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Cell fate polarization in ascidian mesenchyme/muscle precursors by directed FGF signaling and role for an additional ectodermal FGF antagonizing signal in notochord/nerve cord precursors

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Asymmetric cell division plays a fundamental role in generating various types of embryonic cell. In ascidian embryos, asymmetric cell divisions occur in the vegetal hemisphere in a manner similar to those found in *Caenorhabditis elegans*. Early divisions in embryos of both species involve inductive events on a single mother cell that result in production of daughters with different cell fates. Here we show in the ascidian *Halocynthia roretzi* that polarity of muscle/mesenchyme mother precursors is determined solely by the direction from which the FGF9/16/20 signal is presented, a role similar to that of Wnt signaling in the EMS and T cell divisions in *C. elegans*. However, polarity of nerve cord/notochord mother precursors is determined by possible antagonistic action between the FGF signal and a signal from anterior ectoderm, providing a new mechanism underlying asymmetric cell division. The ectoderm signal suppresses MAPK activation and expression of *Hr-FoxA*, which encodes an intrinsic competence factor for notochord induction, in the nerve cord lineage.

KEY WORDS: Ascidian embryo, Mesenchyme, Notochord, Embryonic induction, Asymmetric cell division, FGF, FoxA

INTRODUCTION

How the diversity of cell types is generated from a common set of progenitor cells during animal development is a fundamental issue in the field of developmental biology. Asymmetric cell division plays a crucial role in diversifying cell fates by generating daughter cells specified to have distinct fates (Horvitz and Herskowitz, 1992; Howkins and Garriga, 1998; Goldstein, 2000; Knoblich, 2001; Roegiers and Jan, 2004; Betschinger and Knoblich, 2004). How is the polarity of asymmetry determined and established? From extensive studies using *Caenorhabditis elegans* and *Drosophila* as model systems, asymmetric cell divisions are known to involve both polarized intrinsic factors and inductive signals. As examples of asymmetric cell divisions using external cues, the EMS cell of the *C. elegans* four-cell-stage embryo and the T cell at a later postembryonic stage are known to divide asymmetrically to produce daughter cells with different fates. In these divisions, it is proposed that the position from which Wnt signaling is presented, i.e. from posterior to the cells in both cases, regulates cell fate polarization and spindle orientation (e.g. Thorpe et al., 1997; Goldstein et al., 2006). In the *Drosophila* peripheral nervous system, sensory organ precursor cells undergo several rounds of asymmetric division within the plane of the epithelium. The cell fate polarization and spindle orientation depends on the planar cell polarity within the epithelium (Bellaiche et al., 2004), although daughter cells further communicate with each other through Notch signaling to assume mutually exclusive fates (Rhyu et al., 1994). In contrast, examples using intrinsic cues are found in neuroblasts in the *Drosophila* central nervous system (e.g. Betschinger and Knoblich, 2004). In vertebrate skin development, the basal epidermal cells also use their intrinsic polarity and divide asymmetrically, generating a committed suprabasal cell and a proliferative basal stem cell (Lechler and Fuchs, 2005). Thus, asymmetric cell division is a fundamental system for establishing various cell types during development; however, the way in which mother cells polarize varies.

In ascidian embryos, asymmetric cell divisions occur at the division to the 64-cell stage in the vegetal marginal blastomeres, which encircle the central endoderm located at the vegetal pole (Fig. 1). At this division, four posterior blastomeres (pairs of the B6.2 and B6.4; mesenchyme/muscle mother blastomeres) divide into four outer muscle precursors and four inner mesenchyme precursors. Similarly, in the anterior region, four blastomeres (pairs of the A6.2 and A6.4; notochord/nerve cord mother blastomeres) divide to produce four outer nerve cord precursors and four inner notochord precursors (Nishida, 1987). These asymmetric cell divisions are known to involve the FGF signal from the endoderm, and the differing ways to respond to FGF signal between the anterior and posterior regions are accounted by the presence and absence of maternally localized factor, macho-1 (Fig. 1C) (reviewed by Nishida, 2005). When the inductive influence is inhibited by isolation of the blastomeres or using inhibitors of FGF signaling, both daughters of the mesenchyme/muscle and notochord/nerve cord precursors assume the default muscle and nerve cord fates, respectively (Kim et al., 2000; Minokawa et al., 2001; Kim and Nishida, 2001). In contrast, when the mother precursors are isolated and treated with FGF protein over their entire surface, both the daughter cells adopt the induced mesenchyme and notochord fates (Nakatani et al., 1996; Kim et al., 2000).

The occurrence of the signaling prior to, but not after, cell division that segregates two distinct fates into two daughters, is one of the most important criteria as to whether the division can be regarded as induced asymmetric cell division. Several lines of evidence indicate that induction of notochord and mesenchyme fates by the FGF

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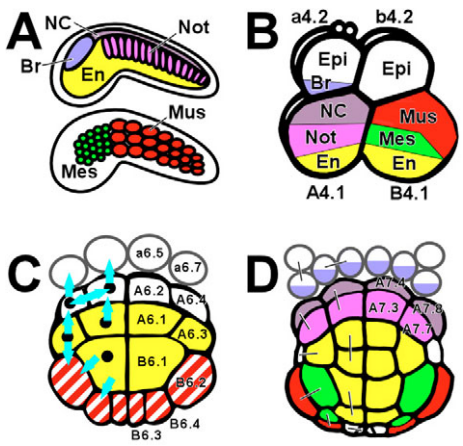

Fig. 1. Diagrams showing the fates of cells in the vegetal hemisphere of ascidian embryos. Endoderm-lineage cells are colored yellow. Those for notochord, nerve cord, the brain, mesenchyme and muscle are shown in pink, purple, blue, green and red, respectively. (**A**,**B**) Lateral views; anterior is to the left. (A) Tailbud embryos. Upper and lower diagrams illustrate midsagittal and parasagittal sections, respectively. (B) An eight-cell-stage embryo. Fate map of major larval tissues and the name of each blastomere are indicated. (**C**,**D**) Vegetal views. Anterior is up. The cells shown by ellipses at the top of each diagram are blastomeres in the animal hemisphere, which are behind the vegetal hemisphere. (C) A 32-cell-stage embryo. Cells expressing *FGF9/16/20* are highlighted by black dots. Light blue arrows indicate direction of FGF signaling. Red hatching indicates the proposed area where macho-1 functions. (D) A 64-cell-stage embryo. The pairs of blastomeres connected with bars are sister blastomeres. Br, brain; En, endoderm; Epi, epidermis; Mes, mesenchyme; Mus, muscle; NC, nerve cord; Not, notochord.

signal takes place during the 32-cell stage, right before the division that separates induced and default fates into daughter cells. In the nerve cord/notochord division, recombination of isolated nerve cord/notochord and endoderm (inducer) precursor blastomeres resulted in notochord formation only when the nerve cord/notochord precursors to be recombined were in the initial two thirds of the cell cycle at the 32-cell stage (Nakatani and Nishida, 1999). The nerve cord/notochord precursors lose their competence at the end of that stage. It is also shown that the inducing ability of the endoderm blastomeres persists even after the 64-cell stage. Consistently, the nerve cord/notochord precursors lose their competence to respond to the treatment of FGF protein at the division to the 64-cell stage (Nakatani et al., 1996). In addition, the sensitive period to the FGF receptor and MEK inhibitors ends at the sixth cleavage, suggesting that notochord induction completes during the 32-cell stage (Kim and Nishida, 2001). As to the division of the muscle/mesenchyme precursors, the precursors acquire the ability of autonomous mesenchyme formation in isolation sometime in the last half of the cell cycle at the 32-cell stage, indicating that mesenchyme induction also completes during that stage (Kim and Nishida, 1999). Accordingly, both precursor blastomeres appear to be polarized by the external signal and divide to produce daughter cells with distinct fates through asymmetric divisions.

