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Early specification of ascidian larval motor neurons

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Abstract

In the tadpole larvae of the ascidian *Halocynthia roretzi*, six motor neurons, Moto-A, -B, and -C (a pair of each), are localized proximal to the caudal neural tube and show distinct morphology and innervation patterns. To gain insights into early mechanisms underlying differentiation of individual motor neurons, we have isolated an ascidian homologue of *Islet*, a LIM type homeobox gene. Earliest expression of *Islet* was detected in a pair of bilateral blastomeres on the dorsal edge of the late gastrula. At the neurula stage, this expression began to disappear and more posterior cells started to express *Islet*. Compared to expression of a series of motor neuron genes, it was confirmed that early *Islet*-positive blastomeres are the common precursors of Moto-A and -B, and late *Islet*-positive cells in the posterior neural tube are the precursors of Moto-C. Overexpression of *Islet* induced ectopic expression of motor neuron markers, suggesting that *Islet* is capable of regulating motor neuron differentiation. Since early expression of *Islet* colocalizes with that of *HrBMPb*, the ascidian homologue of *BMP2/4*, we tested a role of BMP in specification of the motor neuron fate. Overexpression of *HrBMPb* led to expansion of *Lim* and *Islet* expression toward the central area of the neural plate, and microinjection of mRNA coding for a dominant-negative BMP receptor weakened the expression of these genes. Our results suggest that determination of the ascidian motor neuron fate takes place at late gastrula stage and local BMP signaling may play a role in this step.

Keywords: Ascidian; Motor neuron; LIM; BMP; Ion channel genes

Introduction

During animal embryogenesis, development of neuronal characteristics, such as morphology, axonal projection, membrane excitability, and neurotransmitter identity, depends on the timing and localization at which neurons arise. Among a variety of types of neurons, motor neurons are best documented for their function, morphology, and development both in vertebrates and invertebrates.

In the vertebrate spinal cord, the area for motor neuron generation is defined in the context of dorsoventral (D–V) patterning of the neural tube (reviewed in Tanabe and Jessell, 1996; Sasai and DeRobertis, 1997). This patterning is established by proteins secreted from overlaying ectoderm and the ventrally located notochord. Motor neurons are induced by a ventralizing factor Sonic Hedgehog (Shh), which is expressed in the notochord and subsequently in the floor plate of the neural tube (Roelink et al., 1994). A generation of dorsal phenotype of the neural tube involves other secretory factors, such as BMP (Delot et al., 1999; Leim et al., 1995, Neave et al., 1997). Vertebrate motor neuron precursors can be identified by the expression of *Islet*, a member of LIM type homeobox gene family

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(Ericson et al., 1992; Karlsson et al., 1990). *Islet-1* knockout mice fail to differentiate motor neurons, establishing that *Islet-1* is essential for vertebrate motor neuron differentiation (Pfaff et al., 1996). Several LIM type homeobox genes are expressed in the motor neurons and the combinatorial gene expression pattern of this family subdivides motor neurons into groups with different characteristics (Tokumoto et al., 1995; Tsuchida et al., 1994). The critical roles of LIM type homeobox genes in motor neuron differentiation have also been reported in *Drosophila* (Thor and Thomas, 1997; Thor et al., 1999).

The ascidian is a primitive chordate that belongs to a sister group of vertebrates. Anterior–posterior (A–P) patterning of the neural tube through a series of homeobox genes (Katsuyama et al., 1996, 1999) and the neural induction (Okado and Takahashi, 1988, 1993; Nishida, 1991; Inazawa et al., 1998) are both evolutionarily conserved features of ascidians when compared to vertebrates (Meinertzhagen et al., 2004). The CNS of swimming larvae of the ascidian consists of only several hundreds of cells, containing approximately 100 neurons (Nicol and Meinertzhagen, 1991). Despite of this structural and embryological simplicity of the ascidian larval CNS, the exact locations and cell lineage of individual motor neurons have not been defined until recently.

Our observations using lineage tracer and gene expression revealed that larval motor neurons are confined to a specific region of the neural tube that is at the proximal tail region (also called neck region; Okada et al., 2002). Three pairs of descendants of A5.2 blastomeres of the 16-cell stage embryo express neural markers in the proximal tail region of the larval CNS (Katsuyama et al., 2002) and all of these cells extend their axon to the muscle cells (Okada et al., 1997, 2002), indicating that all A5.2-derived neurons are motor neurons. On the other hand, A5.1 blastomeres give rise to putative interneurons, but not motor neurons. Unlike vertebrate spinal cord that contains motor neurons throughout its A–P axis, motor neurons in ascidian embryos are restricted to a part of the neural tube.

Location, morphology, and late development of individual motor neurons were investigated using two gene markers (Okada et al., 2002). One gene marker was *TuNa2*, which putatively encodes for a voltage-gated sodium channel (Okamura et al., 1994) and exhibits motor neuron-specific expression during embryogenesis (Nagahora et al., 2000; Okada et al., 2002). *TuNa2* expression was first detected as two pairs of spots in the lateral cells of the neural tube in the tailbud embryos (Nagahora et al., 2000; Fig. 2B). The anterior spot further divides into two spots during tailbud stage, resulting in three pairs of motor neurons that line along the A–P axis of the neural tube (Okada et al., 2002). The second gene marker was *Hrlim* (or *Lim*), an ascidian LIM type homeobox gene. This gene is expressed both in the motor neuron lineage and in the trunk region of the developing CNS (Okada et al., 2002; Wada et al., 1995; Fig. 2C). Individual motor neurons of *Halocynthia* larvae are designated to Moto-A, -B, and -C, and each has unique characteristics (Okada et al., 2002). Moto-A is most anteriorly located and its axon traverses dorsally toward the posterior end of the tail. Moto-B is the medial pair projecting their axon ventrally and extending it along the ventral muscle cells. It makes synapse only to a proximal region of the muscle bundle in the larval tail. Moto-C, the most posteriorly located pair, has an elongated cell body that projects its axon along the dorsal muscle band. However, it remains unknown how three pairs of motor neurons are specified at specific regions of the larval neural tube during embryogenesis.

