The forkhead genes, Foxc1 and Foxc2, regulate paraxial versus intermediate mesoderm cell fate

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Abstract

During vertebrate embryogenesis, the newly formed mesoderm is allocated to the paraxial, intermediate, and lateral domains, each giving rise to different cell and tissue types. Here, we provide evidence that the forkhead genes, Foxc1 and Foxc2, play a role in the specification of mesoderm to paraxial versus intermediate fates. Mouse embryos lacking both Foxc1 and Foxc2 show expansion of intermediate mesoderm markers into the paraxial domain, lateralization of somite patterning, and ectopic and disorganized mesonephric tubules. In gain of function studies in the chick embryo, Foxc1 and Foxc2 negatively regulate intermediate mesoderm formation. By contrast, their misexpression in the prospective intermediate mesoderm appears to drive cells to acquire paraxial fate, as revealed by expression of the somite markers Pax7 and Paraxis. Taken together, the data indicate that Foxc1 and Foxc2 regulate the establishment of paraxial versus intermediate mesoderm cell fates in the vertebrate embryo.

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Introduction

In the embryo of higher vertebrates (amniotes), the mesoderm is generated during gastrulation when cells delaminate from the epiblast and move through the primitive streak into the region between the endoderm and ectoderm. Several mechanisms are known to control mesodermal cell fate, including the anterior–posterior level at which they traverse the streak, and local signals from neighboring tissues. Fate mapping studies in the mouse and chick indicate that mesendoderm precursors in the most anterior streak contribute to the node, midline notochord, and head mesoderm. Cells that emerge behind the node give rise to the paraxial mesoderm (future somites) adjacent to the midline, while cells that emerge at more caudal levels give rise sequentially to intermediate mesoderm (precursors of the kidney and gonads) and lateral plate mesoderm (precursors of the body wall and the limbs). The most posterior streak cells migrate caudally to generate extraembryonic mesoderm (James and Schultheiss, 2003; Parameswaran and Tam, 1995; Psychoyos and Stern, 1996; Schoenwolf et al., 1992; Smith et al., 1994; Wilson and Beddington, 1996; Yang et al., 2002). However, fate mapping experiments alone do not indicate when mesodermal cells are irreversibly committed to different fates. Heterotopic grafting experiments have revealed that cells within the streak are still developmentally labile (Garcia-Martinez and Schoenwolf, 1992; Parameswaran and Tam, 1995). In the case of paraxial mesoderm cells, it appears that irreversible commitment to somite fate is only achieved after cells have been incorporated into epithelial somites (Tam and Trainor, 1994). The fate of anterior intermediate mesoderm in the chick is determined by Hamburger–Hamilton (HH) stage 8–9, concomitant with onset of expression of the intermediate mesoderm specific transcription factors, Lhx1 and Pax2 (James and Schultheiss, 2003). It is not yet known when the lateral mesoderm is irreversibly committed.
A number of factors have been identified that function in local signaling networks to regulate early mesoderm development. Among these are secreted molecules produced in the node (Fgf4, Nodal, Shh, Noggin), the notochord (Shh, Noggin), the primitive streak (Fgf4, Fgf8, Wnt3a), and the lateral plate mesoderm (Bmp4). Wnt3a is required for the development of all but the most anterior paraxial mesoderm, because Wnt3a-null embryos lack somites posterior to somite 5–7, and instead display ectopic neural tissues on either side of the neural tube (Takada et al., 1994; Yoshikawa et al., 1997). A critical role for FGF signaling in early mesoderm morphogenesis and patterning has been demonstrated in both the mouse (Ciruna and Rossant, 2001; Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994) and chick (Yang et al., 2001 and Pownall et al., 2002).

In this paper, we have investigated the role of Foxc1 and Foxc2 in the early mediolateral patterning of the mesoderm. We show that in the Foxc1; Foxc2 compound homozygous-null embryos, the paraxial mesoderm is respecified so that instead of forming somites, cells express markers characteristic of intermediate mesoderm. Furthermore, Foxc1 and Foxc2 appear to act in a dose-dependent fashion, since progressive loss of functional Foxc2 alleles is associated with increasing lateralization of the paraxial and somitic mesoderm. The expanded intermediate mesoderm domain of Foxc1; Foxc2 compound mutants gives rise to disorganized aggregates of cells expressing markers for nephric duct and mesonephric tubules. Finally, by ectopically expressing Foxc1 or Foxc2 in chick embryos, we provide functional evidence that both genes are able to convert cells from an intermediate to a paraxial cell fate.

