mechanical stimuli and their internal organs were normal, demonstrating the specificity of the effects of the lef1 morpholino oligonucleotide.

Lef1 depleted embryos show developmental defects in derivatives of the ectoderm

At tail bud stages the patterning of the CNS of lef1 depleted embryos did not show gross abnormalities since expression of the pan-neural marker gene *ncam* (Kintner and Melton, 1987) (not shown) and of *pax2* (Fig. 4F) in the CNS was not altered. However, histological analysis showed that only very few nerve

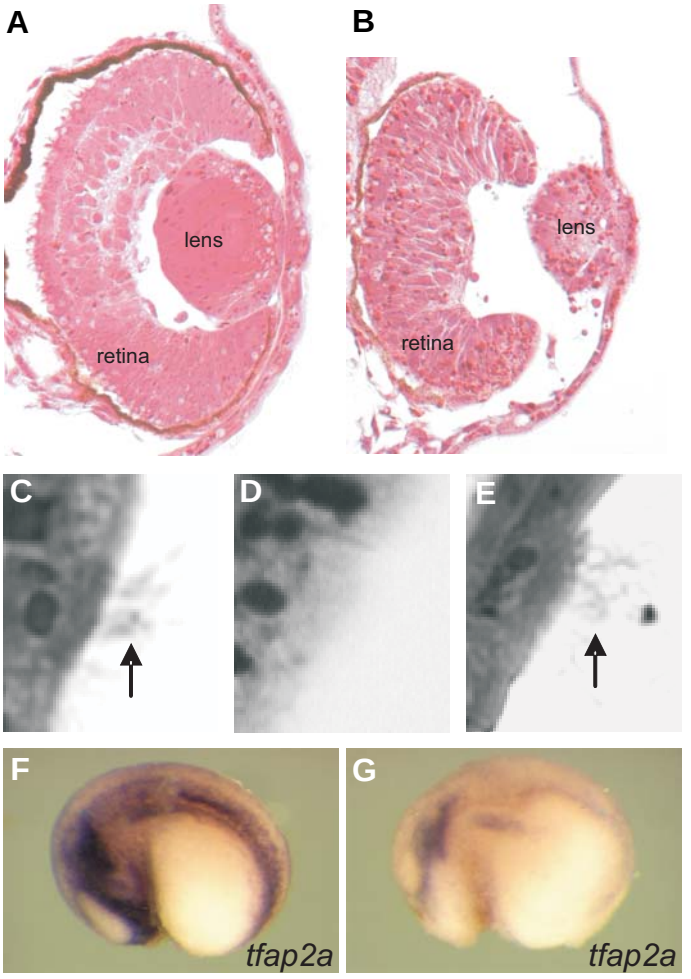

Fig. 3. Ectodermal derivatives are affected by lef1 depletion. *Transverse section of the eye of a control* X. tropicalis *stage 38 embryo* **(A)** *shows differentiation of retina and lens. Transverse section of the eye of a lef1 depleted embryo* **(B)** *shows that the retina and lens have not developed properly and that the lens has not separated from the epidermal ectoderm. Detail of the epidermis showing a ciliated cell (arrow) of a control embryo* **(C)**. *Lef1 depleted embryos* **(D)** *lack ciliated cells in the epidermis. Rescue of ciliated cells (arrow) in the epidermis of embryos injected with* lef1 *MO together with human* LEF1 *RNA* **(E)**. *Expression of the neural crest marker gene* tfap2a *in a control embryo* **(F)** *and reduced expression in a* lef1 *depleted embryo* **(G)**.

fibers were present in the CNS at stage 38 (Fig. 2E) and that nerve fibers where absent in the eyes Fig. 3B). The eyes were smaller and less pigmented compared to those of non-injected controls (Figs 3A,B), while the eye lens remained connected to the epidermis (Fig. 3B), indicating a problem in tissue separation.

