Evolution of Developmental Control Mechanisms

A conserved mechanism for vertebrate mesoderm specification in urodele amphibians and mammals

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A B S T R A C T
Understanding how mesoderm is specified during development is a fundamental issue in biology, and it has been studied intensively in embryos from Xenopus. The gene regulatory network (GRN) for Xenopus is surprisingly complex and is not conserved in vertebrates, including mammals, which have single copies of the key genes Nodal and Mix. Why the Xenopus GRN should express multiple copies of Nodal and Mix genes is not known. To understand how these expanded gene families evolved, we investigated mesoderm specification in embryos from axolotls, representing urodele amphibians, since urodele embryology is basal to amphibians and was conserved during the evolution of amniotes, including mammals. We show that single copies of Nodal and Mix are required for mesoderm specification in axolotl embryos, suggesting the ancestral vertebrate state. Furthermore, we uncovered a novel genetic interaction in which Mix induces Brachyury expression, standing in contrast to the relationship of these molecules in Xenopus. However, we demonstrate that this functional relationship is conserved in mammals by showing that it is involved in the production of mesoderm from mouse embryonic stem cells. From our results, we produced an ancestral mesoderm (m)GRN, which we suggest is conserved in vertebrates. The results are discussed within the context of a theory in which the evolution of mechanisms governing early somatic development is constrained by the ancestral germ line–soma relationship, in which germ cells are produced by epigenesis.

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I n t r o d u c t i o n

Understanding the sequence of events leading to the specification of mesoderm is a fundamental issue in biology whose importance cannot be overstated. For example, it is widely acknowledged that recapitulating the signalling regimes occurring naturally in development is an effective route to the in vitro derivation of selected tissue types from embryonic stem cells (ESCs), maximizing their utility for therapeutic purposes (Irion et al., 2008). Thus, understanding the gene regulatory network (GRN) for mammalian mesoderm specification is essential to the directed derivation of mesodermal cell types in vitro. From another perspective, mesoderm was the last of three primary metazoan germ layers to evolve. Mesoderm movements during gastrulation give shape to the developing embryo (Keller, 2002), which can therefore provide morphological diversity, underscoring the significance of understanding how the mechanisms governing mesoderm specification evolved.

Amphibian embryos have been used as model organisms to study vertebrate development for well over a century. In the last several decades, experiments with embryos from Xenopus laevis have laid much of the foundation for our understanding of the molecular mechanisms that govern vertebrate mesoderm specification. However, the gene regulatory network for Xenopus mesoderm (XMN; Loose and Patient, 2004) is surprisingly complex when compared to other vertebrates. Much of this complexity arises from the presence of two large gene families, the Nodal TGF-beta signalling molecules and the Mix homeobox transcription factors. The Nodal family in vertebrates. Much of this complexity arises from the presence of two large gene families, the Nodal TGF-beta signalling molecules and the Mix homeobox transcription factors. The Nodal family in vertebrates consists of six members, alongside the related TGF-beta Derriere; the Mix family includes seven members (see Table S1) (Wardle and Smith, 2006). With the exception of Xnr-3, all Xenopus Nodal-related molecules have some role in the specification of the mesoderm and endoderm (Hansen et al., 1997; Jones et al., 1995; Onuma et al., 2002; Osada and Wright, 1999). Moreover, it has been shown that 15 distinct copies of Xnr-5 are encoded in the X. laevis genome, and all are expressed and functional (Takahashi et al., 2006). While the acquisition of tetraploidy likely contributes to some of the gene expansion, Xenopus tropicalis, a diploid species, also has multiple copies of each of these genes (D’Souza et al., 2003). Furthermore, this expansion is not peculiar to Xenopus or to frogs in general. Multiple copies of these key genes have been reported in zebrafish, with multiple Nodal genes shown to be a general feature of teleosts (Fan...
The presence of these multiple gene copies stand in contrast to the single Nodal and Mix orthologs found in mice and humans (see Fig. S1A and Table S1), and in the case of mice, it has been demonstrated that expression of both Nodal and Mix is essential to normal mesoderm development (Conlon et al., 1994; Hart et al., 2002). Furthermore, within the amphibious genome, only a single Nodal gene has been identified to date (Yu et al., 2002); thus, the amplified Nodal genes in *Xenopus* and teleost fish were probably not present in the ancestor to chordates. This raises the possibility that the expanded mesendoderm (m)GRN in *Xenopus*, and zebrafish, is a derived trait that evolved within these specific lineages.