Here, we isolated an ascidian homologue of *Islet* and examined regulation of its expression. Overexpression of *Islet* induced ectopic expression of motor neuron markers, which suggests that *Islet* regulates motor neuron differentiation. Comparison of the expression pattern of *Islet* with those of other genes suggests that *Islet* is expressed in all the precursors of three types of motor neurons. Overexpression experiments of *HrBMPb* and dominant-negative BMP receptor raise a possibility that expression of *Islet* and *Lim* in motor neuron precursors in the neural plate depends on BMP signaling.

Materials and methods

Cloning of ascidian Islet cDNA

The cDNA was reverse transcribed from polyA RNA of larvae of the ascidian *Halocynthia roretzi* using a random hexamer. A partial homeobox fragment was amplified from the cDNA. Specific primers used were AAYGARAARCARYTNCAYAC and TTCRCANCKYTTRTTYGRAACCA. A band of expected size was subcloned, sequenced, and found to be of a partial fragment of *Islet* cognate. This PCR fragment was used as a probe to screen *Halocynthia* larva cDNA library and four independent clones were isolated. All cDNA clones were sequenced completely. Two of the clones encoded for full-length Islet protein along with a partial stretch of polyA tail (accession number AB044142). These two clones showed almost identical sequence with only a few SNPs that had no effect on the amino acid sequence (data not shown). One clone lacked a 5′ side sequence along with polyA. Another had an insert of 23 bases between the nucleotide sequences coding for LIM domain and homeodomain, which might be an intron of the immature mRNA.

mRNA detection

Northern and in situ hybridizations were carried out as previously described (Katsuyama et al., 1995; Wada et al., 1995). The *Islet* probe for in situ hybridization was transcribed from ISL3a cDNA clone (Fig. 1; sequence accession number AB044142). Other probes for in situ hybridization were the same as those used previously (Katsuyama et al., 1999; Miya and Satoh, 1997; Okada et al., 2002).

Fig. 1. Comparison of amino acid sequence of Islet family homeoproteins. Ascidian Islet protein (accession # AB044142) is shown as a consensus sequence, which is compared to the Islet proteins of other animals. Islet-1 (chick), Islet-2 (zebrafish), and Islet-3 (zebrafish) are representing vertebrate proteins. Fly (*Drosophila*) and CeLIM-7 (nematode; Islet-related) are invertebrate proteins. Dashes indicate identical residues. Shaded sequences are LIM1 domain, LIM2 domain, and homeodomain from N-terminal side to C-terminal side.

Construction of Islet plasmids

The protein coding region of *Islet* cDNA was amplified using the following primers, GCTAGATCTCGCCAC-CATGGGCGATCAGAGCCAGAAC and GGTGCGG-CCGCTACAAGTCTTCTTCAGAAATAAGCTTTTG-TTCGTAGTTATCGTTCACACG. The PCR product was then digested with *Bgl*II and *Not*I and ligated into an RN3 plasmid (Lemaire et al., 1995) to make RN3-ISL. The synaptotagmin promoter-ISL plasmids were constructed by replacing the *Lim* sequence in Synaptotagmin promoter-Lim-GFP plasmid (Okada et al., 2002) with the *Islet* sequence of RN3-ISL, both of which were cut at *Bgl*II and *Hin*dIII sites.

Microinjection into ascidian embryos

pSP64T-PKI, pSP64T-CQR, and pSP-Zshh (Hammerschmidt et al., 1996) were linearized with *Bam*HI, *Xba*I, and *Bam*HI, respectively. The ascidian BMP plasmids were used as in the previous study (Miya et al., 1997). mRNAs were synthesized using mMESSAGE mMACHINE kit (Ambion), except for the tBRII mRNA (Frisch and Wright, 1998), which was synthesized using RiboMAX RNA Production System (Promega). Microinjection of mRNA into ascidian embryos was carried out as previously described (Katsuyama et al., 1999).

Results

Ascidian Islet gene is expressed in motor neuron lineage

We have previously shown that the ascidian larva contains only three pairs of motor neurons (Moto-A, -B, and -C, a pair of each) in the trunk-tail border region of the neural tube (Okada et al., 2002). To gain more detailed information about the early development of ascidian larval motor neurons, we isolated an ascidian homologue of *Islet*, which is known to determine the cell fate of the motor neuron in other animals (Pfaff et al., 1996; Thor and Thomas, 1997). The *Islet* cDNA clones were obtained through RT-PCR and library screening. The clone contained an insert of 3083 base pairs, which coded for a protein of 432 amino acids in length. The putative protein products of *Islet* display a high degree of identity to the amino acid sequence of the two zinc finger regions called LIM domain, homeodomain, and their vicinities (Fig. 1). Northern hybridization analysis detected a band at approximately 3.0 kb, which was consistent with the length of the cDNA

clones. The band was detected after the gastrula stage, whereas only a faint band was observed at earlier stage and in unfertilized egg (data not shown).

To find out whether *Islet* is expressed in motor neurons, *Islet* expression pattern in young tadpoles was visualized by whole mount in situ hybridization and compared to that of other genes that were reported to be expressed in motor neuron precursors at this stage. *Caudal* is expressed in the lateral wall of the spinal cord region (Katsuyama et al., 1999; Fig. 2E). The expression of all the genes examined here was confined within the bilateral lines of *Caudal* expression, which confirmed that the genes were expressed in the lateral walls of the neural tube. The expression of *Islet* was observed as a pair of spots in the proximal region of the tail neural tube (Figs. 2D and F, indicated by arrows). The expression of a putative sodium channel gene *TuNa2* is specific to motor neurons in the young tadpoles (Nagahora et al., 2000; Okada et al., 2002; Fig. 2B). The expression of *Islet* overlapped with the posterior spots of *TuNa2*, indicating that *Islet* is expressed in some motor neurons, but more posterior level to the *Lim*-positive spots (compare Figs. 2C and D). *Lim*-positive spots at this stage were reported to be comprised of two cells that correspond to Moto-A and Moto-B (Okada et al., 2002), therefore *Islet* is expressed in Moto-C, but not in Moto-A and Moto-B. This comparison also indicates that cells expressing *TuNa1*, another sodium channel gene of the ascidian, in this region of the neural tube are Moto-A and Moto-B (Fig. 2A).