Materials and methods

Mutant mice and embryos

Generation and genotyping of Foxc1; Foxc2 compound heterozygous mice were essentially as described (Kume et al., 2001). Noon of the day of plug was E0.5. Compound homozygous and heterozygous embryos were generated by mating compound heterozygotes. Wild-type control embryos were pooled, and included embryos single heterozygous for either gene. Genotyping was performed on DNA from embryonic yolk sacs. At least two embryos of each genotype were used for expression analysis. Somite number was generally used for staging embryos. In the case of compound homozygous embryos, where no somites are formed, similar-sized littermates were used.

Foxc1 and Foxc2 overexpression constructs and electroporation

Mouse Foxc1 and chick Foxc2 cDNAs were PCR amplified off the complete open reading frame using sequence-specific EcoRI (forward)- and BamHI (reverse)-
flanked primers and PfuTurbo DNA Polymerase kit (Stratagene). Fragments were ligated into the IRES-GFP containing expression vectors (kindly provided by C. Krull, Swartz et al., 2001) using EcoRI and BamHI sites, and constructs were sequenced.

For electroporation of chick embryos, an electro-square porator BTX-830 (BTX) was used in combination with a platinum “bottom” electrode of 1-mm gauge wire mounted in a 5-cm petri dish and a “top” electrode (adapted from Momose et al., 1999) made from flame-sharpened tungsten wire (40 μm at the tip) held by a glass transfer pipette connected to the electro-square porator. A 2-cm glass ring was placed in the petri dish above the “bottom” electrode to support the embryo during electroporation. The final gap between the “top” and “bottom” electrodes during electroporation was approximately 5 mm.

Embryos were collected from fertilized white leghorn chick eggs (Hyline) at stage 5 (Hamburger and Hamilton, 1951) and younger by attaching a 1-in. diameter paper ring (P5, Fisher) to the vitelline membrane overlying the embryo. The embryos were placed onto the glass ring so that the endoderm faced the platinum wire immersed in Tyrode buffer. Through a small hole in the vitelline membrane 1 μl of DNA solution (0.6 μg/μl) was injected into the space between the embryo and the membrane. The “top” electrode was then positioned above the primitive streak using a micro-manipulator (Narishige, Japan), and embryos were pulsed three times for 25 ms at 12 V. Subsequently, embryos were incubated endoderm-up on albumin-agar culture dishes (50% albumin, 1.5% glucose, 0.3% agar, 0.9% NaCl; Sundin and Eichele, 1992) for 24 h.

Whole mount in situ hybridization and histological analysis of mouse embryos

Whole mount in situ hybridization was essentially as described (Hogan et al., 1994). Probes were Meox1 (Candia et al., 1992), Lhx1 (Barnes et al., 1994), Osr1 (So and Danielian, 1999), Fgf8 (Tanaka et al., 1992), Myf5 (Ott et al., 1991), Foxf1 (Mahlapuu et al., 2001), Bmp4 (Winnier et al., 1995), Noggin (Valenzuela et al., 1995), Follistatin (Alban and Smith, 1994), Pax2 (Dressler et al., 1990), Paraxis (Barnes et al., 1997), and a 1.8-kb Ret probe from the pMCRET7.2 plasmid. The Lhx1 probe was cloned by PCR amplification of a 440-bp fragment from Lhx1 exon 2 into the pCRII TOPO vector (Invitrogen). Primers for standard PCR amplification were ATGAGGCCC-GACGTGGAGTCTGCC (forward) and AGAGACGGC-TAGCAGGGGCCAACGC (reverse). Cloned fragments were sequenced. For cryosectioning of stained embryos, specimens were refixed in 4% PFA, embedded in 1.2% Agarose, 5% Sucrose, equilibrated overnight in 30% Sucrose, and frozen in 2-Methylbutane in liquid nitrogen. Frozen sections were made at 20 μm on a Leica CM 1900 Cryostat. Alternatively, stained embryos were paraffin-embedded, sectioned at 10 μm, and counterstained with Eosin.

Whole mount immunostaining and immunohistochemistry

Immunohistochemistry for cleaved Caspase 3 (Cell Signaling Technology) and phospho-Histone H3 (Upstate Biotechnology) was according to standard protocols on sections generated from paraffin-embedded embryos.

Phosphorylated Smad 1/5/8 was detected in mouse embryos essentially as described for di-phosphorylated Erk (from the Rossant lab homepage at www.mshri.on.ca/rossant/protocols.html). Embryos were incubated with a polyclonal phospho-Smad 1/5/8 antibody (Cell Signaling Technology) at 1:1000 and the Biotin-Streptavidin system of the Vectastain Elite ABC kit (Vector Laboratories) was used for detection.

Mouse and chick embryos were processed for immunohistochemistry essentially as described (James and Schulthess, 2003). The following antibodies were used: polyclonal antibody to Pax2 at 1:250 (BAbCo), monoclonal antibody to Pax7 at 1:200 (DSHB), monoclonal antibody to Wt1 at 1:50 (DAKO), monoclonal antibody to GFP (3E6) at 1:500 (Molecular Probes), and polyclonal antibody to GFP (A-6455) at 1:1000 (Molecular Probes).