The evolutionary history of amphibians is well established. Anurans (frogs) and urodeles (salamanders) diverged from a common ancestor with urodele-like traits, over 200 million years ago (Anderson et al., 2008; Rage and Rocek, 1989; Roelants et al., 2007). The fossil record demonstrates that urodeles retained the basic skeletal structure of the tetrapod ancestor (Callier et al., 2009; Niedzwiedzki et al., 2010), while anurans evolved a radical alteration of this, so that the body plan of modern frogs is unique among vertebrates (Handrigan and Wassersug, 2007; Johnson et al., 2003b). An ancestral urodele-like embryology was conserved during the evolution of amniotes (Bachvarova et al., 2009a). This includes fundamental features, such as a surface origin for mesoderm (Smith and Malacinski, 1983), a dorsally restricted blastopore (Shook and Keller, 2008; Shook et al., 2002), the origin of the notochord (Brun and Garson, 1984), and, importantly, the origin of the primordial germ cells (PGCs), which, in amniotes and urodeles, originate by induction within the posterior lateral mesoderm, a trait that is conserved in chordate embryology (Bachvarova et al., 2008a,b). In each of these specific respects, anurans have evolved a divergent embryology (for review, see Shook and Keller, 2008), including the evolution of germ plasm and the repositioning of the germ cell precursors to the endoderm (Johnson et al., 2001, 2003b). Together, these results suggest that the mGRN expressed in urodele embryos might reflect the conserved state for amniobians and for vertebrates at large, including mammals. We tested this by investigating the mGRN in embryos from the axolotl, a representative urodele.

Here we demonstrate that mesoderm induction in axolotl embryos is mediated by a single Nodal and Mix gene, in contrast to the multiple copies of these genes in *Xenopus*. Furthermore, we report that in axolotls Mix functions upstream of Brachury expression, and its expression is necessary to initiate downstream events required for the specification of mesoderm, again in contrast to its role in *Xenopus* (Lemaire et al., 1998). We further show that this unexpected juxtaposition of Mix and Brachury is conserved in the pathway that leads to induction of mesoderm from mouse embryonic stem cells (ESC), indicating that the simplified mGRN that we have identified in axolotls is conserved in mammals. Our results are consistent with the hypothesis that the evolution of germ plasm liberates developmental constraints on the mechanisms that govern somatic development, which we have proposed before (Johnson et al., 2003b). The results are discussed with respect to the germ line–soma relationship and how the change in this relationship evoked by the evolution of germ plasm is a major contributor to species diversity, manifested by the emergence of novel genetic interactions within the mGRN.

**Materials and methods**

**Axolotls**

Natural matings were established as previously described (Armstrong and Malacinski, 1989). One or two cell embryos were placed in 1× MBS + 4% Ficoll (Sigma) and antibiotics and injected in the animal hemisphere with 2×4 nl injections (one per blastomere in two cell embryos). Embryos were staged according to Bordzilovskaya and Dettlaff (1979), which are approximately equivalent to Nieuwkoop and Faber’s (1994) stages of *Xenopus*.

**Morpholino injection**

Morpholino oligonucleotides (GeneTools, LLC, OR) were designed to target splice junctions. Intron/exon boundaries were predicted by homology, and sequence was obtained by PCR from Axolotl genomic DNA prepared from reticulocytes as previously described (Unsal and Morgan, 1995). The morpholino sequences used were as follows: MO: AxNodal-1, 5′-TAGACAGCCTGAGAAGAGACAG-3′ and 5′-TGTAG-GAAAACATCTTACCTGATG-3′; MO: AxNodal-2, 5′-AGATTCAATATTCTTACTCTGATG-3′ and 5′-AGACTCTGAGAAGAAGAGAGACAG-3′; MO: AxMix, 5′-AACTCTTACCTGAAAGAGAGACAG-3′ and 5′-GGCCTATCTACCTGAGAAGAGACAG-3′; and MO: AxNodal-1, 5′-GCAGGGCTTCACCTGAGAAGAGACAG-3′ and 5′-GGCCTATCTACCTGAGAAGAGACAG-3′. A nonspecific morpholino was injected in each experiment at equivalent levels to the specific splice morpholino combinations: MO: Control, 5′-GAAGTTCAGGTTTACCTGCCCCG-3′. Each morpholino experiment was repeated at least three times, and the efficacy of the splice morpholinos was tested by PCR in each experiment. The primers used were as follows: AxNodal-1, FP 5′-AGAAGGACAGTCCACCCCGACAGAGAT-3′ and RP 5′-GGTGCCGCCATACCACTCCCTCCT-3′; AxNodal-2, FP 5′-AGAGCACCCCCGACAGAGAT-3′ and RP 5′-CTCCCTTGATGATGCACT-3′; AxMix, FP 5′-GGATGAGGCTGACAGGCACTCCCGACAGACA-3′ and RP 5′-GGCCGCTACGACAGGACAG-3′; and AxNodal-1, FP 5′-TGCAACAGTATT-GAACCCCGG-3′ and RP 5′-TGCCCCATATTCAACAGACCT-3′.

**cDNA library synthesis and screening**

To isolate AxMix (GU256640), a stage 10 cDNA library was made using a ZAP Express® cDNA Synthesis Kit (Stratagene) and screened using a ZAP Express cDNA Gigapack Gold Cloning Kit (Stratagene). A total of 500,000 clones were screened using a full-length mouse Mix probe (Robb et al., 2000). Screening this same library with *Xenopus* Mix family sequences did not identify any Mix orthologs.