The situation described above in the ascidian is reminiscent of that found in EMS division in *C. elegans*, with the exception that *C. elegans* uses Wnt signaling instead of FGF signaling (Goldstein, 1992; Goldstein, 1993; Goldstein, 1995a; Goldstein et al., 2006). The EMS divides to produce the E cell close to its inducer P_2 cell and the MS cell away from it. Importantly, induction of the E cell by Wnt signaling from the P_2 cell takes place before the EMS divides. The daughters of the EMS adopt default MS fate in the absence of Wnt signaling. Recently, it was shown that the polarity of the EMS is determined by the direction from which Wnt signaling is presented (Goldstein et al., 2006). Therefore, similarly to what has recently been demonstrated in *C. elegans*, a model of directed-signaling-mediated asymmetric division has been proposed in the ascidian, in which the polarity of the asymmetric divisions is determined by the direction from which the FGF signal comes (Nishida, 2002).

It is, however, still unknown whether the polarities are determined solely by the FGF signal, and/or whether their internal state or other cell interactions are also involved. In particular, the notochord fate can not only be induced by endoderm blastomeres but also by neighboring notochord/nerve cord blastomeres, while mesenchyme/muscle precursors do not have an ability to induce the mesenchyme fate mutually (Nakatani and Nishida, 1994; Kim and Nishida, 1999). In agreement with this, *FGF9/16/20* is expressed in nerve cord/notochord precursors as well as in endoderm cells, but not in precursors of mesenchyme/muscle (Fig. 1C) (Imai et al., 2002a; Kumano et al., 2006). These findings suggest that not only the side of the nerve cord/notochord precursor cells on which notochord precursors arise (endoderm side) but also the other sides apart from the endoderm could be exposed to the FGF signal, and yet only one daughter cell is induced to become notochord. In addition, the brain is known to be induced by FGF signal from the nerve cord/notochord precursors (Fig. 1C,D) (Bertrand et al., 2003). As brain precursors are situated on the opposite side of the nerve cord/notochord precursors from the endoderm, this clearly implies that the A6.2 notochord/nerve cord blastomere, for example, would receive the FGF signal from every direction. Therefore, it is difficult to conceive how it could be polarized solely by the FGF signal.

In this study, using molecular and micromanipulative approaches, we investigated whether the asymmetries are determined solely by the direction from which the FGF signal is presented. Our results show that whereas the polarity of the muscle/mesenchyme mother cells is indeed determined in this way, establishment of the polarity in the nerve cord/notochord mother cells depends on a possible antagonistic action between the FGF signal and a suppressive signal from the anterior ectoderm, providing a newly characterized mechanism underlying asymmetric cell divisions.

MATERIALS AND METHODS

Animals and embryos

Eggs of the ascidian *Halocynthia roretzi* were spawned under temperature and light control, and fertilized with a suspension of non-self sperm. Embryos were cultured in Millipore-filtered seawater containing 50 μg/ml streptomycin and 50 μg/ml kanamycin at 9-13°C.

Blastomere manipulation and inhibition of cell division

Fertilized eggs were manually devitellinated with tungsten needles and reared in 0.9% agar-coated plastic dishes filled with seawater. Identified blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope (SZX-12; Olympus). Isolated blastomeres were cultured separately until they developed into partial embryos. For recombination, an isolated group of blastomeres was made to adhere to another group by mutual adhesiveness. For blastomere removal and transplantation at the early 32-cell stage, ectodermal blastomeres (descendants of a4.2 or b4.2) were removed from a host embryo with a fine glass needle. Donor blastomeres that had been isolated from another early 32-cell embryo were immediately transplanted to the region of the host embryo from which the ectoderm cells had been removed. In some experiments, cleavage was permanently arrested

with 2.5 μg/ml cytochalasin B (Sigma) from the 110-cell stage onward. Embryos were cultured and fixed at appropriate stages for immunohistochemistry and in situ hybridization.

Injection of MOs and synthetic mRNAs

An antisense morpholino oligonucleotide (MO; Gene Tools) complementary to *Hr-FGF9/16/20* (5′-TACCATTTGTACTGAAGGCATTTTC-3′) (Kumano et al., 2006) was used to suppress its translation. In control experiments, we used standard control MO supplied by the manufacturer. *Hr-FGF9/16/20* and *Hr-FoxA* plasmids for in vitro RNA synthesis were prepared by PCR-amplifying fragments and subcloning them into the pBluescript-HTB(N) vector (Kumano et al., 2006). As a control for RNA injection, a *venus YFP* fragment was subcloned into pBluescript-RN3 (Lemaire et al., 1995). Capped *Hr-FGF9/16/20*, *Hr-FoxA* and *venus YFP* mRNAs were synthesized with the mMessage mMachine kit (Ambion) and subsequently Poly(A) was added with a Poly(A) Tailing kit (Ambion). MOs (30 pg), synthetic mRNAs (10 pg with the exception of *Hr-FoxA* mRNA: 0.5-1 pg) or both were injected into blastomeres at the eight-cell stage. Microinjection was carried out as described previously (Miya et al., 1997).

Immunohistochemistry and whole-mount in situ hybridization

Formation of mesenchyme was monitored at the equivalent of the larval stage (about 10 hours after the hatching stage) by staining with the anti-Mch-3 monoclonal antibody in embryos whose cleavage was arrested at the 110-cell stage. The antibody specifically recognizes small particles in mesenchyme cells of *Halocynthia* larvae (Kim and Nishida, 1999). The specimens were fixed for 10 minutes in methanol at –20°C. Indirect immunofluorescence detection was carried out by standard methods using a TSA fluorescein system (PerkinElmer Life Sciences) according to the manufacturer's protocol. Immunostaining for activated MAPK (anti-diphosphorylated ERK1/2, M8159; Sigma) and nuclear staining with DAPI were performed as described by Nishida (Nishida, 2003).

Whole-mount in situ hybridization was performed according to Miya et al. (Miya et al., 1997). Specimens were hybridized by using digoxigenin-labeled *Hr-MA4*, *Hr-Bra*, *Hr-FoxA* and *Hr-ETR1* antisense probes. *Hr-MA4*, encoding the muscle *actin* gene, was used to assess muscle specification (Satou et al., 1995). *Hr-Bra*, encoding the *Brachyury* gene, was used for notochord specification (Yasuo and Satoh, 1993). *Hr-FoxA* (formerly *Hr-HNF3-1*) encodes a homolog of class I fork head/HNF-3 (Shimauchi et al., 1997). The expression of these genes was monitored at the 110-cell stage. *Hr-ETR1*, encoding an RNA-binding protein of the Elav family, was used as a molecular marker for nerve cord specification (Minokawa et al., 2001), and its expression was monitored at the neural plate stage in embryos where cleavage was arrested at the 110-cell stage.

RESULTS

Transplanted endoderm blastomeres induce ectopic mesenchyme and notochord formation and suppress muscle and nerve cord fates

To clarify how the polarity of the mesenchyme/muscle and nerve cord/notochord mother cells is established, we first carried out blastomere transplantation experiments. For mesenchyme and muscle, b-line ectoderm cells of the animal hemisphere were removed from the posterior-left region at the early 32-cell stage (precisely at the 24-cell stage just after the formation of B6.2). Then, endoderm blastomeres that had been isolated from another early 32-cell embryo were transplanted to the region from which the b-line ectoderm had been removed (Fig. 2A), such that the mesenchyme/muscle precursor received the inducing signal bi-directionally from the transplanted endoderm and original endoderm cells. Then cleavage of the embryos was arrested at the 110-cell stage and culture was continued until the larval hatching stage.