Islet expression was also detected in cells other than motor neuron precursors, which include mesoderm, anterior neural tube, tail sensory neurons, and sensory neurons in the anterior papillae. However, we did not examine these expressions further in this paper. The overall expression pattern of *Islet* in *Halocynthia* embryos was consistent with a previous observation made in *Ciona intestinalis* (Giuliano et al., 1998).

Islet and Lim exhibit distinct expression pattern

We described the expression pattern of *Lim* in detail from tailbud stage until larval stage in the previous report (Okada et al., 2002). Here, the expression of *Islet* and *Lim* at earlier stages was examined by whole mount in situ hybridization. Distinct signals of *Islet* expression were observed from the late gastrula stage in a pair of blastomeres on the dorsal edge of blastopore (Fig. 3A, green arrow). *Lim* exhibits a similar expression pattern at this stage (Fig. 3F, arrows; Wada et al., 1995). RNA probes of *Islet* and *Lim* were simultaneously hybridized to determine the spatial relationship of expression of the two genes (Figs. 3K, L, M, and N). Only a single pair of spots was observed at the late gastrula stage when stained with the mixture of the two probes (Fig. 3K), indicating that *Islet* and *Lim* were coexpressed in the same blastomeres. The coexpression of *Islet* and *Lim* continued up to the early neurula stage (Figs. 3B and G).

At the neurula stage, a pair of new spots in the more posterior region started to express both *Islet* and *Lim* (Figs. 3C and H, red arrows). *Islet* expression in the anterior region (indicated by a green arrow in Fig. 3C), which attribute to the earliest expression (Fig. 3A), gradually decreased (Figs. 3B and C) and became undetectable after neurula stage (Fig. 3D). *Lim* expression in the same lineage persisted during embryogenesis (Figs. 3F–J, green arrow). Posterior expressions of *Islet* and *Lim* were enhanced during neurula stages (Figs. 3D and I). Continuation of *Islet* and *Lim* expression in each cell lineage was confirmed by observing gene expression in embryos collected every 2 h from the gastrula to the late tailbud stage.

Our previous study showed that posterior spots of *Lim* expression in tailbud stage embryos are Moto-C precursors (Okada et al., 2002; Fig. 3J, red arrow). *Islet* expression in the spinal cord was detected in a similar area to that of posterior expression of *Lim* (Fig. 3E). Simultaneous hybridization of *Lim* and *Islet* probes confirmed that *Islet* is

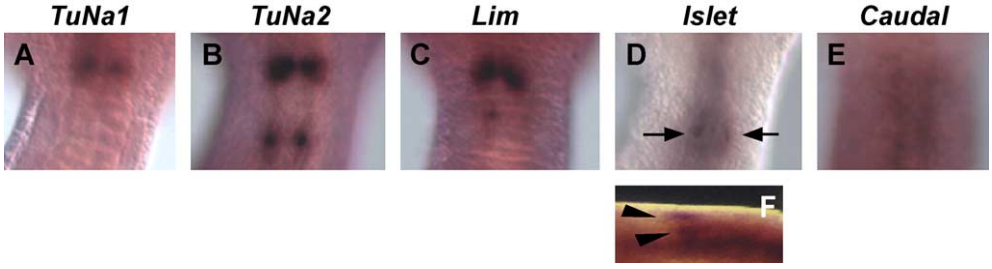

Fig. 2. Gene expression in the motor neurons of young ascidian tadpoles detected by whole mount in situ hybridization. Gene expression is observed in the motor neuron differentiating region, which is located around the border of the trunk and tail region of the tadpole body. (A) Expression pattern of *TuNa1*, an ascidian homologue of type II sodium channel alpha subunit. (B) Expression pattern of *TuNa2*, an atypical sodium channel like gene that exhibits motor neuron-specific expression (Nagahora et al., 2000; Okada et al., 2002). (C) *Hrlim* (*Lim*) is expressed as a pair of spot. Okada et al. (2002) have shown that two pairs of cells are contained in these spots. Expression in the motor neuron-generating region is identical to that of *TuNa1* at this stage. (D) Expression pattern of *Islet*. The notochord expresses high level of *Islet* along the midline of the embryo. Overlapping to this expression, bilateral signals (indicated by arrowheads) showed expression in the motor neuron precursors. (E) Expression of *Caudal*, a lateral wall marker of the posterior neural tube. (F) A lateral view of a specimen hybridized to *Islet* probe shows *Islet* expression clearly (indicated by arrowheads) in the motor neurons above the notochord, which is also positive for *Islet* expression.