The epidermis of lef1 depleted embryos lacked ciliated cells, which are derived from the sensory layer of the ectoderm (Chu and Klymkowsky, 1989) (Fig. 3D). In addition, the epidermis was not well separated from the underlying somites (Fig. 2E), again indicating that tissue separation during early development was abnormal. Several ectodermal placodes, e.g. those for the pituitary and the cephalic ganglia of the sensory system were also affected in lef1 depleted embryos. The pituitary was very small compared to that of control embryos and neural crest derived cephalic ganglia were absent or very small (not shown). Also, in the *lef1* mutant mouse, the ganglia of the trigeminal nerve (NV) were found to be absent (van Genderen *et al.,* 1994). Thus, the sensory system may not be functioning properly in lef1 depleted embryos resulting in the observed lack of response to mechanical stimuli.

The endogenous expression pattern of *Xenopus lef1* shows strong expression in the neural crest (NC) and the branchial arches (Molenaar *et al.,* 1998) suggesting a role for lef1 during development of these tissues. Lef1 depleted embryos lack NC derived pigmented cells (Fig. 2B), indicating a defect in melanophore generation and/or differentiation. The heads of lef1 depleted embryos were small presumably because of disturbed development of neural crest derived mesenchyme. Moreover, the expression of the NC marker gene *tfap2a* (Luo *et al.,* 2003)was strongly reduced in lef1 depleted embryos (Fig. 3G) indicating that lef1 plays a role in neural crest differentiation.

Axial and paraxial mesoderm differentiation is affected in lef1 depleted embryos

Tissues derived from the axial and paraxial mesoderm were also affected in lef1 depleted embryos. The notochord was smaller in diameter and length. The notochordal cells have smaller vacuoles than do wild-type notochords (Figs 2D,E) and did not show the typical epithelial configuration as in wild type (Fig. 2D) and rescued (Fig. 2F) embryos. Signals from the notochord are necessary for the development of the hypochord, which is derived from the endoderm (Cleaver *et al.,* 2000). In lef1 depleted embryos the hypochord was absent (Fig. 2E), suggesting that signaling from the notochord was impaired.

We previously showed that lef1 is required for *myod* expression already during gastrulation (Roël *et al.,* 2002). Therefore we further analysed the formation of the somites and myod expression also later in development, at tadpole stages. The ventral parts of the somites of lef1 depleted embryos did not show distinct intersomitic boundaries (Fig. 4B) and lacked *myod* expression (Fig. 4 D) compared to control embryos (Fig. 4 A,C). Moreover, the ventral parts of the somites did not form hypaxial musculature (Figs 4D), which normally migrates from the somites to form the body wall musculature in control embryos (Fig. 4C; Martin and Harland, 2001). In the posterior part of the embryo the tissue boundaries between the somites and the epidermis and between the somites and the neural tube were also not distinct (Fig. 2E). These results again indicate a role for lef1 in tissue separation.

Derivatives of the lateral plate mesoderm are absent or abnormal in lef1 depleted embryos

The dorsal part of the lateral plate mesoderm (DLP) gives rise to cells that will eventually form the pronephric ducts and some of the major blood vessels (Walmsley *et al.,* 2002). *Lef1* is expressed in the lateral plate mesoderm both in *Xenopus* and the mouse (Molenaar *et al.,* 1998; Galceran *et al.,* 1999). In lef1 depleted embryos the pronephric ducts were absent or incomplete (Figs 2E,H'). Expression of *pax2*, a marker for the pronephric tubules and pronephric duct (Heller and Brändli, 1997; Drawbridge *et al.,* 2003) was absent posteriorly (Fig. 4F). Also, more anterior *pax2* expressing cells did not properly develop into pronephric tubules and proximal pronephric ducts (Figs 2H' and 4G,H). Co-injection of human *LEF1* RNA rescued these effects

(Fig. 2F). These results indicate a role for lef1 in patterning the DLP mesoderm.

Development of the major blood vessels and the heart is affected by lef1 depletion