We used the Mch-3 antigen as a marker of mesenchyme differentiation. When cleavages were permanently arrested at the 110-cell stage, the Mch-3 antigen was expressed in 3.2 out of the four (two on each side) mesenchyme-lineage blastomeres on average (Fig. 2C). In endoderm-transplanted embryos, expression of the Mch-3 antigen was detected not only in cells of mesenchyme lineage, but also ectopically in cells of muscle lineage on the left (transplanted) side (Fig. 2D, arrowhead). The number of Mch-3-positive cells was 4.4 on average. Transplantation of isolated b-line ectodermal (epidermal) cells as a control experiment had no effect on Mch-3 expression (3.1 on average, Fig. 2E). The expression of muscle *actin* was monitored at the 110-cell stage and found to be abrogated when endoderm was transplanted (Fig. 2G), whereas it was unaffected by transplantation of ectodermal cells (Fig. 2H). Therefore, transplantation of endoderm cells to the side opposite from the host endoderm causes muscle lineage cells to assume a mesenchyme fate.

In the case of notochord and nerve cord, a-line ectoderm cells were replaced by endoderm cells of a different embryo at the early 32-cell stage so that the notochord/nerve cord precursors became sandwiched by endoderm cells (Fig. 2B). The expression of *Hr-Bra*, a marker of notochord differentiation, monitored at the 110-cell stage was detected ectopically in cells of nerve cord lineage (Fig. 2J, arrowheads). Transplantation of a-line ectoderm as a control scarcely induced the expression of *Hr-Bra* (Fig. 2K). The expression of *Hr-ETR1*, a marker of neural plate, including nerve cord as well as brain, was abrogated in cells of nerve cord lineage in the cleavage-arrested 110-cell embryos when endoderm was transplanted (Fig. 2M). The expression in brain precursors was also absent because brain-lineage cells were replaced with transplanted endoderm cells. *Hr-ETR1* expression in ectoderm-transplanted control embryos was comparable to that in unoperated control embryos (Fig. 2N). Accordingly, as is the case for muscle/mesenchyme, transplantation of endoderm cells to the side opposite from the host endoderm induces a notochord fate in the nerve cord lineage.

Polarity of asymmetric division to segregate mesenchyme and muscle fates is determined solely by FGF signaling

Our final goal was to reverse the polarity of asymmetric cell divisions by manipulating the position of the signal source, which would work if polarity is indeed determined solely by the direction from which the signal molecule comes. We exploited the recent isolation of the *Halocynthia* ortholog of *FGF9/16/20* (Kumano et al., 2006). Knockdown of *Hr-FGF9/16/20* by injecting antisense MO into the left A4.1 and B4.1 blastomeres of the eight-cell embryo resulted in almost complete loss of Mch-3 antigen expression in mesenchyme lineage cells, with concomitant ectopic expression of muscle *actin* in these cells (Fig. 3A,A′). Consistent with this, transplantation of endoderm cells from such morphants did not alter the patterning (Fig. 3C,C′), whereas transplantation of endoderm cells from embryos injected with control MO sustained the inducing ability (data not shown). In addition, when b4.2 on the left side was injected with synthetic *Hr-FGF9/16/20* mRNA at the eight-cell stage, the cells of the muscle lineage in the left half assumed a mesenchyme fate with concomitant loss of muscle fate (Fig. 3B,B′). These results suggest that FGF9/16/20 is indeed an endogenous inducer for mesenchyme induction.

In an attempt to promote asymmetric division with reversed polarity, we performed a double injection, in which A4.1 and B4.1 were injected with *FGF* MO and b4.2 with *FGF* mRNA, simultaneously. Such embryos, however, had Mch-3 expression in both muscle and mesenchyme lineages (data not shown), probably because the effect of the injected mRNA was too strong. To overcome this problem, we transplanted b-line ectoderm cells that had been injected with *FGF* mRNA at the eight-cell stage to the b-

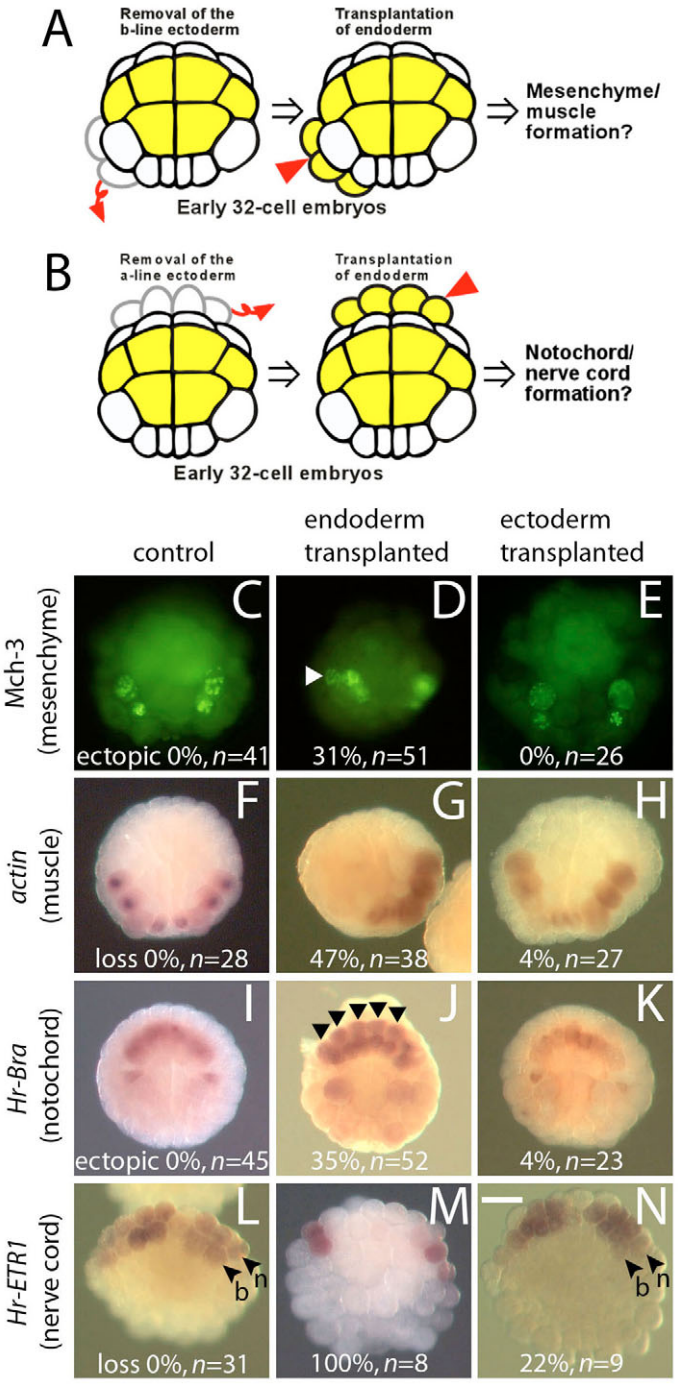

Fig. 2. Effect of endoderm transplantation. (**A**,**B**) Diagrams of the experimental design for endoderm transplantation. Ectoderm cells were removed from early 32-cell host embryos (red twisted arrows). Donor endoderm cells were isolated from early 32-cell embryos. The isolated endoderm cells were transplanted to the ectoderm-depleted region (red arrowheads) so that the presumptive mesenchyme (A) and notochord (B) cells are sandwiched with two groups of endoderm cells. (**C-N**) Expression of tissue-specific markers in un-manipulated control (left column), endoderm-transplanted (middle column) and ectoderm-transplanted control (right column) embryos. (C-K) Vegetal views. (L-N) Animal views. Anterior is up. (C-E) Expression of the mesenchyme-specific Mch-3 antigen in embryos in which cleavage was arrested at the 110-cell stage with cytochalasin B. Arrowhead in D indicates a muscle blastomere that ectopically expressed the Mch-3 antigen. (F-H) Expression of the muscle *actin* gene at the 110-cell stage. (I-K) Expression of notochord-specific *Hr-Bra* at the 110-cell stage. Arrowheads in J indicate cells of nerve cord lineage that ectopically expressed *Hr-Bra*. (L-N) Expression of neural plate-specific *Hr-ETR1* in cleavage-arrested 110-cell embryos. Two rows of expression were observed in brain-lineage (arrowheads b) and nerve cord-lineage (arrowheads n) cells. The percentages at the bottom of each photo represent the proportion of embryos that ectopically expressed the Mch-3 antigen and *Hr-Bra*, and that showed reduced expression of *actin* and *ETR1*. Scale bar: 100 μm.