Results

Somite formation is dependent on Foxc1/c2 gene dosage

Previous work showed that Foxc1; Foxc2 compound null embryos completely lack somites, as judged by morphology and absence or low level expression of markers such as Paraxis, Pax1, and Meox1, normally transcribed at high levels in the paraxial mesoderm (Kume et al., 2001). In this study, we reexamined somite formation in embryos of the Foxc1; Foxc2 allelic series at embryonic day 8.5 (E8.5), and made two important observations: first, somite morphology is dependent on Foxc1/2 gene dosage. In Foxc1 null and compound Foxc1−/−; Foxc2+−/− embryos, somite morphology is normal (data not shown). However, in Foxc2-null embryos, somite shape is slightly abnormal, and in Foxc1−/−; Foxc2−/− compound mutants somites are very narrow and irregular (Figs. 1A, A’, B, B’, C, C’, and 2B, E). Second, we observed that in addition to its high expression in the paraxial mesoderm, Meox1 is also transcribed at low levels in intermediate mesoderm (Figs. 1A and 2A). This raised the possibility that in compound-null embryos, the most medial mesoderm, which expresses Meox1 at low levels (Figs. 1D, D’ and 2C, F), has in fact acquired intermediate mesoderm fate. This might occur as soon as mesoderm cells leave the primitive streak, or after cells have progressed some way along the paraxial mesoderm lineage.
Loss of Foxc1 and Foxc2 leads to medial expansion of intermediate mesoderm markers

To test the above hypotheses, we analyzed expression of intermediate mesoderm markers, Odd skipped-related 1 (Osr1), LIM homeobox protein 1 (Lhx1), and Fgf8. Osr1 is normally expressed in the presumptive and definitive intermediate mesoderm (Figs. 1E and 2G, J) (So and Danielian, 1999). However, in compound-null mutants, expression is medially expanded, so that it extends almost to the neural tube (Fig. 1H). Medial expansion occurs in a gene dosage-dependent manner (Figs. 1F and G). However, this expansion is not seen in the posterior segmental plate region, but only at approximately the level where somite formation would have begun in compound null mutant embryos (Figs. 1H and 2I, L), or has been initiated in other genotypes (Figs. 1F, G and 2H, K). This suggests that Foxc1 and Foxc2 are not required for the early acquisition of prospective paraxial fate. Rather, they become essential later in the process of commitment of paraxial cells to a definitive somite fate. In support of this conclusion, expression of Osr1 is seen in the small and irregular somites of Foxc1+/−; Foxc2+/− mutants, especially in the dorsal region (Figs. 1G and 2H). This shows that Osr1 is being transcribed in mesoderm cells that were able to at least initiate somite formation, and therefore presumably had some characteristics of paraxial mesoderm.

A similar lateralization of paraxial mesoderm in Foxc1; Foxc2 compound mutants is seen using the Lhx1 marker, also normally expressed in the intermediate mesoderm but in more restricted domains than Osr1 (Fig. 1I; Barnes et al., 1994; Fujii et al., 1994). In compound mutants, Lhx1 is ectopically transcribed in cells medial to the intermediate mesoderm in a gene dosage-dependent manner (Figs. 1I and J, and not shown). As with Osr1, evidence for lateralization is not seen in the segmental plate.

Further evidence for lateralization of paraxial mesoderm comes from the analysis of Fgf8 expression. Although expressed at highest levels in the posterior presomitic mesoderm, this gene is also transcribed in the intermediate mesoderm lateral to the newly formed somites. In Foxc1+/−; Foxc2+/− embryos, Fgf8 transcripts can be seen abutting the neural tube (Figs. 1K and L).

Proliferation and cell death in Foxc1; Foxc2 compound mutants

The observed expansion of intermediate mesoderm-specific genes could be secondary to cell death or reduced

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Fig. 1. Gene dosage-dependent expansion of intermediate mesoderm versus paraxial mesoderm in Foxc mutant embryos. Whole mount in situ hybridization of wild-type (A, A', E, I, and K), Foxc2+/− (B, B', and F), Foxc1+/−; Foxc2+/− (C, C', and G), and Foxc1+/−; Foxc2+/− (D, D', H, J, and L) embryos. Anterior is to the top and all views are dorsal except I and J which are ventral. Red arrows mark the border between somite 0 and +1. (A–D) Meox1 is normally strongly expressed in somites, including the forming somite, but at lower levels in intermediate mesoderm (IM) (arrowhead). In Foxc1+/−; Foxc2+/− mutants, no somites are formed, but Meox1 expression is seen adjacent to the neural tube (D). In D, the apparently stronger expression on the right side is caused by a slight tilt of the embryo to its left side, such that the very slim band of cells expressing Meox1 on the left side is partially obscured. (A’–D’) Higher magnification of somitomere I to III, where scale bars = 100 μm.