The lack of blood flow in stage 38 embryos, as well as the abolished expression of the endothelial marker *msr* during gastrulation lead us to further investigate the formation of the vascular system in lef1 deficient embryos. Histological analysis revealed impaired formation of the dorsal aorta and the posterior cardinal veins (pcv) (Figs. 2E,H) and absence of the dorsal longitudinal anastomosing vessel (dlav) (Figs. 2E,H, 5B) when compared with control embryos of the same stage (Fig. 2D,G, 5A). The formation of these structures was rescued by co-injection of human *LEF1* RNA (Fig. 2F). We analyzed the expression of the endothelial marker genes *fli1* (Meyer *et al.,* 1995) and *msr* (Devic *et al.,* 1996), the homologue of the human apelin receptor APJ (O'Dowd *et al.,* 1993). *Fli1* and *msr/APJ* are expressed in overlapping domains in the endothelial precursor cells of the DLP mesoderm (Walmsley *et al.,* 2002) and subsequently in the dorsal aorta, pcv, intersomitic vessels and dlav (Fig. 5C,E and Meyer *et al.,* 1995; Devic *et al.,* 1996). Expression of *fli1* and *msr/APJ* in the prospective dorsal aorta and pcv was strongly decreased (Figs. 5D,F) and the formation of endothelial tubules was blocked in lef1 depleted embryos (Figs 2E,H, 5B). Endothelial precursor cells of the DLP that will form the pcv develop in close proximity to the ventral parts of the somites where angiogenic sprouting of the pcv gives rise to formation of intersomitic vessels (Helbling *et al.,* 2000). *Fli1* and *msr/APJ* expressing cells were also absent between somites (isv, intersomitic vessels) in lef1 depleted embryos (Figs. 5D,F), most likely as a secondary effect of the abnormal formation of the pcv.

As mentioned before, the formation of the hypochord is impaired in lef1 depleted embryos (Fig. 2E). Because the hypochord is required for proper formation of the aorta (Cleaver and Krieg, 1998; Cleaver *et al.,* 2000) our results suggest an indirect effect of lef1 depletion on blood vessel formation in addition to a direct function in vasculogenesis.

Since *Xenopus lef1* is expressed in the presumptive endocardium (Molenaar *et al.,* 1998; Fig. 5G), which also develops by vasculogenic mechanisms (Coffin and Poole, 1991), we examined whether endocardium formation was affected in lef1 depleted embryos. Analysis of the heart of stage 38 lef1 depleted embryos showed severe retardation of the development of both the endocardium and the myocardium (Fig. 5I). Expression of the endothelial marker gene *msr*, which is normally expressed in the endocardium and ventral aorta (Devic *et al.,* 1996), was abolished in the heart of lef1 depleted embryos (Fig. 5F).

Our results indicate that lef1 dependent pathways play an important role in expression of the endothelial-associated gene *msr/APJ* during gastrulation as well as during blood vessel formation and heart development.

Discussion

Our experiments reveal that knockdown of lef1 leads to early developmental defects in the derivatives of the mesoderm and ectoderm in *Xenopus*, as expected from the expression pattern of lef1. In the mouse, targeted inactivation of *Lef1*, or *Tcf1*, did not produce early phenotypes, only null mutations in both *Lef1* and *Tcf1* caused a severe defect in the differentiation of paraxial mesoderm at the same time leading to the formation of additional neural tubes (Galceran *et al.,* 1999). A redundant role of Lef1 and Tcf1 in Wnt signaling during early mouse development explained these results (Galceran *et al.,* 1999). Since *tcf1* is also expressed during early development in *Xenopus* (Roël *et al.,* 2003), redundancy between lef1 and tcf1 functions is less or absent during *Xenopus* development. This difference may relate to the differences in the timing of paraxial mesoderm differentiation between mouse and *Xenopus* (Pownall *et al.,* 2002). Also, differences in the expression of Lef1 iso-forms between mouse and *Xenopus* may be important. Human *LEF1* exon VI is naturally differentially spliced (Arce *et al.,* 2006). Furthermore, natural dominant negative LEF1 is present in normal human and murine thymus tissue (Arce *et al.,* 2006; Travis *et al.,* 1991). We showed that the genomic sequences of *Xenopus* and *Fugu lef1* do not contain exon VI. Only a LEF1 isoform containing exon VI (Arce *et al.,* 2006) can efficiently induce formation of an ectopic axis and