line ectoderm-removed region of another embryo in which FGF9/16/20 had been knocked down (Fig. 3, diagram above panels D-G). Although transplantation of all the descendants of left-side b-line blastomeres still had an excessive effect (Fig. 3D,D′), when part of the descendants was used, it resulted in perfect reversal of the fates (Fig. 3E,E′), as shown on the left side of Fig. 3E″, although the percentage was not so high (approximately 30%). Control embryos, in which control MO and *venus YFP* mRNA were used, never showed such reversal (Fig. 3G,G′). In another control, transplantation of uninjected b4.2 ectoderm cells did not induce expression of the mesenchyme marker in FGF-knockdown embryos, nor did it block muscle fate (Fig. 3F,F′). Finally, as a simple control, embryos with b-line ectoderm cells just removed at the early 32-cell stage differentiated mesenchyme and muscle cells in their normal positions (data not shown). Taken together, these results suggest that the polarity of asymmetric division of muscle/mesenchyme mother cells depends simply on the direction from which the FGF9/16/20 signal is presented. The FGF9/16/20 signal is interpreted equally on the future mesenchyme and muscle sides during the 32-cell stage, suggesting that no intrinsic cues are involved in the process.

Ectopic expression of FGF9/16/20 does not fully induce notochord fate in nerve cord lineage cells

We next tried to reverse the positions of notochord and nerve cord. It has been shown that knockdown of *Hr-FGF9/16/20* in entire embryos results in complete loss of *Hr-Bra* expression and ectopic *Hr-ETR1* expression in the notochord lineage (Kumano et al., 2006). However, knockdown of *Hr-FGF9/16/20* locally only in the descendants of one A4.1 blastomere by injecting the left-side A4.1 with MO at the eight-cell stage resulted in partial loss of *Hr-Bra* expression in the descendant cells (Fig. 4A, black arrowheads). The cells that still expressed *Hr-Bra* were, in most cases, medial notochord precursors (yellow arrowheads) derived from a single A6.2 at the 32-cell stage. It is likely that the medial cell was induced at the 32-cell stage across the midline by the right-side A6.2 blastomere, as notochord blastomeres can induce each other (Nakatani and Nishida, 1994). This is not consistent with our hypothesis that the FGF signal alone determines the polarity, because the left-side A6.2 cell received the FGF signal only from the right side and not from the endodermal side but still underwent asymmetric division with normal polarity. As for the nerve cord marker, only two lateral cells of the notochord lineage that did not express *Hr-Bra* showed ectopic *Hr-ETR1* expression (Fig. 4A′, arrowheads).

Injection of the left-side a4.2 blastomere with *FGF* mRNA at the eight-cell stage resulted in only two lateral cells of nerve cord lineage expressing *Hr-Bra* weakly (Fig. 4B, red arrowheads), and resulted in great reduction in *Hr-ETR1* expression in nerve cord