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proliferation in paraxial mesoderm cells that normally express the highest levels of Foxc1 and Foxc2. We therefore assayed for cell death and proliferation by immunohistochemistry against cleaved Caspase 3 and phospho-Histone H3, respectively (Fernandes-Alnemri et al., 1994; Gurley et al., 1978; Nicholson et al., 1995). We did not observe any difference in cell proliferation between compound mutant and wild-type control embryos of matched stages (3–4 somites and 7 somites; Table 1 and data not shown).

Similarly, the number of cells undergoing apoptosis was not significantly increased in compound null mutant embryos compared with wild type.

The lateral mesoderm marker Foxf1, and Bmp4 signaling in compound mutants

Since intermediate mesoderm markers are medially expanded in compound mutants, we asked whether the same applies to a lateral mesoderm marker, Foxf1 (Mahlapuu et al., 2001; Peterson et al., 1997). Foxf1 expression neither appears to be ectopically expressed in the intermediate mesoderm of mutant embryos of any genotype, nor is expression abnormal in the segmental plate (Figs. 3A–D and data not shown). However, the normal low level of transcript in the medial ventral portion of the paraxial mesoderm is moderately increased in compound mutants compared with wild-type embryos (Figs. 3A and B).

Mediolateral patterning of the mesoderm is influenced by Bmp4 signaling. Therefore, we asked whether Bmp4 transcripts and other components of this signaling pathway are affected in mutant embryos. However, there was no significant change in expression of either Bmp4 (in the lateral mesoderm) or Noggin (in the floor plate and notochord) in Foxc1; Foxc2 compound null embryos (data not shown).

Since somites are absent or reduced in compound mutants, it was likely that expression of the gene encoding another Bmp antagonist, Follistatin (Fst), which is restricted to the somites at E8.5, is also absent or reduced, and this was confirmed by whole mount in situ hybridization (Figs. 4A and B). This raised the possibility that Bmp4 signaling had expanded medially, thereby affecting mesoderm differentiation, and we addressed this by analyzing the distribution of phosphorylated Smad1/5/8 protein, which represents a direct read-out of Bmp signaling. As shown in Fig. 4, the signal was clearly detected in the lateral plate mesoderm, in the dorsal rim of the neural folds, and in scattered cells in the intermediate and lateral paraxial mesoderm both in wild-type control embryos and in Foxc1; Foxc2 compound homozygous mutants (Figs. 4C, D, and F–K). However,
there was no significant medial expansion of signal in compound mutants of any genotype. This suggests that the absence of neither Foxc1 and Foxc2 nor of Follistatin extends the spatial domain of Bmp signaling medially from the lateral plate.

Mediolateral differentiation of somites is abnormal in Foxc1; Foxc2 compound mutants

In compound mutants, intermediate mesoderm markers are ectopically expressed in somites at E8.5. To address whether the subsequent patterning and differentiation of the somites is affected, we analyzed mutant embryos for expression of both Myf5, a marker of epaxial muscle progenitor cells at the medial lip of the differentiating dermomyotome (Ott et al., 1991), and Lbx1, a marker of migratory hypaxial muscle progenitors in the lateral dermoyotomal lip (Dietrich, 1999; Jagla et al., 1995; Mennerich et al., 1998).

Expression of Myf5 is almost completely lost in Foxc1+/--; Foxc2−/− compound mutants, while in Foxc2−/− embryos, expression is weaker than in wild type, and onset of expression is delayed (Figs. 5A–C and G–I). By contrast, the domain of Lbx1 expression is expanded medially in Foxc2−/− embryos, and in Foxc1+/--; Foxc2−/− embryos, Lbx1 is transcribed throughout the dorsal aspect of the differentiating somite, abutting the neural tube (Figs. 5D–F and J–L). Moreover, expression is initiated in younger somites compared with wild-type embryos. In compound null mutants, Lbx1 expression is strongly downregulated (data not shown), presumably because somites are completely absent. Thus, we conclude that in embryos where somites are formed, progressive loss of Foxc gene function leads to increasing lateralization of somite tissue.