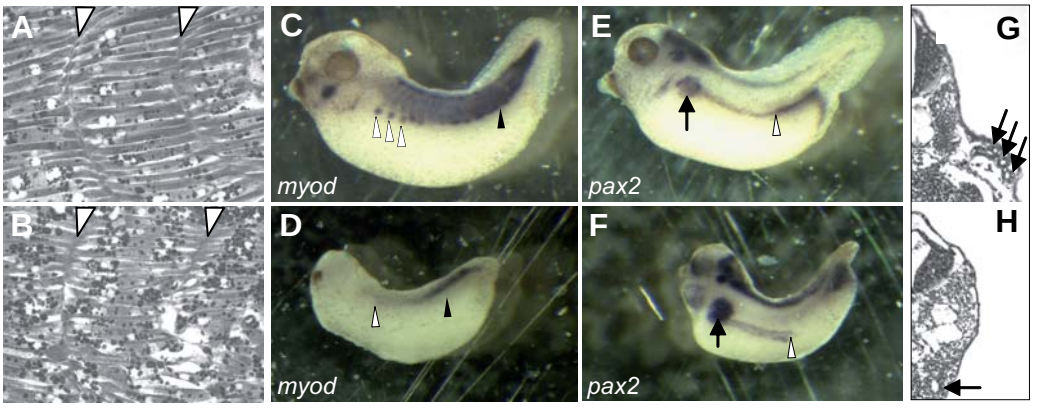

Fig. 4. Mesoderm formation is affected by lef1 depletion. *Somitic boundaries of a control* X. tropicalis *embryo* **(A)**. *The intersomitic boundaries (white arrow heads) are affected by lef1 depletion* **(B)**. *At the ventral side boundaries are lost.* Myod *expression in a control* X. tropicalis *stage 38 embryo* **(C)** *in the ventral part of somites (black arrowhead) and migrating hypaxial musculature (open arrowheads).* Myod *expression is downregulated in the ventral part of somites (black arrowhead) and hypaxial musculature is absent in lef1 depleted embryos* **(D)**. *Control embryos stained for* pax2 *expression* **(E)** *in the pronephros (black arrow), pronephric duct (open arrowhead indicates junction between pronephric duct and rectal diverticulum) and rectal diverticulum. In lef1 depleted embryos* **(F)** *the caudal part of the pronephric ducts did not grow out properly and did not join the rectal diverticula, which were rudimentary (open arrowhead indicates the posterior end of an aberrant pronephric duct). Transverse section of the pronephros* **(G)** *showing pronephric tubules in a control embryo (arrows). The pronephros of lef1 depleted embryos* **(H)** *remains a single tube (arrow), with an S-shape (cf. Fig. 4F).*

enhance *siamois* expression (Gradl *et al.,* 2002). Our rescue experiments in which the human LEF1 isoform was used lacking the activation domain encoded by exon VI demonstrate that this domain is not important for activation of downstream targets, like *myod* (Roël *et al.,* 2002) and *msr/APJ* (this study) in then*Xenopus* embryo. The β-catenin binding domain on the other hand is essential for lef1 signaling since ectopic expression of a *lef1* construct lacking the β-catenin BD abolishes *myod* expression (Roël *et al.,* 2002).

The phenotype of lef1 knockdown embryos as described here, is different from that obtained after depletion of tcf3 (Houston *et al.,* 2002) confirming non-redundant functions of these proteins during early development of *Xenopus* (Roël *et al.,* 2002).

Lef1 is required to pattern the ventral and dorsal mesoderm

We show that lef1 is required for the proper expression of *myod, msr/APJ* and *nodal related 3* in the ventral and dorsal mesoderm. *Myod* (Roël *et al.,* 2002) but not *myf5* expression (this study) requires lef1 in the mesodermal myotomal progenitors in the paraxial mesoderm during gastrulation. Furthermore, our results indicate that lef1 is not only required for expression of *myod* during gastrulation in the earliest myotomal progenitors but also in their hypaxial migratory derivatives during body wall formation. We noticed that the ventral parts of the somites showed abnormal intersomitic boundaries and that somites were not distinctly separated from surrounding tissues. The latter results suggest that lef1 is required in the (pre)somitic mesoderm, as in other locations (see below), for proper tissue separation and/or boundary formation.