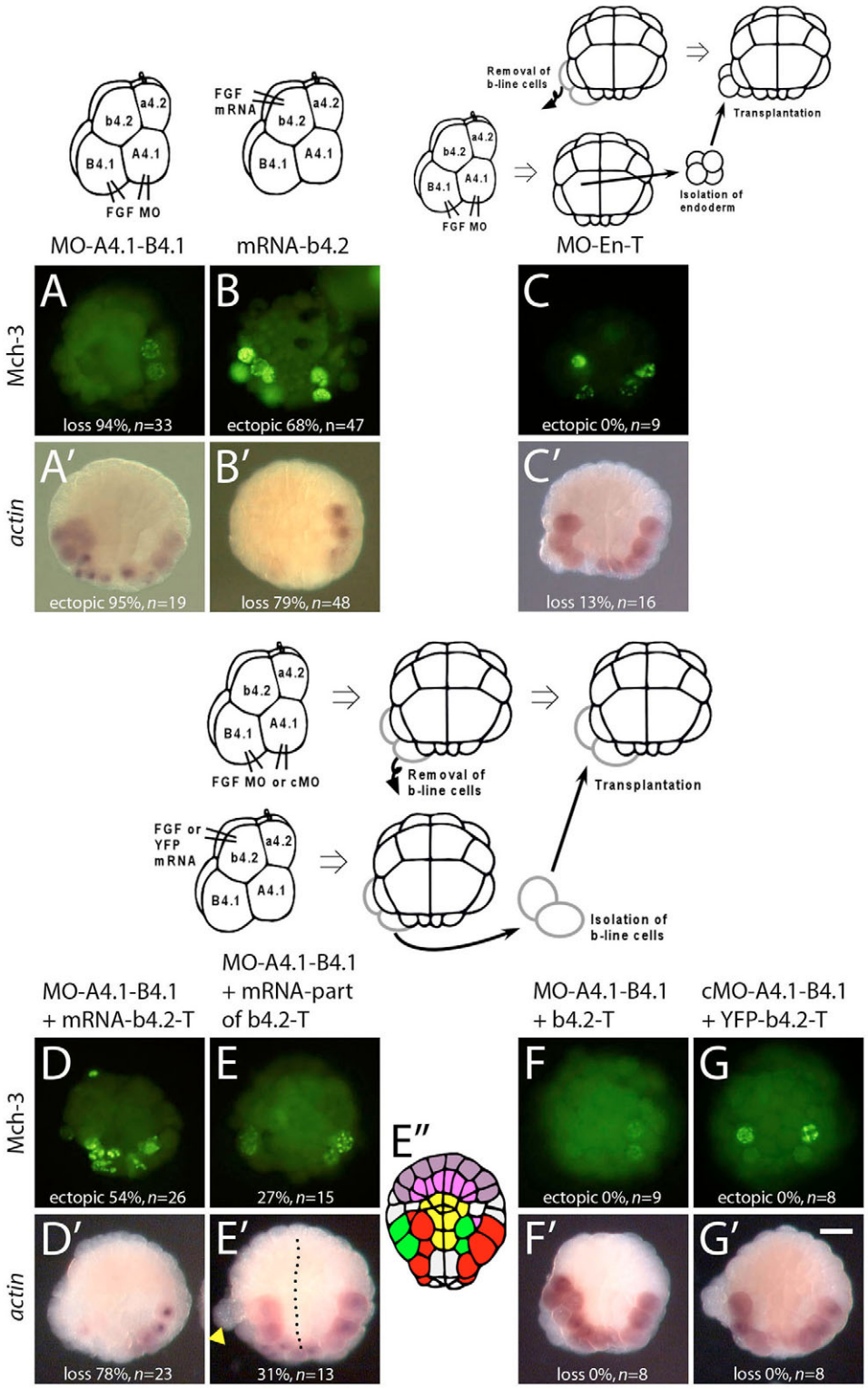

Fig. 3. Reversal of FGF signal direction causes reversal of mesenchyme/muscle asymmetric division. Expression of mesenchyme marker (Mch-3) in cleavage-arrested 110-cell embryos (**A-G**), and of muscle *actin* in 110-cell-stage embryos (**A′-G′**). All photos are vegetal pole views; anterior is up. Only the left side of each embryo was manipulated. Diagrams of the experimental designs are shown at the top of the panels. (A,A′) The A4.1 and B4.1 blastomeres on the left side were injected with *Hr-FGF9/16/20* MO at the eight-cell stage. (B,B′) A b4.2 blastomere was injected with *FGF* mRNA at the eight-cell stage. (C,C′) Endoderm-transplanted embryos. Donor endoderm cells were injected with *FGF* MO (MO-En-T) at the eight-cell stage. (D-G,D′-G′) b-line ectoderm-transplanted embryos. b-line donor cells were injected with either the *FGF* mRNA (D,E, mRNA-b4.2-T) or *venus YFP* mRNA (G, YFP-b4.2-T), and were transplanted. The host embryos had *FGF* MO (D,E, MO-A4.1-B4.1) or control MO (G, cMO-A4.1-B4.1) injected. (E,E′) A subset of the b4.2 descendant cells injected with *FGF* mRNA (arrowhead in E′) were transplanted. In E′, the midline of the embryo is indicated by a broken line. (E″) Schematic representation of the results in E and E′. Normal positions of mesenchyme (green) and muscle (red) fates (right half) and altered positions of the fates on the manipulated side (left half) are shown. (F,F′) b-line cells were transplanted without injection as a control. The percentage in E represents the proportion of embryos in which muscle-lineage but not mesenchyme-lineage cells expressed the Mch-3 antigen. The percentage in E′ indicates the proportion of embryos in which mesenchyme-lineage but not muscle-lineage cells expressed *actin*. Scale bar: 100 μm.

lineage (Fig. 4B′). The expression of *Hr-ETR1* was observed in a broad area in the animal hemisphere, probably because of broad induction of brain fate by overexpressed FGF. Double injection of MO and mRNA in an attempt to reverse the polarity, in which the left-side A4.1 and a4.2 blastomeres were injected with *FGF* MO and mRNA, respectively, at the eight-cell stage did not work again (Fig. 4C,C′) and resulted in the same expression pattern as that observed after single *FGF* mRNA injection. Overexpression of FGF in the animal hemisphere restored *Hr-Bra* expression mostly in notochord precursors without promoting it in the nerve cord lineage in the FGF-less background (compare Fig. 4C with 4A). Embryos injected with control MO and *venus YFP* mRNA showed normal gene expression (Fig. 4D,D′).

We then carried out transplantation procedures as we did for analysis of the muscle/mesenchyme mother cells. Surprisingly, however, transplantation of endoderm cells that had been injected

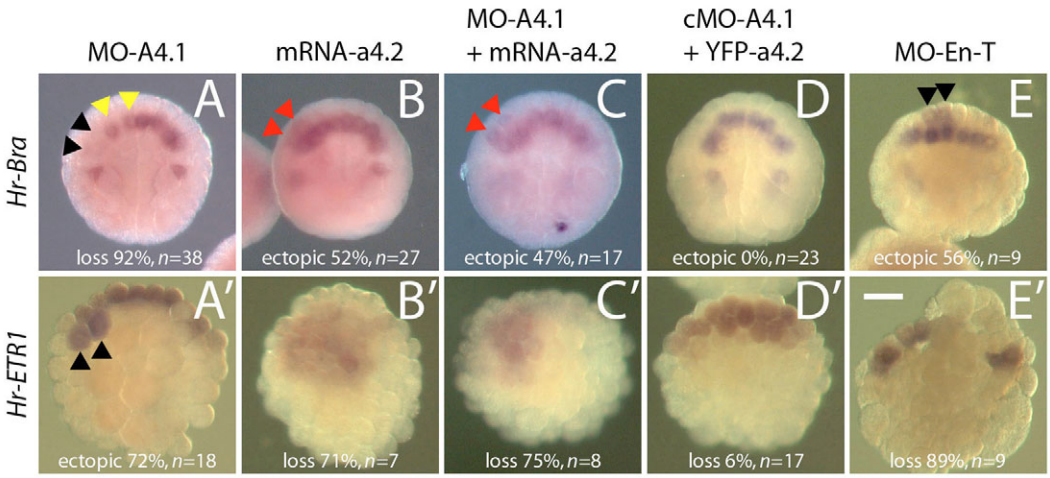

Fig. 4. Robust fate specification in nerve cord/notochord precursors. Expression of a notochord marker (*Hr-Bra*) in 110-cell embryos (**A-E**), and of a nerve cord marker (*Hr-ETR1*) in cleavage-arrested 110-cell embryos (**A′-E′**). Anterior is up. (A-E,A′) Vegetal views. (B′-E′) Animal views. Manipulation was carried out on the left side of embryos with the exception of those in E and E′, where both sides were manipulated. (A,A′) 110-cell embryos developed from eight-cell embryos where A4.1 was injected with *Hr-FGF9/16/20* MO. Black arrowheads indicate notochord blastomeres that did not express *Bra* but expressed *ETR1*. Yellow arrowheads represent notochord blastomeres that weakly expressed *Bra*. (B,B′) a4.2 was injected with *FGF* mRNA. Red arrowheads represent nerve cord blastomeres that weakly expressed *Bra*. (C,C′) A4.1 and a4.2 blastomeres were injected with *FGF* MO and mRNA, respectively. *ETR1* is expressed broadly in the anterior-animal hemisphere in B′ and C′. (D,D′) Control embryos to C and C′ in which cMO (A4.1) and *venus YFP* mRNA (a4.2) were injected. (E,E′) Endoderm-transplanted embryos. Donor endoderm cells were injected with *FGF* MO at the eight-cell stage. Black arrowheads indicate nerve cord blastomeres that ectopically expressed *Bra*. Scale bar: 100 μm.

with the *FGF* MO still induced ectopic *Hr-Bra* expression in cells of nerve cord lineage and suppressed the nerve cord fate (Fig. 4E,E′). The cells that showed ectopic *Hr-Bra* and no *Hr-ETR1* expression were, in most cases, found in the medial position.

Anterior ectoderm cells suppress expression of *Brachyury* in nerve cord blastomeres

To explain the inconsistent results obtained above in the case of nerve cord/notochord mother cells, we speculated that some suppressive influence might emanate from the ectodermal side, and that replacement of ectoderm cells with endoderm cells in the transplantation experiments might remove this suppressive influence. To investigate this possibility, we simply removed the blastomeres of a-line ectoderm from early 32-cell stage embryos. The resulting embryos ectopically expressed *Hr-Bra* in the four medial (two bilateral) cells of nerve cord lineage in 38% of cases (Fig. 5A, arrowheads), and lost *Hr-ETR*1 expression in the same cells (Fig. 5A′). In contrast, later removal of ectoderm cells at the early 64-cell stage (at the 44-cell stage, to be precise) had no effect (Fig. 5B,B′). These results suggest that the a-line ectoderm cells, in