Abnormal development of nephrogenic tissue in compound mutants

The intermediate mesoderm in the trunk region, from around somite 5 posteriorly, normally develops into the pronephric duct. This extends posteriorly and forms the mesonephric duct, while the adjacent nephrogenic mesenchyme is induced to give rise to mesonephric tubules, which are most prominent between somites 8 and 15. To ask whether the medial expansion of Lhx1 and Osr1 has functional significance for the later development of the intermediate mesoderm, we examined E9.5 compound mutant embryos for markers of developing nephric tissues. These included Pax2 RNA and protein, normally present in the maturing nephric duct and tubules, Wt1 (Wilms tumor homolog 1), expressed in the proximal end of the mesonephric tubules and the surrounding mesenchyme, and Ret RNA, expression of which is restricted to the mesonephric duct (Dressler et al., 1990; Pachnis et al., 1993; Torres et al.,

Table 1
Proliferation and cell death in compound homozygous embryos

<table>
<thead>
<tr>
<th>Genotype/embryo</th>
<th>Wild type (7s)</th>
<th>−/−;−/− (7s)</th>
<th>Wild type (3–4s)</th>
<th>−/−;−/− (3–4s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primitive streak</td>
<td>1.63 ± 0.38%</td>
<td>1.66 ± 0.86%</td>
<td>2.91 ± 2.21%</td>
<td>2.71 ± 0.86%</td>
</tr>
<tr>
<td>(n = 4; 17/1043)</td>
<td>(n = 6; 36/1324)</td>
<td>(n = 6; 42/1533)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presomitic/SI-II</td>
<td>1.25 ± 0.5%</td>
<td>1.58 ± 0.54%</td>
<td>2.17 ± 0.84%</td>
<td>2.2 ± 0.99%</td>
</tr>
<tr>
<td>region</td>
<td>(n = 5; 9/670)</td>
<td>(n = 5; 13/988)</td>
<td>(n = 5; 24/1104)</td>
<td>(n = 5; 25/1132)</td>
</tr>
<tr>
<td>Cell death assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primitive streak</td>
<td>0/747 (n = 3)</td>
<td>0/706 (n = 3)</td>
<td>2/1313 (n = 6)</td>
<td>0/1292 (n = 6)</td>
</tr>
<tr>
<td>(n = 4; 17/1043)</td>
<td>(n = 6; 36/1324)</td>
<td>(n = 6; 42/1533)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presomitic/SI-II</td>
<td>1/1161 (n = 7)</td>
<td>1/1270 (n = 7)</td>
<td>3/838 (n = 5)</td>
<td>1/879 (n = 5)</td>
</tr>
<tr>
<td>region</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Shown are average percentage of labeled cells ± standard deviation (number of sections analyzed; total number of labeled cells/total number of cells counted).

<sup>b</sup>Shown are the total number of labeled cells/total number of cells counted (number of sections analyzed).

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![Fig. 3. Histological analysis of the lateral plate mesoderm marker Foxf1.](image-url)
Compared with wild type, the Pax2 expression domain is significantly enlarged in the region of the mesonephros, especially in compound null mutant embryos (Figs. 6A–G). Moreover, the anterior boundary of the Pax2 domain is located more anteriorly than in wild-type embryos (in the case of compound null embryos, which have no somites along the A–P axis, this boundary was determined with respect to the position of the otic vesicle (Fig. 6C)). From whole mount in situ hybridization, it appeared that...
excess Pax2-expressing cells are aggregated into structures resembling large mesonephric tubules (Figs. 6C, D, and G). However, immunohistochemical analysis of sections from compound null embryos showed that although cells express Pax2 and Wt1 protein, they are not organized into well-polarized proximal and distal mesonephric tubules (Figs. 6H–K). Analysis of compound mutant embryos with a probe for Ret, a marker of nephric duct tissue, provided similar evidence for excess intermediate mesoderm cells and disorganization of the nephric tissue (Figs. 6L–Q). In addition, analysis of sectioned embryos showed cells in the more medial mesoderm close to the neural tube, that express Pax2, Wt1, and Ret (Figs. 6I, P, and O), suggesting that these medial cells have acquired a nephric tissue fate.

**Misexpression of Foxc2 or Foxc1 in the presumptive intermediate mesoderm of chick embryos leads to a change to somite cell fate**

To investigate whether expression of Foxc genes is sufficient to regulate trunk mesoderm differentiation, we performed gain of function experiments in chick embryos. Chick Foxc2 (cFoxc2) and mouse Foxc1 cDNAs were each cloned into an expression construct (Swartz et al., 2001) containing an internal ribosome entry sequence linked to green fluorescent protein (Foxc-GFP). The resulting cFoxc2-GFP and Foxc1-GFP plasmids were separately electroporated into HH stage 4 chick embryos in the region of the primitive streak that is normally fated to form intermediate and lateral plate mesoderm (James and Schultheiss, 2003). Embryos were analyzed at stages HH 10 to 11, when mesonephric duct morphogenesis is normally initiated. Nephric tissue was identified with antibodies to Pax2, and somitic tissue was analyzed by examining expression of Paraxis (Barnes et al., 1997) and Pax7 (Ericson et al., 1996).