**Degenerate PCR**

Degenerate PCR was carried out using stage 10.5 cDNA. Primers: AxNodal, forward primer 5′-TGTACTRTYACCCVMARMAGTWC-3′ and reverse primer 5′-GGCAVCCRCAYTCBTSBACRAYCA-3′. The primers used were as follows: AxNodal-1, FP 5′-AGAAGGACAGTCCACCCCGACAGAGAT-3′ and RP 5′-GGTGCCGCCATACCACTCCCTCCT-3′; AxNodal-2, FP 5′-AGAGCACCCCCGACAGAGAT-3′ and RP 5′-CTCCCTTGATGATGCACT-3′; AxMix, FP 5′-GGATGAGGCTGACAGGCACTCCCGACAGACA-3′ and RP 5′-GGCCGCTACGACAGGACAG-3′; and AxNodal-1, FP 5′-TGCAACAGTATT-GAACCCCGG-3′ and RP 5′-TGCCCCATATTCAACAGACCT-3′.

**In situ hybridization**

Embryos were fixed in 4% PFA at 4 °C for 1 week, then washed twice in 100% methanol, and stored at −20 °C. In situ hybridization on hemisectioned embryos was carried out as previously described for *X. laevis* (Lee et al., 2001). Hemisectioned embryos were stored in 100% methanol at −20 °C until use. DIG-labelled probes were prepared as previously described (Sive et al., 2000); see Table S2 for probe details.

**Quantitative RT–PCR**

qPCR was performed using the ABI 7500 Sequence Detection System (Applied Biosystems) with TaqMan probes and primers as described in Table S3. RNA was isolated from a minimum of 5 whole embryos or 10 cap explants depending on the experiment. Each assay was performed in three independent experimental replicates. Data shown are from one representative experiment each time. Gene
expression during developmental time was measured in two independent sets of embryos with at least five embryos at each stage. Measurement of AxMix in AxMix morphants used primers targeted specifically to exon 2, all other AxMix qRT–PCR used primers targeted to 3′ UTR. qPCR data are analyzed in Microsoft Excel by the comparative C_t method (Livak and Schmittgen, 2001). Mouse sequences were assayed using the following standardized PCR assays from Applied Biosystems (UK): Mixl1 (Mm00489085_m1), Brachyury/T (Mm00436877_m1), and Actin (Mm02619580_g1).

Cell culture and manipulation

CGR8 mouse ES cell lines were maintained on gelatin-coated dishes (0.1%) in ESC medium as described in Turksne (2006). Embryoid bodies were generated via the hanging drop method, cultivating 600 cells in a 20-μl drop. ES cells were expanded and differentiated as previously described (Tada et al., 2005). Mixl1-specific shRNA sequences were designed as previously described (Izumi et al., 2007).
Southern blots

About 30 µg of genomic DNA was digested for each lane with all possible combinations of PstI, BsrGI, and MscI (NEB). Primers used to generate each probe are shown in Table S4. Hybridizations were performed according to standard methods (hybridize at 55 °C overnight, two washes in 2× SSC, 0.1% SDS at room temperature, once in 1× SSC, 0.1% SDS at room temperature, and once in 1× SSC, 0.1% SDS at 50 °C.

Results

Nodal and Mix orthologs

Based on our hypothesis that the conserved mGRN is expressed in axolotl embryos, we investigated the Nodal and Mix families, which are amplified in Xenopus. Using a combination of degenerate PCR and library screening, we identified two orthologs of Nodal, AxNodal-1 and AxNodal-2 (Fig. S1), and a single Mix ortholog (Fig. S2), AxMix. Southern blotting to genomic DNA confirms these to be the only copies of these genes in the axolotl genome (Figs. S3 and S4). Phylogenetic analysis shows that AxNodal-1 is most closely related to a Nodal gene identified in Cynops, another urodele, and then to the Xenopus genes Xnr1, 2, 3, 5, and 6 (Fig. S1). AxNodal-2 clusters with Xnr4 from Xenopus (Fig. S1). Interestingly, AxMix is more closely related to Mix orthologs from human or mouse than it is to any individual Xenopus gene (Fig. S2). We analyzed expression of these genes using a combination of quantitative real-time PCR (qPCR) and whole-mount in situ hybridization (WISH) to hemisected embryos, a method that allows two different probes to analyzed in equivalent tissue from the same embryo.

