Developmental Biology 2010 (2010) 138–152

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

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

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ARTICLE INFO

Article history:
Received for publication 7 December 2009
Revised 2 April 2010
Accepted 6 April 2010
Available online 13 April 2010

Keywords:
Mesoderm induction
Gene regulatory networks

ABSTRACT

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.

Introduction

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 *Xenopus* 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

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doi:10.1016/j.ydbio.2010.04.002

and Dougan, 2007). 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 amphioxus 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., 2009a,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 amphibians 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 Brachyury 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 Brachyury 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 (Gene Tools, 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′-TAGACAGGCTGTGGGAAGAGAAGAC-3′ and 5′-TTGATGAAAGCATCTTACCTGCATG-3′; MO:AxNodal-2, 5′-AGATTCCATATTTCTTACCTGCATG-3′ and 5′-AGACTCTGAAGAAGAAAAGGAGAAG-3′; MO: AxMix, 5′-AACCTCCTACTGCAAAAGAAGAGAC-3′ and 5′-GGCCTATCCACGGGTCTCACCTGGA-3′; and MO:AxBra, 5′-TGATCTGTAGAGAGAGAAGGACAGT-3′ and 5′-TCCCCCACCACCACTCACCGCTCCT-3′. A nonspecific morpholino was injected in each experiment at equivalent levels to the specific splice morpholino combinations: MO:Control, 5′-GGATTTCAAGGTTGTTTACCTGCCG-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′-AAGCCCCACCTGCTCTTGCGTTCA-3′ and RP 5′-GGTGGCGCATCACCACCTCCCCATTCT-3′; AxNodal-2, FP 5′-AGAGCACCCCGCCGCCAGAGAAGAT-3′ and RP 5′-CTCCTCGTGGTGATGAACCACAACCTG-3′; AxMix, FP 5′-GGATGAGCAGGATGCCCGCAGACA-3′ and RP 5′-GCGGGACTTGGCACGCCTATTCT-3′; and AxBra, FP 5′-TGCACAAGTATGAACCCCG-3′ and RP 5′-TCGCCATTATCCAGAACATC-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′ TGGATCRTYYACCCVMARMAGTWC 3′ and reverse primer 5′ GGCAVCCRCAYTCBTSBACRAYCA 3′. 5′ and 3′ RACE was carried out using a BD SMART RACE Kit (Clontech). RACE-specific primers for 5′ RACE were AxNodal-1 5′ GGTGGCGCATCACCACCTCCCCATTCT 3′ and AxNodal-2 5′ CTCCTCGTGGTGATGAACCACAACCTG 3′ and those for 3′ RACE were AxNodal-1 5′ TACCGCTGTGATGGAAAGTGTCCCAGC 3′ and AxNodal-2 5′ ATGCTTACAGATGCGAAGGGCTGTGCC 3′. AxNodal-1 (GU256638) and AxNodal-2 (GU256639).

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