In mouse paraxial mesoderm explants both *Myod* and *Myf5* are Wnt responsive (Tajbakhsh *et al.,* 1998) and several studies have suggested that *myf5* expression may be regulated by β-catenin/tcf in *Xenopus* (Yang *et al.,* 2002; Shi *et al.,* 2002). Although depletion of lef1 in *Xenopus* does not lead to inhibition of *myf5* expression, we noticed that co-injection of human *LEF1* RNA without exon VI sequences leads to ectopic *myf5* expression across the dorsal midline (DM). In the DM *myf5* expression may be repressed by Tcf factors (Yang *et al.,* 2002), whereas *myod* expression requires an activator function of lef1 (Roël *et al.,* 2002). Indeed, knockdown of tcf3 leads to ectopic expression of *myf5* across the dorsal midline at early gastrula stages (G. Roël, unpublished). These results indicate different functions for lef1 and tcf3 in the regulation of *myod* and *myf5* expression.

We observed ectopic expression of nodal related 3 in the dorsal mesoderm upon lef1 depletion. This suggests a repressor function for lef1, which would be in line with results obtained in cell lines (Brantjes *et al.,* 2001). Although *nodal related 3* is thought to be directly regulated by Wnt/Tcf (McKendry *et al.,* 1997) the observed effects in lef1 depleted embryos could also be indirect. Thus, whether the observed effects of lef1 knockdown on the expression of *myod, msr/APJ* or *nodal related 3* in the ventral and dorsal mesoderm are direct or indirect remains to be established.