Analysis of the Nodal-related genes (Fig. 1) shows that both commence expression at the midblastula stage (stage 9), with transcript levels peaking in early gastrulae (stage 10) (Fig. 1A). A direct comparison of expression levels, both time courses are normalised to AxNodal-1 at stage 9. At all stages, AxNodal-1 is expressed at least two-fold higher than AxNodal-2, and this is confirmed by WISH (Fig. 1B). At stage 9, weak expression of AxNodal-1 and -2 is detected in the animal cap, although the signal is strongest in the marginal zone (Fig. 1B, i). The expression of AxNodal-1 at this stage is on the future dorsal side of the embryo, confirmed by comparison with Goosecoid in hemisectioned embryos (Fig. S5A). Both AxNodal genes are expressed in the dorsal lip of early gastrulae (stage 10, Fig. 1B, ii and v), and by stage 12, AxNodal-1 is mainly found in the mesoderm with some weak expression in the endoderm (Fig. 1B, vii). At later stages, AxNodal-1, but not AxNodal-2, is detectable in the left lateral plate mesoderm, consistent with the well-characterised role for Nodal in the left–right asymmetry (Fig. 1C) (Shen, 2007). Thus, the expression pattern of AxNodal-1 is equivalent to the additive expression profile of Xenopus Xnr1, 2, 5, and 6, with the exception of the pre-MBT expression unique to Xenopus (Yang et al., 2002). AxNodal-2, lacking the later asymmetrical expression, has an expression pattern similar to Xnr-4, in agreement with the phylogeny (Fig. S1) (Joseph and Melton, 1997). Together, these data suggest that Nodal was duplicated before the divergence of anurans and urodèles from their last common ancestor, with subsequent amplification of the AxNodal-1 grouping in anurans.

AxMix transcripts are first detected by qPCR at stage 9 (Fig. 2A). Expression persists until tailbud stage (stage 25), contrasting with Xenopus embryos, in which the cumulative expression of Mix genes is complete by stage 14 (Ecochard et al., 1998; Henry and Melton, 1998; Lemaire et al., 1998; Tada et al., 1998). We compared expression of the axolotl Brachyury ortholog, AxBrA (Johnson et al., 2003a), with AxMix. Interestingly, the relative timing of expression of AxMix and AxBrA is altered compared to their Xenopus orthologs. In Xenopus, Brachyury and Mix family gene expression commences at the start of gastrulation (Rosa, 1989; Smith et al., 1991). However, in axolotl embryos, AxMix precedes AxBrA expression by several hours, with the initiation of AxBrA expression being delayed until midgastrula stages (Fig. 2A; also see Johnson et al., 2003a). We confirmed the unexpected relationship in the timing of these genes’ expression using WISH (Fig. 2B). AxMix is first detected in the mesoderm of the blastopore lip as early as stage 10, and this can clearly be seen there by stage 10.5 (Fig. 2B, i). By stage 10.75, expression is retained in the involuted dorsal mesoderm and at the leading edge of the involuting mesoderm in the blastopore lip (Fig. 2B, iii). At this stage, AxBrA first becomes detectable in presumptive mesoderm on the embryo’s surface (Fig. 2B, iv), as well as in a fraction of the dorsal mesoderm previously marked by AxMix (Fig. 2B, iii and iv). Lower-level AxBrA expression can also be detected in ventral mesoderm at this stage (Fig. 2B, iv). By stage 12, AxMix expression is extinguished in the dorsal mesoderm, where AxBrA RNA is now abundant, and AxMix is now found in ventral mesoderm, as well as endoderm (Fig. 2B, v and vi). Coexpression of the two genes is retained in a fraction of the ventral mesoderm compartment at this stage (Figs. 2B, v and vi, and S5B). By stage 14, ventral AxBrA expression is maintained, while AxBrA transcripts are found only in the posterior mesoderm and in the roof of the archenteron, which contains the notochordal precursors (Fig. 2B, vii and viii). To summarize, AxMix expression precedes that of AxBrA, beginning in the dorsal mesoderm, and largely covers the cumulative expression domains of all the Xenopus Mix family members. However, in contrast with Xenopus embryos, AxMix and AxBrA are not coexpressed in cells found at the mesoderm/endoderm boundary, rather AxMix expression precedes that of AxBrA in this domain (Figs. 2A and B).

Knockdown of Nodal-related genes

Our results suggest that the underlying axolotl mGRN is simplified compared to Xenopus. To test this hypothesis directly, we employed a morpholino (MO) knockdown approach in which we first cloned the introns of candidate genes then designed morpholinos that span the intron/exon boundaries to disrupt slicing (splicing MOs). Since intron sequence is not conserved between family members of related genes, this approach avoids any possibility of cross-reactivity. Also, disrupted splicing is readily detectable, indicating successful knockdown of target genes.

The initiation of mesoderm specification by Nodal gene family members is conserved in vertebrates (Shen, 2007; Swalla, 2006). In Nodal\(^{-/-}\) mouse embryos, for example, the primitive streak fails to form, indicating the absence of mesoderm (Conlon et al., 1994). To investigate roles for AxNodal-1 and -2 in mesoderm specification, we first established the phenotype of a Nodal null signalling mutant. Axolotl embryos were treated with the soluble Nodal signalling inhibitor SB431542, which has been shown to completely disrupt mesoderm development in Xenopus and zebrafish embryos (Ho et al., 2006). As expected, embryos treated with SB431542 fail to form dorsal lips and do not gastrulate (100%, n = 3×15) (Fig. 3A), phenocopying embryos from Xenopus. We further characterised this phenotype in embryos by investigating the expression of AxMix, AxBrA, AxGf8, and AxSox17. As expected, all four markers are significantly downregulated, indicating a block to the formation of both the mesoderm and endoderm (Fig. 3B).