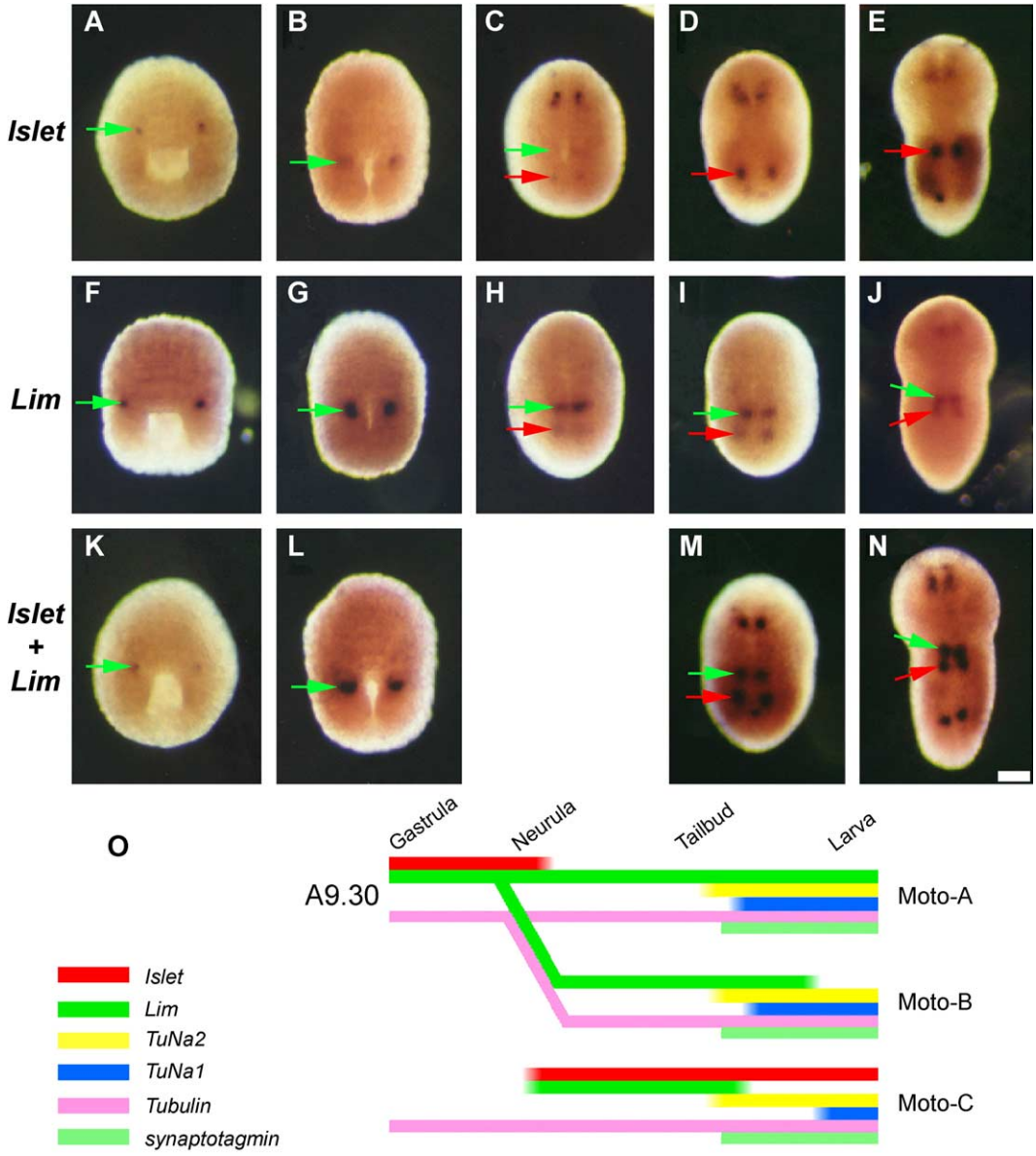

Fig. 3. Expression pattern of *Islet* (A–E) and *Lim* (F–J) in the motor neuron lineages. Anterior pole is toward the top of the photographs. Either vegetal view of late gastrula (or neural plate stage) embryos (A, F, and K) and dorsal view of the early (B, G, and L), the mid (C and H), and the late (D, I, and M) neurula stage embryos and tailbud stage embryos (E, J, and N) are shown. (K–N) Expression of *Islet* and *Lim* was detected simultaneously by hybridizing with both probes, showing coexpression of these two genes in the early stage of motor neuron lineages. Green arrow indicates Moto-A/B precursor and red arrow indicates Moto-C precursor (see the text). Scale bar indicates 50 μm. (O) The temporal expression pattern of *Islet* and *Lim* is summarized comparing to other neural genes; *TuNa1* (Okada et al., 2002; Okada unpublished data; Okamura et al., 1994), *TuNa2* (Nagahora et al., 2000), *tubulin* (Miya and Satoh, 1997; Y.K., unpublished data), and *synaptotagmin* (Katsuyama et al., 2002). Pan-neuronal genes (*Tubulin* and *synaptotagmin*) exhibit the same time course of expression in three motor neuron lineages, whereas LIM type homeobox genes have the expression pattern different from each other. Expression of LIM genes predates that of ion channel genes in the motor neuron lineages.

actually expressed in Moto-C precursors from beginning of the expression. Anterior *Lim*-positive spots in tailbud embryos (green arrow in Fig. 3J) consist of two cells, the precursors of Moto-A and Moto-B (Okada et al., 2002). Taken together, *Islet* is expressed in the precursors of all three types of motor neurons from early stage in ascidian embryos, suggesting its role in regulating motor neuron differentiation. The expression patterns of *Islet*, *Lim*, and other neural genes from the late gastrula stage are summarized in Fig. 3O with results of the previous papers (Okada et al., 2002; Katsuyama et al., 2002).

At the tailbud stage, *Islet* was also expressed transiently as bilateral pairs posterior to Moto-C (Fig. 3E, posteriormost spots in the tail that are not indicated by arrows). Since our previous studies revealed that neurons with axonal projection to muscles are confined to the proximal tail region, it is unlikely that cells of posteriormost expression of *Islet* are motor neurons and are probably tail sensory neurons.

In examination of *Lim* and *Islet* expressions during embryogenesis, we noted that the position of Moto-C precursors varies between embryos (Fig. 4). In some tailbud embryos, a posterior spot of *Lim* expression (Moto-C) was located just adjacent to the anterior spot (Figs. 3J and N). However, in other tailbud embryos, there is a space for a single cell between the anterior and posterior spots (Figs. 4F, G, and H). Similarly, the position of cells expressing *Islet* varied in each tailbud embryo (Figs. 4C and D). The variability of *Islet* expression in motor neuron precursors was evident referring to the position of the anterior end of the mesoderm that also expresses *Islet* (Figs. 4A–D). A simultaneous hybridization of *Islet* and *Lim* probes did not show any other signals besides those in tailbud stages (Fig. 4H) in all in situ specimens, which confirmed that all Moto-C precursors express both *Islet* and *Lim*. In some embryos, the *Islet* expression or posterior *Lim* expression was detected at different levels between left and right sides of the neural tube (Figs. 3C, D, E, F, and G). The expression of *TuNa2* in tailbud embryos showed a pattern similar to *Islet* and *Lim* expression (data not shown). These make contrast with the position of Moto-A/B precursors that were detected by expression of *Lim*, *TuNa1*, and *TuNa2* at a constant level along the A–P axis of the neural tube. In all in situ specimens, Moto-A/B precursors were observed lateral to the anterior edge of blastopore in neural plate stage embryos (Figs. 3A, B, F, G, K, and L) at the same level to the posterior end of the closing blastopore in the neurulae (Figs. 3H, I, and M) and at the anterior end of the mesoderm in tailbud stage embryos (Fig. 3N). These observations raise a possibility that one of two cells lining anteroposteriorly in the posterior neural tube differentiates into Moto-C. By referring to the cell lineage map of *C. intestinalis* by Nicol and Meinertzhagen (1988), it is likely that these potent