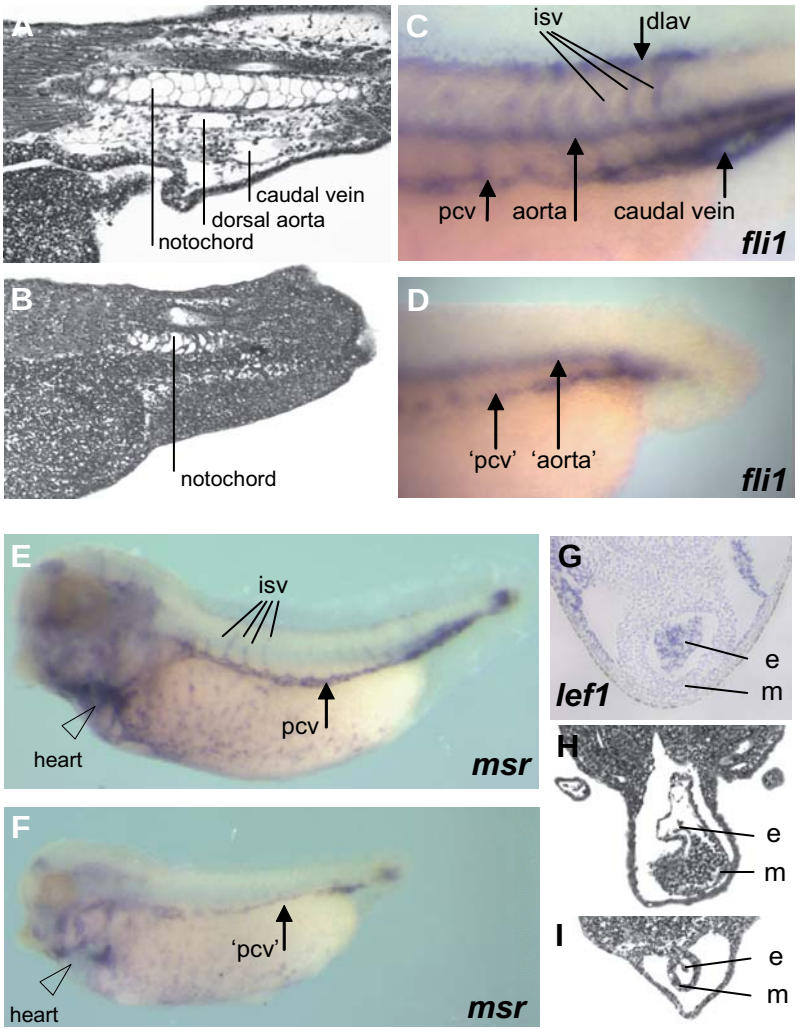

Fig. 5. The cardiovascular system is affected by lef1 depletion. *Sagittal section of a stage 38 control embryo* **(A)** *showing the dorsal aorta and the caudal vein. In lef1 depleted embryos* **(B)**, *the tail lacks the major blood vessels, the caudal vein and the dorsal aorta. Fli1 expression in the endothelium of a control embryo* **(C)**. *isv, intersomitic vessels. dlav, dorsal longitudinal anastomosing vessel. pcv, posterior cardinal vein. aorta, dorsal aorta. Lef1 depleted embryos show strongly reduced expression of* fli1 **(D)** *at the position of the pcv and the aorta and no indication of intersomitic vessels, dlav or caudal vein.* Msr/APJ *expression in control embryo* **(E)** *and in lef1 depleted embryo* **(F)** *showing reduced expression. Endogenous expression of* lef1 *in the heart of a stage 34 control embryo* **(G)**. *e, endocardium. m, myocardium. Transverse section of the heart of a control embryo* **(H)** *and of a lef1 depleted embryo* **(I)** *which shows severe retardation of heart development both for the endocardium and myocardium.*

Lef1 functions in the neural crest

The endogenous expression of *lef1* in neural crest cells (Molenaar *et al.,* 1998) indicates a function in the differentiation of these cells. Indeed, lef1 depleted embryos showed developmental defects in several derivatives of the neural crest like melanocytes and sensory ganglia. Formation and expansion of the neural crest depends on Wnt signals (Yanfeng *et al.,* 2003). In *Wnt1/Wnt3a* double mutant mice *tfap2a* expression was down regu-

lated (Ikeya *et al.,* 1997) as we also observed in lef1 depleted *X. tropicalis* embryos. *Tfap2a* expression was recently found to be responsive to Wnt signals (Luo *et al.,* 2003) and its expression may be directly regulated by lef1 in the neural crest. However, we cannot discriminate between direct effects of lef1 depletion in the neural crest cells and indirect effects e.g. through the paraxial mesoderm (Monsoro-Burq *et al.,* 2003).

A role for Xenopus *lef1 in cell adhesion*

An iterative feature in lef1 knockdown embryos that we observed seems to be that different cell types derived from different germ layers are not able to separate from their initial context. Examples are neural crest cells, which need to delaminate before they can migrate (Yanfeng *et al.,* 2003); the eye lens, which buds of and separates from the surface ectoderm (Grimes *et al.,* 1998); ciliated cells of the epidermis, which migrate from the inner, sensorial layer into the outer layer (Deblandre *et al.,* 1999); hypaxial muscle precursors, which leave the somites and migrate into the ventral body wall (Martin and Harland, 2001); the hypochord which separates from the endoderm (Cleaver *et al.,* 2000) and dorsal aorta precursor cells, which need to escape from the lateral plate mesoderm before they can migrate towards the hypochord (Cleaver and Krieg, 1998). In these processes lef1 may play a role in the regulation of differential cell-cell adhesion.

Xenopus *lef1 is required for development of the cardiovascular system*

We showed that lef1 depletion results in the impaired development of major blood vessels and the heart. The dorsal aorta and the posterior cardinal veins arise from cells of the dorsal side of the lateral plate mesoderm (Walmsley *et al.,* 2002) by different cellular processes. The precursor cells of the future dorsal aorta require signals from the hypochord to migrate from the lateral plate mesoderm towards the hypochord (Cleaver and Krieg, 1998; Cleaver *et al.,* 2000). The lack of the hypochord in lef1 depleted embryos may be causative for the absence of the dorsal aorta but not for the absence of the posterior cardinal veins which are normally formed directly from the lateral plate mesoderm. Since *lef1, fli1* and *msr/APJ* are co-expressed in the lateral plate mesoderm and since *msr/APJ* expression requires lef1, our data suggest that lef1 functions in the specification of angioblasts when they still reside in the lateral plate mesoderm. In addition to impaired cell fate specification, the effects of lef1 depletion on blood vessel development may be caused by blocking the separation and subsequent expansion of the number of precursor cells in the lateral plate mesoderm.

From results of endoderm-depleted embryos it was concluded that signals for angioblast specification are coming from the mesoderm, which is in line with our data, and that signals required for endothelial tube formation come from the endoderm (Vokes and Krieg, 2002). These signals are presently unknown except for the VEGF excreting hypochord, which is an endoderm derivative and which is absent in lef1 depleted embryos. This may explain why endothelial tubes were not formed in lef1 depleted embryos while still some *fli1* and *msr/APJ* positive precursor cells were present. Another indirect effect on vasculogenesis in the lef1 depleted embryos may be caused by the somites that did not differentiate correctly and therefore may not function properly as a signaling compartment to induce blood vessel formation (Cleaver and Krieg, 1998). Because development of the posterior cardinal vein is impaired in lef1 depleted embryos we think that the lack of angiogenic sprouting of the intersomitic vessels in lef1 depleted embryos is secondary to the effects of lef1 depletion on vasculogenesis possibly in combination with impaired signaling from the affected somites (Rossant and Howard, 2002).