To specifically disrupt Nodal gene expression, we used two splicing MOs for each gene (Fig. 4A). Morpholino sets were injected into one-cell embryos, and the effect on the respective target RNAs was monitored by RT–PCR (Fig. 4B). To test for nonspecific and off-target effects, we used a mistargeted control MO (MO:Control). As the two AxNodal genes have subtly different expression patterns, we investigated the consequences of the knockdown of each gene individually or both in combination. Control MOs injected at equivalent or greater levels had no discernible effect on the embryos, other
than slowing development. In contrast, knockdown of AxNodal-1 results in complete developmental arrest at the onset of gastrulation (88%, n = 3 × 20). These morphant embryos are unable to form a dorsal lip (compare Fig. 4C, i–iv with v–viii), phenocopying the effects of SB431542 treatment (Fig. 4C, v–viii). By stage 14, sibling embryos have gastrulated normally, while the AxNodal-1 morphants are halted at a pregastrula stage, resembling embryos at stage 9 (Fig. 4C, vii). This phenotype suggests a complete loss of mesoderm. In the same
AxNodal-1 and AxNodal-2 gene knockdown. (A) Schematic illustrating the action of the two splice morpholinos targeted to AxNodal-1 and AxNodal-2 (shown as M:A and M:B). Approximate location of PCR primers indicated by arrows. (B) PCR demonstrates effectiveness of AxNodal-1 and AxNodal-2 morpholinos (MO:AxNodal-1 and MO:AxNodal-2). MO: Control = Control. About 80 ng of each of M:A and M:B, 160 ng in total; 160 ng of MO:Control. (C) AxNodal-1 and AxNodal-2 morphant embryos. Vegetal views, except uninjected (iii and iv) and MO:AxNodal-2, stage 28 (xii), lateral view. AxNodal-2 morphants gastrulate, subsequent axial patterning is disrupted. AxNodal-1 morphants fail to gastrulate, remaining phenotypically at stage 9. Each morpholino combination is 80 ng of two splice morpholinos, 160 ng in total. Dorsal lips indicated by arrows. (D) qPCR analysis of MO:AxNodal embryos at stages 12 and 15.
experiments, AxNodal-2 morphants successfully completed gastrulation, although they were delayed with respect to uninjected siblings (100%, n = 3 × 20) (compare Fig. 4C, xi with ii–iii). Later, AxNodal-2 morphants are disrupted with respect to axial patterning, having reduced head and tail structures (100%, n = 3 × 20). Nevertheless, the ability of these embryos to complete gastrulation indicates that AxNodal-2 is dispensable for mesoderm induction. Coinjection of both sets of morpholinos has no additional effects over injecting MOs targeted only to AxNodal-1 (n = 3 × 20) (Fig. 4C, xiii–xvi), substantiating that only AxNodal-1 is required.

We next examined gene expression in morphants by qPCR. In all cases, gene expression was normalized to embryos injected with the control MO. At stage 12, AxNodal-2 morphants show a mild decrease in expression of AxMix, AxBra, AxFGF-8, and AxSox17 (Fig. 4D), but by stage 15 (neurula), expression levels reach those in controls. In contrast, AxNodal-1 morphants display an almost complete loss of expression from all four genes when assayed at stage 12, and expression is never recovered. These results are similar to those obtained with SB431542 treatment. Again, the phenotype of AxNodal-2 and -1 MOs combined is equivalent to the AxNodal-1 phenotype alone (Figs. 4C and D). Remarkably, these results indicate that only AxNodal-1 is required to initiate mesoderm development, a marked contrast to Xenopus embryos, in which functional redundancies override a specific requirement for any single Nodal-related gene to produce mesoderm.

Knockdown of AxMix

A priori, the consequence of the loss of all Mix activity in axolotl embryos is difficult to predict. In Xenopus, Mix.1 and Brachyury negatively regulate each other’s expression to drive the segregation of endoderm and mesoderm (Lemaire et al., 1998). In contrast, Mixer depletion results in a mild down-regulation of Brachyury, indicating that at least some level of Mix activity is required for normal Brachyury expression (Kofron et al., 2004). However, knockdown of all seven mix/bix family members in X. laevis is technically
challenging and, to date, has not been achieved. Finally, mouse embryos carrying a targeted deletion of Mixl1 do not express T/Brachyury in the primitive streak (Hart et al., 2002), indicating a direct role in mesoderm specification.