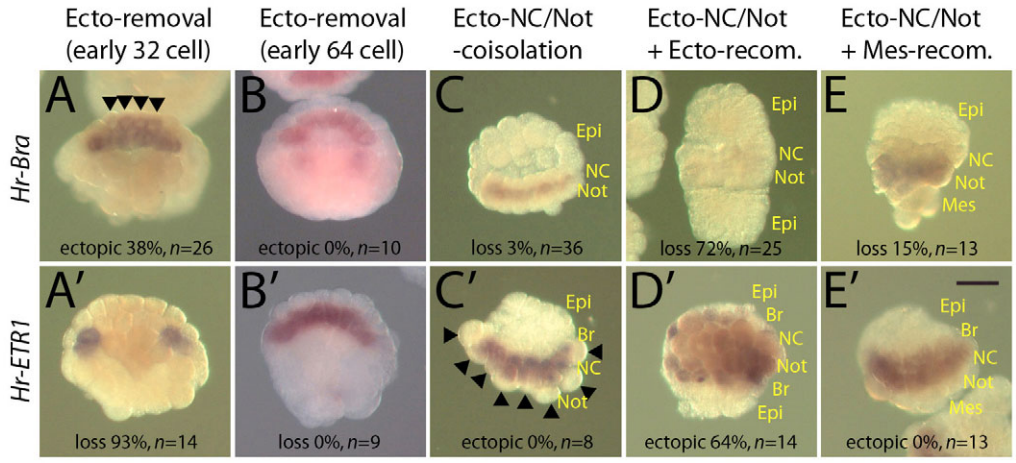

Fig. 5. Suppression of notochord fate by a signal from ectoderm. (**A-B′**) Expression of *Hr-Bra* in 110-cell embryos (A,B; vegetal views) and of *ETR-1* in cleavage-arrested 110-cell embryos (A′,B′; animal views). Anterior is up. (A,A′) Embryos with a-line cells removed at the early 32-cell stage. Arrowheads indicate cells in the nerve cord lineage that ectopically expressed *Bra*. (B,B′) Embryos with a-line cells removed at the early 64-cell stage. (**C-E′**) Expression of *Hr-Bra* and *ETR-1* in partial embryos. (C,C′) Every a-line cell (Ecto) and nerve cord/notochord precursor was co-isolated from early 32-cell embryos and cultured until the 110-cell stage. Arrowheads in C′ indicate cells of notochord lineage. They did not express *ETR1*. (D,D′,E,E′) The same co-isolate as in C was recombined at the notochord side with a-line cells (D,D′) or mesenchyme precursors (E,E′). In D′, four rows of *ETR1*-expressing cells were observed, consisting of two rows of notochord and nerve cord precursors flanked by two rows of brain cells. Br, brain; Epi, epidermis; Mes, mesenchyme; NC, nerve cord; Not, notochord precursors. Scale bar: 100 μm.

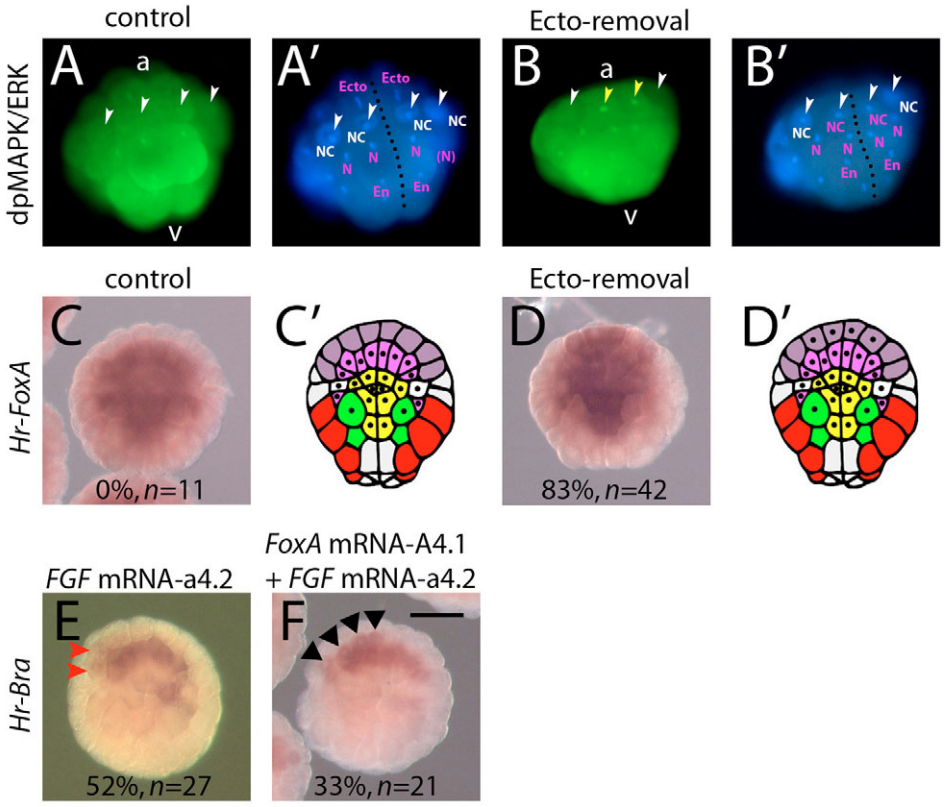

Fig. 6. Suppression of MAPK activation and *FoxA* expression by an ectoderm signal. (**A**,**B**) Activation of MAPK (ERK) in an unmanipulated control 44-cell-stage embryo (A) and a 44-cell-stage embryo with a-line cells removed at the early 32-cell stage (B). Anterior views. Arrowheads indicate nuclei of the four nerve cord lineage cells to be compared. Yellow arrowheads represent ectopic activation of MAPK in the medial nerve cord precursors. a, animal pole; v, vegetal pole. (**A′**,**B′**) Nuclear staining with DAPI to show the position of blastomeres and their nuclei. Midlines are indicated by broken lines. Pink and white letters below nuclei show blastomeres with and without activated MAPK in their nuclei, respectively. (**C-F**) Expression of *Hr-FoxA* (C,D) and *Hr-Bra* (E,F) in 110-cell embryos. Vegetal views. Anterior is up. (C) An unmanipulated 110-cell-stage embryo. (D) An embryo with a-line cells removed at the early 32-cell stage. Note that the uppermost layer of nerve cord cells is ectopically stained. Dots in the diagrams (C′,D′) signify the blastomeres that expressed *FoxA*. (E) A a4.2 blastomere on the left side was injected with *Hr-FGF9/16/20* mRNA at the eight-cell stage. Red arrowheads indicate weak ectopic expression of *Bra*. (F) A4.1 and a4.2 blastomeres on the left side were injected with *FoxA* and *FGF* mRNAs at the eight-cell stage, respectively. Black arrowheads indicate nerve cord lineage cells that ectopically expressed *Bra*. The percentages shown in C-F represent the proportion of embryos that showed ectopic expression of *FoxA* (C,D) and *Bra* (E,F) in cells of nerve cord lineage. Scale bar: 100 μm. Ecto, ectoderm; En, endoderm; N, notochord; NC, nerve cord.

contact with nerve cord precursors, suppress notochord fate and promote nerve cord fate, and that this process is complete by the early 64-cell stage.

To further clarify this suppressive activity, we carried out co-isolation and recombination experiments. A sheet of cells containing rows of the a-line ectoderm and a single row of the four A-line nerve cord/notochord precursors (Ecto-NC/Not) was co-isolated at the early 32-cell stage. The resulting partial embryos showed a single row of *Hr-Bra* expression in the notochord lineage at the equivalent of the 110-cell stage (Fig. 5C) even without endoderm, due to mutual and lateral induction between nerve cord/notochord precursors. *Hr-ETR1* expression was observed in two rows of cells, one of which was nerve cord lineage and the other brain lineage originating from the a-line ectoderm, but never in notochord lineage cells (Fig. 5C′, arrowheads). Thus, the presence of endoderm cells is again not crucial for normal segregation of notochord and nerve cord fates. Next, a-line ectoderm cells were isolated from another embryo and recombined with the Ecto-NC/Not co-isolates, such that the NC/Not precursors were sandwiched between a-line ectoderm. These partial embryos showed reduced *Hr-Bra* expression in the notochord lineage cells (Fig. 5D), and four rows of *Hr-ETR1* expression, corresponding to two of brain and one each of notochord and nerve cord lineages (Fig. 5D′). When mesenchyme precursors (Mes) were recombined with the Ecto-NC/Not co-isolates as a control, the partial embryos showed expression of *Hr-Bra* and *Hr-ETR1* comparable to that in normal embryos (Fig. 5E,E′). These results strongly support the idea that a signal from the a-line ectoderm suppresses notochord fate in nerve cord lineage cells and ensures that they assume a nerve cord fate.