The early steps in cardiogenesis are believed to be regulated by activation of the Wnt/Ca^{2+} pathway as well as both activation and repression of the Wnt/β-catenin pathway, during different stages of heart development (reviewed in Eisenberg and Eisenberg, 2006). In addition, recent studies show that msr/APJ and its ligand apelin are both required for proper development of the heart and blood vessels during *Xenopus* development (Inui *et al.,* 2006). This is in line with our results showing that lef1 is required for the expression of *msr/APJ* during gastrulation and also for the development of the endocardium and myocardium during later stages.

Materials and Methods

Analysis of Xenopus *and* Fugu *lef1 genomic sequences*

X. laevis genomic DNA was purified by standard methods (Davis *et al.,* 1986). Primers used: exon V fwd: 5'-GGCCAGATGACACCACCATTGG, exon VII: 5'-CCCGGAGGACCAGAAACCATATG. A genomic DNA fragment was amplified using the Advantage PCR kit (Clontech) and ligated into the pGEM-T easy vector (Promega) and sequenced using a 373 DNA sequencer (Perkin Elmer). *Fugu* genomic sequences were derived from the *Fugu rubripes* database of the DOE Joint Genome Institute (www.jgi.doe.gov).

RNA synthesis for capped mRNA and RNA antisense probes

Capped RNA encoding the human LEF1 isoform without exon VI (van de Wetering *et al.,* 1996) was synthesized using mMessage mMachine T7 (Ambion). For synthesis of DIG labeled (Roche) antisense RNA probes plasmid DNAs were linearized and used as template. RNA was purified using RNeasy columns (QIAGEN). Plasmid DNAs were kindly provided by A. Brändli *(Xenopus pax2)*, M. Baltzinger *(Xenopus fli1)*, S. Hoppler *(Xenopus wnt8)*, C. Kintner *(Xenopus ncam)* and J. Smith *(Xenopus brachyury)*. *X. tropicalis* specific probes, *lef1, myod, msr/APJ* and *tfap2a*, were constructed by RT-PCR using primer sequences designed from the *X. tropicalis* EST database (Sanger Center). *X. tropicalis myf5* probe was generated according to Polli and Amaya (2002). PCR fragments for probes were ligated into the pGEM-T easy vector (Promega).

Embryo manipulation

X. tropicalis embryos were obtained by *in vivo* fertilization and injected with 20 ng *lef1* morpholino antisense oligonucleotide (Gene Tools LLC, Roël *et al.,* 2002), which is complementary to the translation start site of *X. tropicalis lef1* or 20 ng *lef1* MO together with 180 pg RNA encoding human LEF1 to restore Lef1 protein levels in the embryo. Human *LEF1* isoform without exon VI was used (van de Wetering *et al.,* 1996). The human *LEF1* RNA is not sensitive to the *lef1* MO since its sequence contains 6 mismatches. Embryos were injected in both blastomeres at the 2-cell stage and raised at 23°C in 12% MMR (Peng *et al.,* 1991). Developmental stages of *Xenopus* embryos were determined according to Nieuwkoop and Faber (1967). Embryos were fixed in MEMPFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1mM MgSO4, 4% paraformaldehyde) for whole-mount *in situ* hybridization and sectioning.

Whole mount in situ *hybridization, sectioning and 3D-reconstructions*

Whole mount *in situ* hybridization was performed as described before (Molenaar *et al.,* 1998) with modifications for *X. tropicalis*: hybridization at

65°C without RNase treatment for all probes except *X. tropicalis myf5* (65°C, with RNase treatment). Embryos were embedded in Technovit 8100 and sectioned at 7μm for histological analysis. Serial sections of transversally sectioned embryos were used as input for the TDR-3dbase (Verbeek, J. *et al.,* 1995), which reconstructs serial sections into a 3 dimensional image.

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