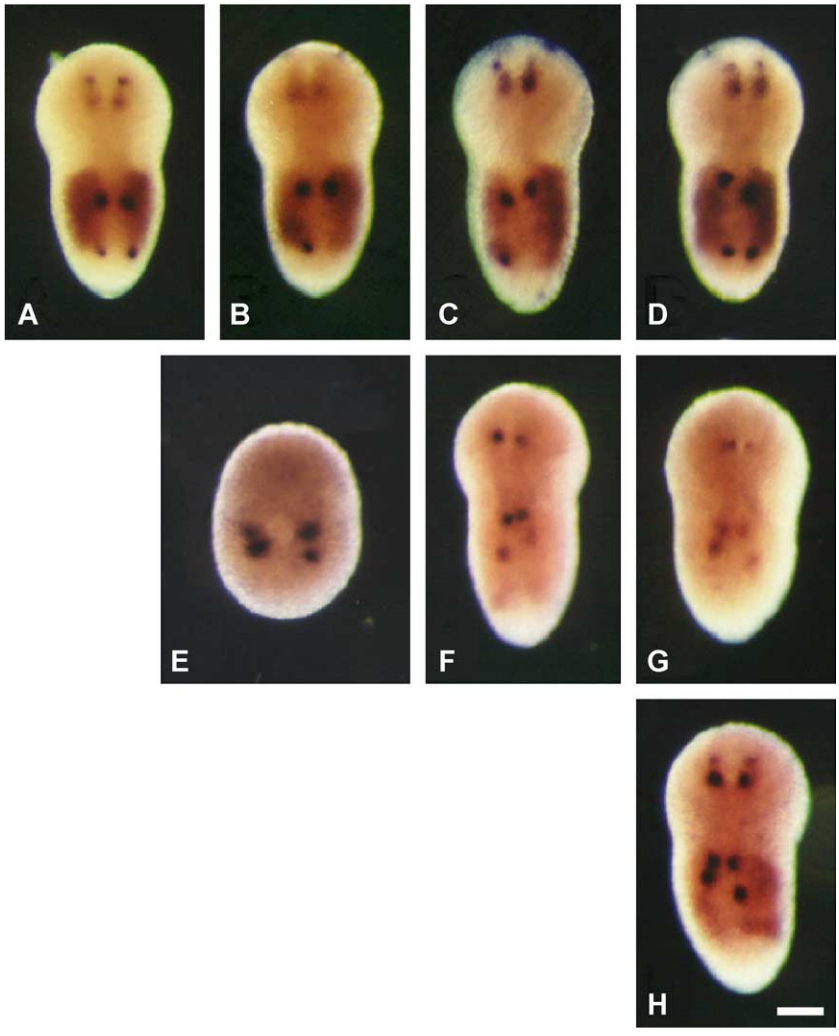

Fig. 4. Position of Moto-C precursor marked by *Islet* and *Lim* expression. Variation of expression pattern of LIM type homeobox genes in tailbud stage embryos. Red arrowheads indicate Moto-C precursors marked by *Islet* (A–D) and *Lim* (E–G). Green arrowheads indicate Moto-A/B precursors. (E) Cells at different positions between left and right sides of the early neurula become Moto-C as shown by *Lim* expression. Fig. 3H shows the expression pattern of *Lim* different from this at a similar stage, where spots on the left and right sides locate at the same level along the A–P axis. (F and G) Alternative expression of *Lim* between the left and right sides of tailbud stage embryos. (H) A simultaneous hybridization to *Islet* and *Lim* probes confirms that *Islet* and *Lim* are expressed in the same Moto-C precursor.

Moto-C cells are daughters of A9.29, namely A10.57 and A10.58.

Islet overexpression induced ectopic expression of motor neuron markers

To examine the role of *Islet* in motor neuron differentiation, we microinjected synthetic *Islet* mRNA into the ascidian embryos. However, the cleavage pattern was disrupted and the development of injected embryos was arrested at an early stage of gastrulation (data not shown). Such a nonspecific abnormality caused by *Islet* mRNA injection was reported in zebrafish (Kikuchi et al., 1997). Because endogenous *Islet* expression begins at the gastrula stage, we expected that forced expression of *Islet* from a later stage by a stage-specific and cell-type-specific gene promoter may avoid this problem. For this purpose, we utilized a synaptotagmin 3.6-kb promoter fragment, which drives a gene expression in epidermal and neural cells in similar fashion to endogenous *synaptotagmin* in ascidian embryos (Katsuyama et al., 2002; Ono et al., 1999). The *synaptotagmin* promoter was ligated with Islet-GFP fusion sequence and the resulting plasmid was injected into fertilized eggs. The expression of exogenous *Islet* protein was visualized by the fluorescent signal of tagged GFP in epidermis from the neurula stage and in the neural tube from the tailbud stage. As reported in other promoter analyses that used ascidian embryos, the injected DNA was mosaically integrated, thus the expression pattern and level of GFP signals were different among embryos. Nevertheless, all injected embryos developed into larvae with normal morphology (Fig. 5). In the normal and uninjected larvae, strong expression of *TuNa2*, which indicates motor neurons, was observed only in the region around the boundary of the tail and trunk parts, accompanying weak expression in the brain and the sensory neurons at the anterior top of the epidermis (Nagahora et al., 2000; Fig. 5C). The *Islet*-overexpressing larvae (n = 10/12) expressed *TuNa2* in ectopic regions, such as the CNS in the trunk (Fig. 5A) and epidermis (Fig. 5B). Although ectopic expression of *Lim* was not observed at the larval stage (Fig. 5D; n = 4), we did see ectopic expression in tailbud stage embryos (Fig. 5E; n = 7/9). This result suggests that ectopic expression of *Lim* induced by *Islet* overexpression is transient in nature. The expression patterns of the pan-neural markers *synaptotagmin* (Katsuyama et al., 2002) and *TuNa1* (Okamura et al., 1994) were not affected by *Islet* overexpression (data not shown). As a control experiment, GFP alone was overexpressed under the control of *synaptotagmin* promoter. In these embryos, there was no change in the levels of expression in any of the neural markers we examined.