Ectodermal signal antagonizes activation of MAPK and *FoxA* gene expression

In order to identify what might be suppressed by the signal from the ectoderm, we examined MAPK (ERK) activation with an antibody against diphosphorylated MAPK in a-line ectoderm-removed embryos. In normal control 44-cell embryos, activation of MAPK was observed in notochord precursors in all cases (pink letters in Fig. 6A,A′, n=42), whereas it was observed in nerve cord precursors only in 26% of cases (white letters and arrowheads in Fig. 6A,A′), as reported previously (Nishida, 2003). This is consistent with the fact that FGF signaling transduces the signal via MAPK. The diphosphorylated MAPK signal observed in nerve cord, if any, was weaker than that in notochord and was restricted to the two medial precursors in most cases. In contrast, in embryos depleted of a-line ectoderm, MAPK was activated in nerve cord precursors (yellow arrowheads) as well as in notochord precursors in 89% of cases (pink letters in Fig. 6B,B′, n=44, P<0.01). The signal in nerve cord precursors was evident in the two medial cells and weaker in the two lateral cells. These observations indicate that the ectodermal signal antagonizes FGF signaling by suppressing activation of MAPK in nerve cord precursors.

Previous studies have shown that transcription factors, FoxA (formerly HNF3-1) and ZicN (ZicL in *Ciona savignyi*), are essential for induction of *Brachyury* by FGF as intrinsic competence factors in signal-receiving cells (Shimauchi et al., 1997; Wada and Saiga, 2002; Imai et al., 2002b; Imai et al., 2006; Yagi et al., 2003; Kumano et al., 2006). These factors are expressed in notochord/nerve cord precursors at the 32-cell stage. At the 64- and 110-cell stages,

however, *Hr-ZicN* is continuously expressed in both the notochord and nerve cord precursors and required for execution of both fates, while expression of *Hr-FoxA* is downregulated in nerve cord precursors (Kumano et al., 2006). To clarify if the ectoderm signal affects *Hr-FoxA* expression in nerve cord lineage cells, we examined the expression of *Hr-FoxA* in a-line ectoderm-removed embryos. In contrast to control 110-cell embryos, in which *Hr-FoxA* was expressed in precursors of notochord, endoderm and trunk lateral cells (Fig. 6C), embryos without a-line ectoderm cells showed *Hr-FoxA* expression in cells of nerve cord lineage in addition to the precursors mentioned above (Fig. 6D). These results suggest that the ectodermal signal suppresses *Hr-FoxA* expression in the nerve cord lineage. As overexpression of *Hr-FoxA* mRNA did not cause ectopic *Hr-Bra* expression by itself in nerve cord lineage (Kumano et al., 2006), activation of *Hr-Bra* expression by MAPK and its downstream Ets is also likely suppressed by the ectodermal signal.

Injection of *FGF* mRNA into a4.2 merely caused weak ectopic expression of *Hr-Bra* in lateral cells of nerve cord lineage (Fig. 6E, Fig. 4B). Ectopic expression of *Hr-FoxA* in these embryos was similar to that in *Hr-Bra*. Only weak ectopic expression of *Hr-FoxA* was observed in lateral cells of nerve cord lineage (data not shown). These results suggest that such injection could not overcome the inhibitory effect of ectoderm on *Hr-Bra* and *Hr-FoxA* expression. However, when we next injected the left-side A4.1 and a4.2 blastomeres with *Hr-FoxA* and *FGF* mRNAs, respectively, at the eight-cell stage, ectopic expression of *Hr-Bra* was observed in cells of nerve cord lineage at a level as strong as that in notochord precursors in 33% of cases (Fig. 6F, arrowheads). Therefore, increased levels of both FGF signal and *Hr-FoxA* expression are able to overcome and bypass the inhibitory effect from the ectoderm.

DISCUSSION

Our results demonstrated that the asymmetric segregation of mesenchyme and muscle fates in the posterior marginal zone is simply determined by the direction from which the FGF9/16/20 signal is presented. It does not seem to involve pre-localized intrinsic factors or external signals other than FGF. However, the polarity of notochord/nerve precursor cells in the anterior marginal zone is determined by a possible antagonistic action between the FGF signal and a signal from anterior ectoderm. The latter signal suppresses FGF signal transduction in cells of nerve cord lineage, and blocks the expression of *Hr-FoxA*, an essential transcription factor for notochord formation (Fig. 7).

Direction of FGF9/16/20 signal polarizes asymmetric division of mesenchyme/muscle blastomeres

In this study, we successfully reversed the polarity of muscle/mesenchyme precursors by manipulating the position of FGF signal sources (Fig. 7B). Thus, a directed FGF9/16/20 signal from endoderm is the key regulator of the polarity of the asymmetric division that segregates mesenchyme and muscle fates. Our previous work has shown that all the neighboring blastomeres in contact with mesenchyme/muscle precursors at the 32-cell stage, with the exception of endoderm precursors, do not have the ability to induce a mesenchyme fate (Kim and Nishida, 1999). In accordance with this, only endoderm precursors express *FGF9/16/20* at that stage (Fig. 1C). Therefore, mesenchyme/muscle precursors receive the FGF signal only from the endodermal side. The asymmetric divisions of the EMS and T cells in *C. elegans* are known to utilize similar mechanisms, whereby the position from which Wnt

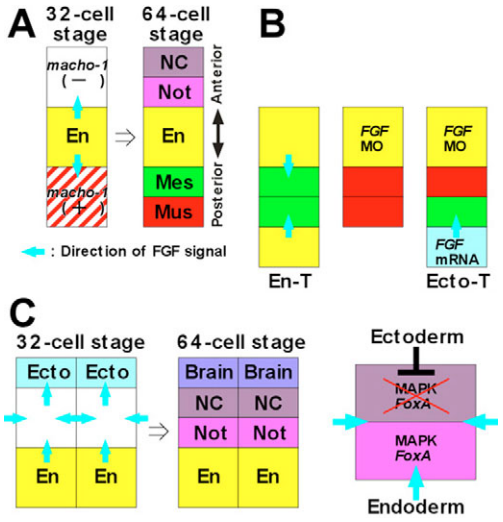

Fig. 7. A model for patterning of the vegetal hemisphere in ascidian embryos. (**A**) Patterning in normal embryos. Cell types are highlighted by the same color code as in Fig. 1. At the division to the 64-cell stage, two kinds of asymmetric division take place in the anterior (NC vs. Not) and posterior (Mes vs. Mus) marginal zones. macho-1 is a maternal and intrinsic competence factor for mesenchyme induction. Light blue arrows indicate FGF signal. (**B**) Direction of asymmetry for segregation of muscle and mesenchyme fates is determined by the direction from which the FGF signal comes. (**C**) Polarity of asymmetric division that produces nerve cord and notochord precursors is determined by the direction from which the inhibitory signal to suppress notochord fate comes. The signal inhibits activation of MAPK by the FGF signal and expression of *FoxA* on the nerve cord side. Presence of the ectodermal signal seems to be more crucial for generation of the medial nerve cord precursors (A7.4 cells in Fig. 1) than that of the lateral precursors (A7.8 cells). See text for details. Ecto, ectoderm; En, endoderm; Mes, mesenchyme; Mus, muscle; NC, nerve cord; Not, notochord.

signaling is presented determines the cell polarities (Goldstein et al., 2006). As ascidians and *C. elegans* have similar modes of asymmetric cell division, both involving fate determination by inductive signals immediately before division, directed-signaling-mediated asymmetric cell division could be a basic mechanism for producing a variety of cell types during animal development, especially when the embryos consist of a small number of cells.