Injection of MOs targeted to the two splice junctions surrounding exon 2 of 

\( AxMix \) (Fig. 5A) completely disrupts splicing (Fig. 5B). Morphants reach early gastrula stage, but then development halts (97%, \( n = 3 \times 20 \)). Dorsal lips fail to form and involution does not occur (Fig. 5C, v and vi), suggesting a defect in mesoderm specification. As before, sibling embryos injected with a control MO gastrulate normally, although early development is slower than in uninjected embryos (100%, \( n = 3 \times 20 \)) (Fig. 5C, iii and iv). We next assayed marker gene expression in morphants. \( AxMix \) RNA is barely detectable in these embryos (Fig. 5D). Again, small changes in gene expression are seen in embryos injected with control MO, which are consistent with the delay in development. More importantly, we assumed that \( AxBr \) expression would be enhanced in \( AxMix \) morphants, based on the role of Mix activity in \( Xenopus \), but this was not the case; \( AxBr \) expression is completely lost in \( AxMix \) morphants (Fig. 5D). Unlike Nodal morphant embryos, however, \( AxFGF-8 \) and \( AxSox17 \) expression is maintained and later upregulated (Fig. 5E). Therefore, the loss of \( AxBr \) RNA results from the loss of \( AxMix \) and is not a consequence of the disruption of a Brachyury/FGF feedback loop (Schulte-Merker and Smith, 1995). This novel relationship between \( AxMix \) and \( AxBr \) is supported by our earlier observation that \( AxMix \) expression precedes that of \( AxBr \) in the mesoderm.

We next asked if the loss of mesoderm in \( AxMix \) morphants is a direct consequence of the loss of \( AxBr \). We therefore designed morpholinos targeted to disrupt splicing in the \( AxBr \) gene (Figs. 6A and B). As with \( AxNodal \) and \( AxMix \), \( AxBr \) morphants fail to gastrulate (100%, \( n = 3 \times 20 \)) (Fig. 6C, v and vi). However, in this case, although \( AxMix \) expression is initially downregulated, it is...
ultimately upregulated to higher than normal levels, as would be expected if AxBra negatively regulates AxMix (Fig. 6D). Moreover, in contrast with AxMix morphants, these embryos show a loss of FGF-8 expression, raising the possibility that AxMix itself is a repressor of FGF-8 activity, and explaining why FGF-8 is increased in AxMix morphants (Figs. 5D and E). Lastly, AxSox17 expression is upregulated in these embryos, suggesting that an increase in endoderm occurs at the expense of the production of mesoderm (Fig. 6E).

**AxMix is required for AxBra expression**

Taken together, our observations demonstrate that the mGRN in axolotls contains a single Mix gene and two Nodal genes. Surprisingly, Mix acts upstream of Brachyury in the specification of mesoderm, in contrast to the relationship of these genes in *Xenopus*, suggesting that injection of AxMix RNA would expand the AxBra domain. To test this in axolotl embryos, one of the two ventral blastomeres at the four-cell stage was injected with RNA encoding either AxBra or AxMix, along with a lineage tracer (miniruby) to mark the site of RNA injection. Endogenous gene expression was then assayed at stage 12 by WISH. Similar to results with *Xenopus* (Lemaire et al., 1998), the injection of RNA for AxBra (200 pg) inhibits Mix expression at the site of injection (Fig. 7), indicating that negative regulation of Mix expression by Brachyury is conserved. However, injection of AxMix RNA (200 pg) induced ectopic expression from the zygotic AxBra gene. This is the opposite of predictions based on *Xenopus* and supports the positioning of AxMix in the mGRN upstream of AxBra.

To further elucidate the pathway to mesoderm specification in axolotls we turned to animal cap assays, a standard experimental regime for amphibian embryos (Fig. 8A). By titration, we established that 1 pg of RNA encoding activin (mimicking the effects of Nodal) was sufficient to induce elongation (Fig. 8B), an indicator for the induction of mesoderm (Green et al., 1992). Elongation, and so mesoderm induction, in response to activin RNA can be completely blocked by coinjection of the AxMix morpholino (100%, n = 3 × 10). However, mesoderm induction in the morphants can be rescued by injection of 20 pg of RNA encoding AxMix. At a higher level of 100 pg AxMix RNA, the caps no longer elongate, rather they appear to produce endoderm (100%, n = 3 × 10), as expected (Green et al., 1992). We next analyzed gene expression in each group of caps. In accord with our previous results, the inclusion of the AxMix MO dramatically reduces Brachyury induction by activin (Fig. 8C).

Brachyury expression is rescued in caps coinjected with a low level of AxMix mRNA. However, high levels of AxMix upregulate AxSox17 (about twofold) and not AxBra, supporting the observation that high levels of AxMix induce endoderm at the expense of mesoderm. AxFGF-8 expression can be induced in the presence of AxMix MO, indicating a Brachyury-independent pathway, and expression is decreased by overexpression of AxMix, as expected.

To further investigate the hierarchical relationship between AxMix and AxBra, we rescued the failure of mesoderm induction in AxMix morphants downstream of activin signalling by overexpressing AxBra. Overexpression of AxBra alone in axolotl animal cap explants induces elongation (100%, n = 3 × 10) typical of mesoderm (Fig. 9A). Mesoderm induction by activin, blocked by the AxMix MO, can be rescued (100%, n = 3 × 10) by overexpression of AxBra (Fig. 9A). At the level of gene expression, AxBra overexpression reduces AxSox17 levels, suggesting that the loss of Brachyury expression in AxMix morphants is a significant cause of the loss of mesoderm (Fig. 9B). However, as we see with the AxMix rescue (Fig. 8C), AxBra overexpression fails to restore normal expression of AxFGF-8, supporting the presence of a Brachyury-independent pathway regulating AxFGF-8 activity (Fig. 9B). Taken together, these data confirm that AxMix is required for the induction of mesoderm, acting downstream of Nodal and upstream of AxBra.