Two interesting points have emerged from these studies. First, Foxc gene expression negatively regulates Pax2 expression and intermediate mesoderm development. In control experiments (n = 8) in which GFP vector alone was electroporated, Pax2 expression was normal (Figs. 7A and B) and often co-localized with the GFP (Fig. 7B). In contrast, Pax2 never co-localized with GFP in embryos electroporated with cFoxc2-GFP (n = 12; Figs. 7C and D). In several embryos (n = 4), cFoxc2-GFP was expressed unilaterally, and in these cases, we observed decreased expression of Pax2 on the affected side (Figs. 7E and F) concomitant with loss of mesonephric duct morphology.

Second, Foxc genes positively regulate somite fate in the intermediate mesoderm. Paraxis is normally expressed in the somites and the most anterior part of the segmental plate (Fig. 8E), while Pax7 is normally expressed in epithelial somites and the dorsal neural tube (Figs. 8J and K). Expression of cFoxc2-GFP, but not GFP alone, resulted in ectopic expression of Paraxis and Pax7 in the intermediate mesoderm (Figs. 8A-D and F-I) in all embryos analyzed (Paraxis, n = 9; Pax7, n = 11). Significantly, both markers were expressed only in a small percentage of GFP-positive cells in the intermediate mesoderm and were never expressed in the lateral plate mesoderm. Similar results were obtained from electroporation experiments using the Foxc1-GFP plasmid (data not shown).

These data suggest that in addition to being necessary for trunk paraxial mesoderm development, Foxc1/2 are sufficient to promote paraxial cell fate, as judged by expression of the somite markers Paraxis and Pax7, at the expense of intermediate, but not lateral plate, mesoderm cell fates.

**Discussion**

Mesodermal patterning in the vertebrate embryo proceeds through a series of stages, starting when cells transit the primitive streak at different anterior–posterior levels. During translocation to their final position, they are exposed to multiple signals from surrounding tissues that influence their fate and their differentiation into paraxial, intermediate, and lateral plate mesoderm. Here, we present two lines of evidence that Foxc1 and Foxc2 are synergistically required for cells to specify a paraxial rather than intermediate mesoderm fate. First, analysis of compound null mouse mutants shows that loss of Foxc1 and Foxc2 leads to a dose-dependent conversion of paraxial into intermediate mesoderm in the absence of significant changes in cell death or cell proliferation. Second, ectopic misexpression of either Foxc1 or Foxc2 in the prospective intermediate mesoderm of the chick embryo leads to upregulation of the somite markers, Paraxis and Pax7, while intermediate mesoderm patterning is impaired. Moreover, our results suggest that while Foxc genes may play a role earlier in mesoderm development, they are absolutely required in cells that have already progressed along mesodermal lineages but are not yet committed to paraxial cell fates.

**Gene dosage-dependent effect of Foxc1 and Foxc2 on early mesoderm patterning**

In Foxc1; Foxc2 compound null embryos, mesoderm is able to initiate development towards paraxial, intermediate, and lateral mesodermal fates, since Osr1 and Foxf1 expression patterns are not changed from wild type. However, intermediate mesoderm-specific markers begin to be ectopically expressed in the presumptive paraxial mesoderm at the level of somitogenesis where commitment to somite fate normally takes place (Figs. 1 and 2). This suggests that as mesodermal progenitors progress towards commitment to their final fates, they pass through a stage in which their differentiation can be altered by changes in environmental cues or the activity of downstream signaling pathways. Our results imply that the decision whether to differentiate into somite or interme-
mediate mesoderm depends on both the concentration of Foxc1/c2 proteins the cells express, and the time over which these proteins are present after the nascent, naive mesodermal cells leave the primitive streak.

A similar mechanism for cell fate regulation by cumulative expression of a forkhead transcription factor has been suggested for the role of pha-4, a Foxa2 ortholog, in pharynx formation in *Caenorhabditis elegans*. PHA-4 has
been shown to specify pharyngeal identity and to orchestrate pharynx formation through differential affinity to binding sites of target genes (Gaudet and Mango, 2002). During early phases of pharynx formation, when *pha-4* is expressed at low levels, it is thought to bind only to target genes with high affinity sites in their promoter regions. At later stages of pharynx development, *pha-4* expression increases and the protein binds to target sites with lower affinity. Promoter analysis of *Paraxis*, a gene normally expressed in the presumptive and definitive paraxial mesoderm and not expressed in *Foxc1, Foxc2* compound null mutant embryos (Kume et al., 2001), reveals several Fox binding sites, suggesting that Foxc1 and Foxc2 directly regulate *Paraxis* expression (B.W. and B.L.M.H., unpublished results). A dose-dependent effect of *Foxf1* RNA has recently been shown to be important for the proper vascularization of the lung in newborn mice heterozygous for a *Foxf1* null mutation (Kalinichenko et al., 2001, 2002). Taken together, these data indicate that dosage levels of forkhead proteins are crucial for correct differentiation and patterning of tissues during embryonic development.