In another experiment, both the full-length form and the dominant-negative form of *Islet*, from which homeobox sequence was deleted (Kikuchi et al., 1997), were overexpressed simultaneously in the embryos. In those embryos, ectopic expression of *TuNa2* was not observed (n = 6/6).

Fig. 5. Overexpression of *Islet* induces ectopic expression of motor neuron markers. Expression pattern of *TuNa2* (A–C) and *Lim* (D and E). *Islet* was overexpressed in ectodermal cells utilizing a *synaptotagmin* promoter (Katsuyama et al., 2002) from the neurula stage (A, B, D, and E). As a control, GFP was expressed in ascidian embryos by the promoter (C). (A) Dense staining of ectopic *TuNa2* expression was observed throughout the brain of an *Islet*-overexpressing larva. (B) Ectopic expression of *TuNa2* was detected in the dorsal side of an *Islet*-overexpressing larva. (C) Overexpression of GFP did not alter the expression pattern of a motor neuron marker *TuNa2*. Normally, *TuNa2* is expressed in anterior top where adhesive papillae forms, weakly in the small number of cells located posterior to the otolith (middle part of the trunk), and the motor neurons (the region around the junction of the tail and trunk) of the hatched larvae. *Islet* overexpression did not alter expression pattern of a pan-neural gene *synaptotagmin* (data not shown). (D) *Islet* overexpression did not change the expression pattern of *Lim*, when gene expression was analyzed at swimming larva stage. Compare this picture to Fig. 6A of Wada et al. (1995). (E) Ectopic expression of *Lim* induced by *Islet* overexpression was observed in tailbud stage embryos. As shown in Figs. 2, 3, and 4, the epidermis never expresses *Lim* normally. Scale bar indicates 50 μm.

This indicates that ectopic expression of *TuNa2* induced by *Islet* overexpression is mediated by the role of *Islet* as the transcription factor.

Involvement of BMP signaling in expression of motor neuron markers

In vertebrate embryos, the ventral phenotypes, such as expression of motor neuron markers, are induced by Shh. To test whether Shh signaling is involved in the specification of motor neurons, Shh was overexpressed in *Halocynthia* embryos. The overexpression had no effect on the expression pattern of *Islet*, *Lim*, and *TuNa2* (data not shown), although morphology of larvae was slightly abnormalized when very high dose mRNA was injected. In vertebrates and *Drosophila*, Hedgehog signaling is transduced by suppression of PKA activity and thereby affects gene expression

(reviewed in Ingham and McMahon, 2001). Consistent with the result of Shh overexpression experiment, neither PKI (a dominant negative PKA) nor forskolin induced ectopic *Islet* expression in ascidian embryos at gastrula, neurula, and tailbud stages. In addition, overexpression of constitutively active PKA did not block expression of *Lim* and *Islet*, whereas it affected cell cleavage pattern and gastrulation (data not shown).

Since *Islet*-positive Moto-A/B precursors in the gastrula are descendants of a blastomere that expresses *HrBMPb* (the ascidian homologue of BMP2/4) at the 110-cell stage (Miya et al., 1997), we tested the possible role of BMP signaling in specification of the motor neuron fate. *HrBMPb* mRNA was microinjected into fertilized eggs and the expression pattern of motor neuron markers *Lim* and *Islet* was examined at the gastrula and neurula stages (Fig. 6). At these stages, the BMP-overexpressing embryos were morphologically indistinguishable from uninjected normal embryos. At the gastrula stage, the expression of *Islet* and *Lim* was observed in all cells that lined along the anterior edge of the blastopore in the neural plate region in BMP-overexpressing embryos (Figs. 6B and F). Ectopic expression of *Lim* induced by BMP overexpression seems to be in the region corresponding to A9.14, A9.16, or their next descendants after one cell division. While nuclear staining was strong for *Lim* expression, in situ signals of *Islet* were weak and distributed in the cytoplasm at this stage. The difference of intensity of in situ signal is consistent to the normal expression of *Islet* and *Lim* at this stage (Fig. 6A). BMP overexpression suppressed *Islet* expression in the presumptive brain region of the neurulae and enhanced *Islet* expression in the presumptive spinal cord region (Figs. 6C and D, arrowheads). Inhibition of anterior *Islet* expression is consistent with the reduction of gene expression in the anterior neural tube in BMP-overexpressing embryos, which was reported by Miya et al. (1997). BMP overexpression significantly suppressed *Lim* expression at the neurula stage (Fig. 6H). These results suggest that BMP upregulates both *Lim* and *Islet* in gastrula embryos, but only *Islet* in neurula embryos.