However, the asymmetric cell divisions in ascidians and *C. elegans* differ on two points. First, both daughter cells in ascidian embryos assume induced fate when the mother cells receive the inductive signal bi-directionally from opposite sides. This happens when isolated blastomeres are treated by FGF protein over the entire surface (Nakatani et al., 1996) and when mother cells are sandwiched by the host and transplanted endoderm cells as well as by host endoderm and transplanted FGF-over-expressing cells (present study). In *C. elegans*, in contrast, if EMS is signaled from opposite sides by flanking it with two P_2 blastomeres, only rarely do both daughters adopt the induced E fate; rather, only one daughter adopts an E fate (Goldstein, 1995a). Goldstein has proposed a model whereby a signal from P_2 may cause a segregation of cytoplasmic components, making one daughter differ from the other. Accordingly, induction on one side might interfere with induction on the other side. The second difference is whether the directed signals regulate spindle orientation during the asymmetric divisions. In *C. elegans*, orientation of the mitotic spindle is coordinated with

the polarity of cell fate asymmetry and ensures that different fates are segregated into different daughters (Goldstein, 1995b; Goldstein, 2000). In ascidians, however, blockade of FGF signaling by treatment with a MEK inhibitor or an FGF receptor inhibitor, or by injection of *FGF* MO does not alter the cleavage pattern up to the 64-cell stage (Kim and Nishida, 2001; Kumano et al., 2006) (present study). Thus, it is likely that FGF signaling is not involved in orientation of the mitotic spindles and cleavage planes in ascidian embryos.

Suppression of notochord fate in nerve cord precursors by an ectodermal signal

In contrast to the situation in muscle/mesenchyme mother cells, that in notochord/nerve cord blastomeres was thought to be more complicated because the mother cells are likely to be exposed to the FGF signal not only from the endoderm side but also from every tangential direction (Fig. 7C). The present results also support the idea of the dispensability of the FGF signal from endoderm. Therefore, the embryos must have evolved other ways of accomplishing polarization of notochord/nerve cord blastomeres by additional signals. In the present study, we demonstrated the presence of an additional signal that is secreted from the anterior ectoderm and controls the polarization. This influence from ectoderm completes its role by the early 64-cell stage. The discovery of this signal clarifies the complex situation required for asymmetric division and is still consistent with findings from previous experiments. For example, when an isolated notochord/nerve cord blastomere receives the signal by treatment with basic FGF protein, both daughter cells adopt a notochord fate (Nakatani et al., 1996; Minokawa et al., 2001), probably because of exposure to the FGF signal over the entire cell surface in the absence of the ectodermal signal. Furthermore, in a simplified experimental situation, namely in the absence of the ectodermal signal, notochord/nerve cord blastomeres seem to be polarized solely by the direction from which the FGF signal comes, as observed in mesenchyme/muscle blastomeres. When a single notochord/nerve cord blastomere was co-isolated or recombined with a single endoderm blastomere, only half of the descendants of the notochord/nerve cord blastomere expressed *Hr-Bra* (Nakatani et al., 1996). In this case, the notochord/nerve cord blastomere receives an FGF signal only from the endodermal side but not from the other sides, and is likely to be polarized solely by the FGF signal.

In normal embryos, the medial notochord/nerve cord blastomere (A6.2 in Fig. 1C) is likely to receive more FGF signal than the lateral one (A6.4) at the 32-cell stage when induction occurs because the medial cell is in contact with three FGF-expressing cells, namely another medial cell across the midline, the lateral cell and an endoderm cell, while the lateral cell is only adjacent to two, the medial cell and an endoderm cell. Consistent with this, weak activation of MAPK was occasionally observed in two medial nerve cord precursors at the 44-cell stage. This might be a reason why, in anterior ectoderm-removed embryos, ectopic diphosphorylated MAPK and *Bra* expression was often detected only in the descendants of the two medial nerve cord precursors and *Hr-ETR1* remains expressed in the lateral nerve cord precursors in most cases (Fig. 4E, Fig. 5A,A′, Fig. 6B). Similarly, there appeared to be a difference in the intensity of, or sensitivity to, the ectodermal signal between the medial and lateral sides for some unknown reason, because when we overexpressed FGF in animal blastomeres, only lateral nerve cord cells showed weak *Hr-Bra* expression (Fig. 4B, Fig. 6E). These observations suggest that the presence of ectodermal signal is more crucial for generation of the medial nerve cord precursors than that of the lateral precursors.

Ectodermal signal suppresses activation of MAPK and expression of *FoxA*

The signal from anterior ectoderm suppressed the activation of MAPK in nerve cord lineage cells. The FGF signal activates the Ras-MEK-MAPK-Ets signaling pathway in ascidian embryos (Kim and Nishida, 2001; Miya and Nishida, 2003; Bertrand et al., 2003). Thus, it is plausible that the ectodermal signal antagonizes a step(s) of FGF signal transduction.

Our results suggested that the ectodermal signal also downregulates *FoxA* expression in nerve cord lineage. Hr-FoxA has recently been identified as an intrinsic competence factor for notochord induction (Kumano et al., 2006). When *FoxA* or *FGF* mRNA was solely overexpressed, the nerve cord precursors scarcely expressed *Bra*. In contrast, overexpression of FoxA in the A-line cells and FGF in the a-line cells was able to fully induce an ectopic notochord fate in nerve cord lineage cells (Fig. 6F). Therefore, the ectodermal signal blocks information from both intrinsic and extrinsic cues indispensable for *Bra* expression to ensure the asymmetry of *Bra* expression after asymmetric division.

It remains to be determined whether this suppression of *Hr-FoxA* is achieved through inactivation of MAPK, or of FGF signaling that does not involve MAPK activation, or even an unknown pathway that is independent of FGF signaling. It is noteworthy that expression of *FoxA* starts cell-autonomously at the 32-cell stage, whereas its maintenance at the 64-cell stage depends on FGF signaling (Kumano et al., 2006). Also, it has been proposed that the regulation of *Hr-Bra* expression by FGF signaling involves two distinct pathways: FoxA-dependent and -independent pathways (Kumano et al., 2006). As *Bra* expression was fully induced in the nerve cord lineage only when FoxA was co-expressed in the background in which FGF was overexpressed, the threshold over which FGF signaling is able to activate *Bra* expression in the FoxA-independent pathway might be lower than that for the activation of *FoxA* expression. Likewise, expression of *ETR-1* is more sensitive to FGF signaling than that of *FoxA*, because *ETR-1* expression was eliminated when FGF was overexpressed in a-line ectoderm without *FoxA* expression fully activated in the nerve cord lineage.

In conclusion, the polarities of the two kinds of asymmetric cell division in ascidian embryos are determined by extracellular cues from neighboring cells. In mesenchyme/muscle precursors, the polarity is simply specified by the direction from which the FGF signal is presented. In notochord/nerve cord precursors, the mechanism is more complicated, as the polarity depends on a yet-unknown signal from ectoderm that antagonizes the FGF signal. The presence of this additional signal in the asymmetric division of nerve cord/notochord mother cells reflects the fact that the notochord/nerve cord precursors need to secrete FGF in order to induce brain in the adjacent ectoderm cells.

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