Analysis of *Foxc1, Foxc2* compound mutant embryos indicates that *Foxc2* heterozygous embryos lacking all *Foxc1* activity can form normal somites and display normal intermediate mesoderm patterning (not shown). By contrast, in the reverse situation, *Foxc1* heterozygous embryos lacking *Foxc2* activity develop smaller somites than normal and show medial expansion of intermediate mesoderm specific markers, and eventually have more mesonephric tubule tissue than normal (Figs. 1, 2, and 6). Therefore, the activity of one functional *Foxc1* allele is sufficient to allow somitogenesis, although somites are morphologically similar to those in wild type embryos (white arrow). In transverse sections, the mesonephric tissue (arrow) in compound mutants is slightly expanded at the anterior of the nephric cord (arrowhead: nephric duct, P, and Q). The boxed area in P is enlarged in Q. Broken lines in A, B, and D, and L, M, and N indicate level of respective transverse sections. Scale bars = 500 μm (A–C), 300 μm (D), 50 μm (E–G), 40 μm (H–K), 250 μm (L–N), 50 μm (O and Q), and 100 μm (P). da, dorsal aorta; g, gut; h, heart; nd, nephric duct; nt, neural tube; ov, otic vesicle; so, somite; t, nephric tubule.

Evidence suggests that Bmp signaling controls early mediolateral and dorsal–ventral patterning of the mesoderm (Tonegawa and Takahashi, 1998; Tonegawa et al., 1997). However, our data indicate that neither *Bmp4* gene expression nor the spatial signaling of the protein are changed in

- **Fig. 6.** Loss of *Foxc1* and *Foxc2* results in expansion and disorganization of mesonephric tissue at E9.5. Expression of *Pax2* RNA (A–G), *Pax2* and *Wt1* protein (H–K), *Ret* RNA (L–Q) in E9.25–9.5 embryos. In wild-type embryos, *Pax2* is expressed in the mesonephric duct and adjacent mesonephric tubules (arrowhead; A); the anterior end of expression domain is at level of somites 14–15 (white arrow). In *Foxc1*+/–; *Foxc2*–/– embryos (B and F), the anterior limit is at level of somites 12–13 (white arrow; B), and expression of *Pax2* is slightly expanded at the anterior of the nephric cord (F; arrowhead: nephric duct, arrow: mesonephric tissue). In *Foxc1*+/–; *Foxc2*–/– mutants (C, D, and G), the expression domain is dramatically enlarged (arrowheads) and extends more anteriorly relative to the otic vesicle (ov) than in wild type (white arrow). In transverse sections, the mesonephric tissue (arrow) in compound mutants is aggregated (F and G). Bracket in D indicates neural tube, and in G indicates disorganized non-somitic cells between nephrogenic tissue and neural tube. Sections of wild-type (H and J) and double mutant (I and K) embryos analyzed with immunohistochemistry (H and I) for *Wt1* (red) and *Pax2* (green) and with the nuclear stain DAPI (I and K). In the wild type, *Pax2* marks distinct ductal (nd) and tubular (t) structures, while *Wt1* is found in the proximal end of the tubule and surrounding mesenchyme. In the mutants, the pattern of *Pax2* and *Wt1* expression is disorganized, and *Pax2* and *Wt1* expression extends into the ‘paramedial’ mesoderm. In wild type (L and O), *Ret* RNA is expressed in the nephric duct (nd) and neural tube (nt). In two views of the same *Foxc1*+/–; *Foxc2*–/– embryo, *Ret* is present in a large, anterior domain (arrowhead), and also in mesodermal cells close to the neural tube (white double arrowhead; M, N, P, and Q). The boxed area in P is enlarged in Q. Broken lines in A, B, and D, and L, M, and N indicate level of respective transverse sections. Scale bars = 500 μm (A–C), 300 μm (D), 50 μm (E–G), 40 μm (H–K), 250 μm (L–N), 50 μm (O and Q), and 100 μm (P). da, dorsal aorta; g, gut; h, heart; nd, nephric duct; nt, neural tube; ov, otic vesicle; so, somite; t, nephric tubule.