Because *TuNa2* expression in tailbud stage embryos is specific to motor neurons, we examined the effects of BMP overexpression on *TuNa2*, as well as other neural markers, at the tailbud stage. Consistent with the previous study (Miya et al., 1997), BMP overexpression caused agenesis of anterior neural structures such as anterior adhesive papillae, otolith, and ocellus (data not shown). At

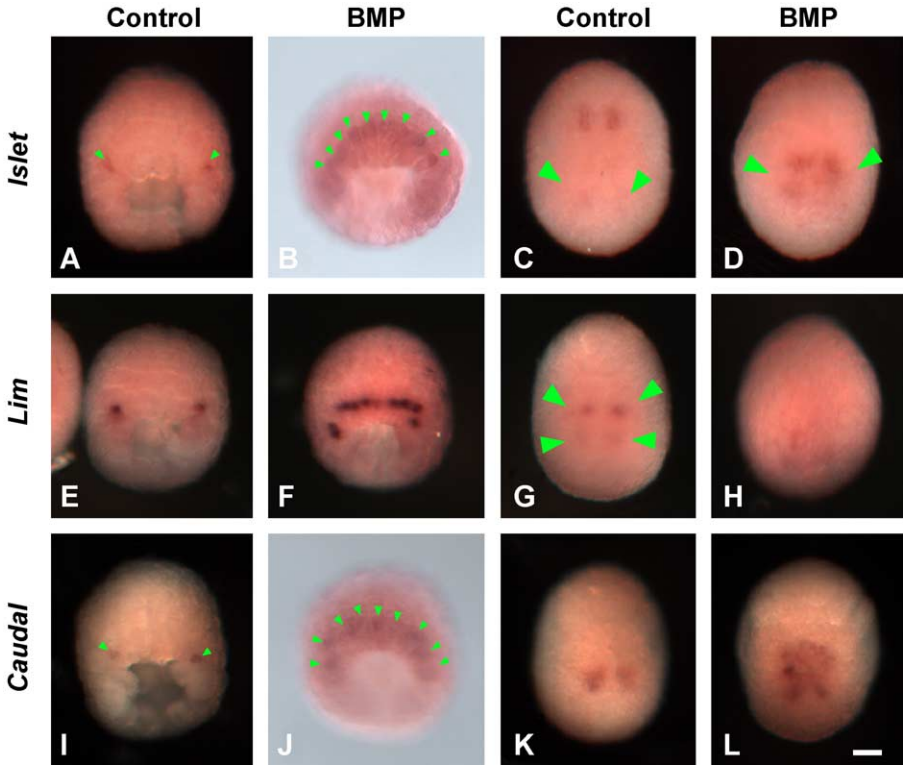

Fig. 6. Effects of *BMP* overexpression on the expression of *Islet*, *Lim*, and *Caudal* in gastrula and neurula embryos. Specimens hybridized to *Islet* (A–D), *Lim* (E–H), and *Caudal* (I–L) probes. Embryos were injected with either 0.1 μg/μl beta-galactosidase mRNA as negative control (A, C, E, G, I, and K) or 0.1 μg/μl *HrBMPb* mRNA (B, D, F, H, J, and L). Anterior pole is toward the top of the photographs. Either vegetal view of gastrula (A, B, E, F, I, and J) or dorsal view of neurula embryo (C, D, G, H, K, and L) is shown. In the gastrulae, ectopic expressions of *Islet* (B), *Lim* (F), and *Caudal* (J) were detected in a line in the neural plate at the anterior edge of the blastopore. Faint signals of expression of *Islet* and *Caudal* are indicated by arrowheads. In the neurulae, expressions of *Islet* (D) and *Caudal* (L) were enhanced, whereas expression of *Lim* was reduced by BMP overexpression. Arrowheads in C, D, and G indicate motor neuron region in the dorsal ectoderm. Scale bar indicates 50 μm.

the tailbud stage, closure of the neural tube was not made in BMP-overexpressing embryos, and the boundary between the trunk and tail region of the embryos became ambiguous (Fig. 7). Whereas the expression of *tubulin* was suppressed in the anterior region, as previously reported (Miya et al., 1997), it was enhanced in the posterior region by BMP overexpression (Fig. 7B). Ectopic expression of *TuNa2* was observed in the midline of the overexpressing embryos (Fig. 7H). *Islet* expression was observed as a band in the dorsal surface of the proximal tail region of BMP-overexpressing embryos, while *Islet* expression in the anterior neural tube (brain region) was eliminated by BMP overexpression (Fig. 7D). Thus, ectopic expression of *TuNa2* and *Islet* observed in the tailbud stage is consistent to that observed in the gastrula stage (Fig. 6) in terms of that expression of motor neuron makers expanded into the ventral side of the posterior neural tube. In contrast, *Lim* expression was reduced upon BMP overexpression, but not completely abolished (Fig. 7F). The *Lim*-expressing cells were found only along the dorsal midline of the embryos, which is probably the presumptive floor plate in a normal embryo.

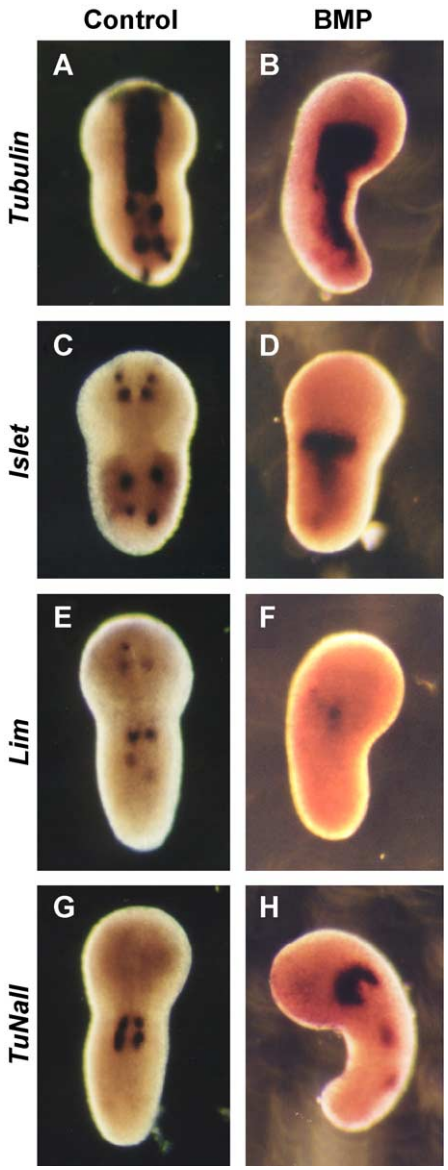

Fig. 7. Effects of BMP overexpression on the motor neuron markers in tailbud stage embryos. The dorsal view of the embryos was shown. Probably uneven distribution of overexpressed BMP was responsible for irregular bending and length of the tail part. Expression of *tubulin* (A and B), *Islet* (C and D), *Lim* (E and F), and *TuNa2* (G and H) was examined by whole mount in situ hybridization in the embryo injected with either *GFP* (control) or *HrBMPb* mRNA. Scale bar indicates 50 μm.