**Mixl1 in ES cell differentiation**

Our results reveal a role for Mix activity in axolotl embryos that is very different from its function in *Xenopus* (Henry and Melton, 1998; Kofron et al., 2004; Lemaire et al., 1998). We sought to determine which role for Mix is conserved in mammals, where conflicting conclusions from a variety of studies have not clearly defined a role for Mix1 in the production of mesoderm (Hart et al., 2002; Izumi et al., 2007). We used RNA interference-mediated knockdown to block Mix1 activity in murine embryonic stem cells, using the Mix1 knockdown 1 sequence described by Izumi et al. (2007). ESC lines were produced after stable transfection with Mix1 shRNA, a scrambled Mix1 control, or the vector alone, and these were used to produce embryoid bodies (EBs) to test the consequence of Mix1 knockdown on the expression of Brachyury/T. As expected, the Mix1 shRNA leads to a substantial inhibition of Mix1 compared with nontransfected, scrambled, or vector-only lines (Fig. 10A). However, in agreement with our findings in axolotls, Brachyury/T expression is
dramatically decreased by Mixl1 shRNA under the same conditions (Fig. 10B).

Taken together, the data demonstrate a conserved role for Mix in the induction of the mesoderm that must reflect its ancestral functions in vertebrates, indicating that the role of Mix in the suppression of mesoderm is derived. Further, we confirm the model proposed by Izumi et al. (2007), so that at high levels, Mix induces endoderm, but at low levels, Mix activity induces mesoderm (Figs. 8B and C and 10B). Importantly, the results of Mix knockdown experiments in axolotl embryos and mouse ESC are in agreement with the reported absence of Brachyury/T in the primitive streak of Mixl1−/− mouse embryos (Hart et al., 2002). These results suggest that the ectopic Brachyury/T expression observed in Mixl1−/− embryos results from alternative Mixl1-independent pathways for Brachyury activation in mice.

Discussion

Here we show that the mGRN of axolotls is simplified compared to that of Xenopus but resembles that of mammals. When considered within the context of the profound differences in early morphogenesis of these two amphibian species (Johnson et al., 2003b; Shook and Keller, 2008), it is not surprising that the GRNs governing early development diverged; nor, given the conserved embryological features of urodeles and amniotes (Bachvarova et al., 2009a,b), is it surprising that the mGRN of axolotl embryos is apparently conserved in mammals. Indeed, the absence of amplified copies of Nodal in amphioxus (Yu et al., 2002), as well as mammals, suggests that the simplified network we uncovered is conserved in chordates.

We used antisense morpholinos targeted to the splice junctions of AxNodal1 and AxMix to unambiguously disrupt their expression. Knockdown of AxNodal1 blocks the induction of mesoderm, phenocopying the effects of chemical inhibition of Nodal signalling at both a morphological and a molecular level. Unexpectedly, knockdown of AxMix also blocks the induction of mesoderm, demonstrating that these two factors act together in a pathway for mesoderm specification. This is supported by rescue of the mesodermal phenotype by AxMix RNA injection in animal cap explants. Similar results are not possible with Xenopus embryos due to the gene amplifications that evolved in the Nodal and Mix gene families. Indeed, morphants of several of the Xenopus Mix genes gastrulate with no failure in mesoderm specification, although FGF signalling is upregulated (Colas et al., 2008; Kofron et al., 2004; Trindade et al., 2003). Similarly, although mesoderm specification in Xenopus can be prevented by chemical inhibition of Nodal signalling, there is no evidence that expression of any of the 25 or so Nodal gene family members is crucial to the production of mesoderm (Ho et al., 2006; Osada and Wright, 1999; Takahashi et al., 2006). Thus, amplification of the Nodal and Mix genes renders
**Fig. 9.** Rescuing mesoderm induction with AxBra overexpression. (A) Axolotl animal caps injected with 1 pg of Activin mRNA induce mesoderm in the presence and absence of Mo:AxMix. The elongation phenotype, characteristic of mesoderm, can be rescued by overexpression of 200 pg of AxBra mRNA. (B) qPCR analysis of AxBra, AxSox17, and AxFGF-8 expression in animal caps. The AxBra primers detect endogenous, not exogenous Brachyury. The up-regulation of AxSox17 is rescued by the overexpression of AxBra. However, AxFGF-8 levels are not significantly reduced, supporting the existence of a Brachyury-independent FGF-8 pathway.