- **Fig. 7.** Misexpression of *Foxc2* represses intermediate mesoderm formation in chick embryos. Immunohistochemistry of transverse sections of chick embryos after electroporation of *Foxc2* into the intermediate–lateral plate mesoderm domain. DAPI staining (A, C, and E) and merged photographs of immunohistochemistry for *GFP* and *Pax2* (B, D, and F). Electroporation of control GFP construct into the intermediate–lateral plate mesoderm domain has no effect on histology of the tissue (A), and *Pax2* protein co-localizes with GFP expressing cells (B). In embryos electroporated with *cFoxc2*-GFP (C–F), *Pax2* never co-localizes with GFP-expressing cells (C and D). Unilateral expression of *cFoxc2*-GFP construct can result in extensive decrease in *Pax2* expression on the GFP-expressing side (F, right side). The nephric duct rudiment on the left (control) side (arrow) is absent on the right (electroporated) side (arrowhead, E, F). im, intermediate mesoderm; np, neural plate; nt, neural tube; s, somite.
Foxc1; Foxc2 compound mutant embryos. Foxc1 and Foxc2 therefore do not appear to regulate Bmp4 activity in the lateral patterning of the mesoderm.

Foxc1 and Foxc2 are involved in mediolateral patterning of the somitic mesoderm

Similar to early mesoderm patterning events, the mediolateral and dorsal–ventral somite patterning of the somite, and the differentiation of the sclerotome and dermamyotomal populations, are regulated by signaling pathways involving Shh, Bmp, and Wnt proteins (Hirsinger et al., 1997; Johnson et al., 1994; Marcelle et al., 1997, 1999; Pourquie et al., 1996; Teillet et al., 1998; Watanabe et al., 1998).

Our results reveal that dermomyotome patterning of the differentiating somites is abnormal in embryos lacking functional Foxc2 alleles. While the epaxial domain is reduced, the hypaxial domain is enlarged. This phenotype is particularly striking in Foxc1 +/−; Foxc2−/− embryos in which the dermomyotome appears to represent almost exclusively the hypaxial domain. We therefore conclude that loss of Foxc2 function leads to lateralization of somite mesoderm.

Mesonephric duct and tubule development are dependent on Foxc1 and Foxc2 gene dose

Previous analysis of Foxc1 homozygous null and Foxc1; Foxc2 compound heterozygous embryos and newborn mice had revealed an expansion of mesonephric tubules and ectopic ureter buds associated with an anterior enlargement of Gdnf and Eya1 expression domains in the mesonephric mesenchyme (Kume et al., 2000). Here, due to the complete absence of Foxc1 and Foxc2, we show an even more pronounced effect on the development of the mesonephric region at E9.5. In the anterior intermediate mesoderm of compound null mutant embryos, large disorganized aggregates of cells expressing nephric duct and tubule markers such as Pax2, Wt1, and Ret are accumulated. Furthermore, these markers are also ectopically expressed in the medial mesoderm close to the neural tube. Our findings indicate that Foxc genes are involved in pro- and mesonephric kidney development at several stages: first, Foxc1 and Foxc2 play a role in the establishment of the intermediate mesoderm. Later, Foxc1 and Foxc2 are required for correct patterning of the nephrogenic region into duct and tubule tissue and adjacent mesenchyme. Unfortunately, because the
compound null embryos die before E10.0, the role of Foxc genes in development of the ureter buds and metanephric kidney could not be followed.

Foxc1 or Foxc2 are sufficient to induce somitic fate in intermediate mesoderm in the chick embryo

Studies on the early patterning of the mesoderm in chick and mouse embryos indicate that similar mechanisms and molecular interactions regulate mesoderm specification and fate determination in both species. The expression of two chick Foxc genes also appears to be similar to that of the mouse orthologs (Buchberger et al., 1998; Sudo et al., 2001).

Our studies reported here provide evidence that misexpression of Foxc1 or cFoxc2 induces expression of the somite markers Paraxis and Pax7 in the intermediate mesoderm, while expression of Pax2, a marker for cells committed to nephric duct and nephrogenic cord (James and Schultheiss, 2003), is abolished. Furthermore, misexpression of Foxc1 or cFoxc2 disturbs normal morphology in the intermediate mesoderm. However, misexpression in the lateral plate mesoderm does not lead to ectopic expression of somite or intermediate markers.

Taken together, our results suggest that Foxc proteins are necessary for the establishment of paraxial mesoderm fate, and are sufficient to induce expression of at least a subset of paraxial mesoderm markers in the prospective intermediate mesoderm. Secondly, Foxc proteins appear to function only in the decision between paraxial versus intermediate mesoderm cell fates, but not between intermediate and lateral plate mesodermal fates; the latter decision appears to require an independent mechanism. In summary, our data provide evidence for a novel, critical role of the transcription factors Foxc1 and Foxc2 in the fate commitment process of paraxial and intermediate mesoderm.

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References


onistic regulation by SHH and BMP4 proteins. Development 125, 2631–2639.