**Fig. 10.** The consequence of Mixl1 knockdown on mouse ES cells. (A) ES cells aggregated into embryoid bodies by hanging drop go on to express Brachyury/T in the presence or absence of exogenous Activin. (B) qPCR demonstrating the Mixl1 knockdown obtained by shRNA knockdown. (C) qPCR demonstrating the loss of Brachyury/T in Mixl1 shRNA, but not scramble or vector alone, differentiated embryoid bodies.
the mesodermal GRN of Xenopus resistant to perturbations that would be lethal in axolotl.

The resistance of the mGRN to genetic perturbation offers a mechanistic explanation for the accumulation of amplified Nodal and Mix genes in the Xenopus genome, as it fulfils Waddington’s concept of a canalized developmental process. The robustness that results from canalization is generally considered a selective advantage (Kitano, 2004); however, this can only be true under conditions that do not compromise development of the germ line. We suggest that gene expansion within the mesodermal GRN could not have not been tolerated before the evolution of predetermined germ cells in frogs. The induction of PGCs in ventral mesoderm is the ancestral condition for amphibians (Bachvarova et al., 2009a) and would likely have been disrupted as a consequence of Mix and Nodal expansion. Therefore, it is logical to propose that the evolution of germ plasm liberated constraints on the mechanisms of mesoderm specification in anurans, in accord with previous hypotheses (Crother et al., 2007; Johnson et al., 2003b). Furthermore, expansion of the Nodal and Mix genes in teleosts (Fan and Dougan, 2007), which also contain germ plasm, suggests this may be a generalized mechanism leading to canalized development.

The evolution of gene expansion within a GRN is likely to include subfunctionalisation of genetic interactions within the network. Here, we have revealed a previously unrecognised role for Mix in the axolotl that may have been obscured by subfunctionalisation in Xenopus. In Xenopus embryos, Nodal signalling induces coexpression of the Mix genes and Brachyury in the mesendoderm (Lemaire et al., 1998; Wardle and Smith, 2006). The subsequent mutual antagonism of these factors causes Brachyury to segregate with the mesoderm and the Mixes to segregate with endoderm. However, we have detected limited coexpression of AxBra and AxMix in axolotl embryos and even then only in ventral mesoderm. Furthermore, our results place AxFGF-8, AxSox17, and AxBra downstream of Nodal signalling, but activated by two independent pathways, with the activation of AxBra being dependent on AxMix activity. Based on the prevailing interpretation of evidence from Xenopus, we would have expected a Mix morphant to promote mesoderm and suppress endoderm (Lemaire et al., 1998). However, we see the converse, increased AxSox17 expression in AxMix morphants (Fig. 5E), with a loss of mesoderm. This identifies a requirement for AxMix in mesoderm induction before any role in its suppression, and this is not conserved in Xenopus. We demonstrated this directly, showing that the AxBra domain is expanded in response to forced AxMix expression (Fig. 7). Studies with mouse embryos have lead to conflicting results, with some studies implicating Mix in mesoderm production, and others in its repression. However, we knocked down Mix1 in EBs and showed a clear inhibition of Brachury expression. This is consistent with the absence of Brachury expression in the primitive streak (the site of nascent mesoderm production) of Mix1−/− mouse embryos, suggesting that the role for Mix at the top of a hierarchy or transcription factors leading to mesoderm specification is conserved in vertebrates.

On the basis of these findings, we have constructed a gene regulatory network for mesoderm specification in axolotl embryos containing a key change from the Xenopus network in which AxMix activates AxBra and, consequently, the mesoderm (Fig. 11). This presumed mesodermal GRN for the axolotl, including dashed lines for links not yet confirmed, will probably require alteration in the future, since, for example, the role of the localized determinants that initiate the mesoderm GRN in Xenopus are uncertain in axolotls (Nath and Elinson, 2007). Nevertheless, our observations suggest a two-step process for mesoderm induction in the axolotl. Firstly, Nodal, via Mix, induces a population of mesendodermal cells, the bipotential precursors of the mesoderm and endoderm. In the second step, Brachury expression, triggered by Mix, induces the mesoderm and suppresses Mix. The loss of mesoderm in the Nodal and Mix morphants reflects the loss of the bipotential mesendoderm that accounts for the mesodermal defects we observe.

It is straightforward to understand why canalization of the mGRN would evolve as an adaptive response to selection, but it is not obvious why the less robust simplified mGRN would be conserved through

Fig. 11. The presumptive GRN for the axolotl. (A) Solid lines indicate experimentally verified links; dashed lines indicate presumed links from Xenopus. Models comparing the relative roles of Nodal, Mix, and Brachury in Xenopus (B) and axolotl (C) development.
vertebrate evolution. However, development directed by the simplified mGRN, in our view, is slower and more deliberate, with a less rapid specification to restricted cell fate than is afforded by more robust gene networks; this may have accommodated the co-option of early regulatory mechanisms in the mammalian lineage. The integrated development of the embryonic germ line and soma that is required of the process of epigenesis is a major constraint that conserves the tetrapod body plan (Johnson et al., 2003b), which has been a central concern of biological natural history for well over a century.

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Appendix A. Supplementary data


References


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