To test if BMP signaling is required for normal expression of *Lim* and *Islet*, the *Xenopus* dominant-negative BMP receptor (dnBMPR) mRNA was injected into a blastomere at the two-cell stage. The injected side of the embryos was visualized by fast green dye upon fixation at gastrula and neurula stages. The signal of *Islet* and *Lim* was detected in the uninjected side (Figs. 8A–D arrowheads), while the signal of neither *Islet* nor *Lim* was detected on the injected side at both gastrula and neurula stages. Overexpression of dominant-negative BMP receptor did not affect expression of a neural marker *beta-tubulin* (Fig. 8), which is consistent to the effect of overexpression of *Chordin*, an anti-BMP molecule (Darras and Nishida, 2001). This suggests that BMP signaling is required for expression of *Islet* and *Lim* in motor neuron precursors.

Caudal, a homeobox gene, is expressed specifically in bilateral pairs of blastomeres, A9.30, of the gastrula embryo (Katsuyama et al., 1999). These blastomeres correspond to cells expressing *Islet* and *Lim* at later gastrula stage (Figs. 3 and 9). The blockade of *Caudal* gene function by antisense oligonucleotide or dominant-negative molecule suppresses the expression of *Lim* and *Islet* in the motor neuron lineage, whereas expressions in other cells such as *Islet* in the notochord and *Islet* and *Lim* in the anterior neural tube are unaffected (Katsuyama et al., 1999). It is also known that *Xenopus* BMP regulates the expression of a *Caudal* family gene (Pillemer et al., 1998). Thus, we test a possibility that *Caudal* gene function mediates the regulation of *Islet* and *Lim* expression by BMP. The BMP overexpression caused ectopic expression of *Caudal* in a similar fashion as it did on *Lim* and *Islet* (Figs. 6J and L). dnBMPR suppressed the expression of *Caudal* on the injected side (Figs. 8E and F). These observations suggest that activation of *Caudal* gene could be involved in activation of motor neuron markers by BMP signaling.

Discussion

This study examined the expression pattern of *Islet* and *Lim* in ascidian embryos to gain insights into early specification of the motor neuron lineage. Each precursor exhibits a distinct temporal pattern of *Islet* and *Lim* expression. Overexpression of *Islet* induced ectopic

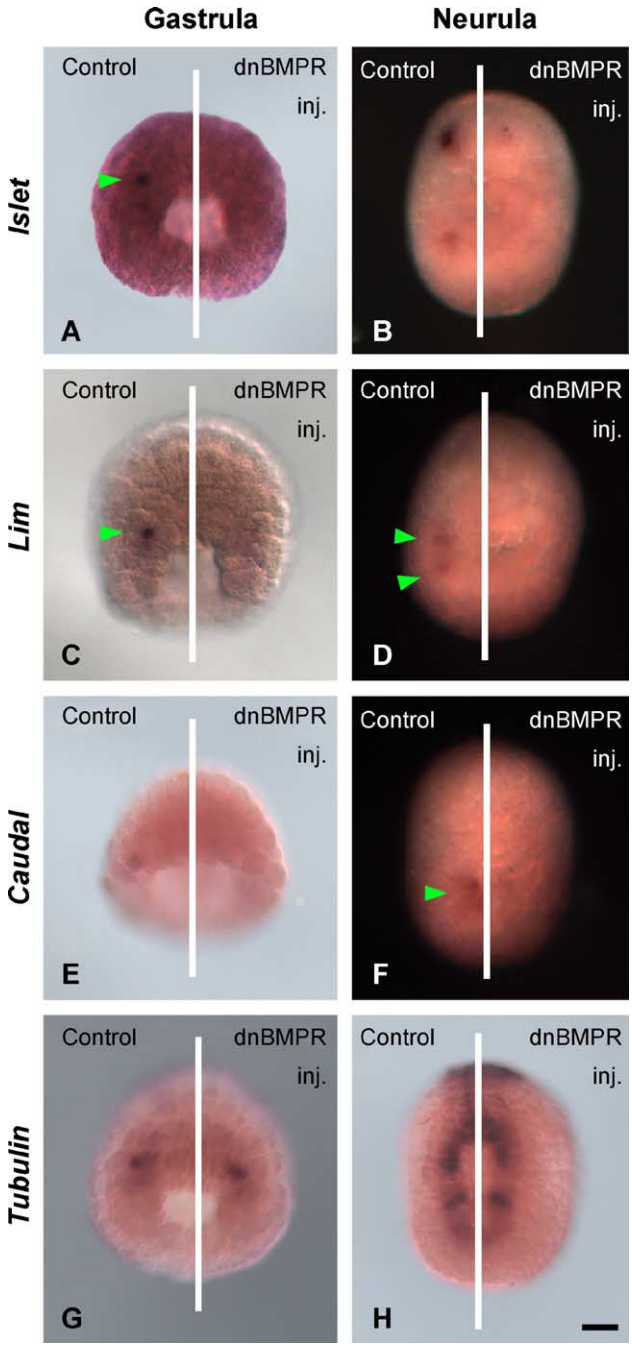

Fig. 8. Effect of blocking BMP signaling on the gene expression revealed by in situ hybridization. Embryos were unilaterally injected with a dominant-negative form BMP receptor. Only embryos that were injected on the right side are shown in this figure. Anterior pole is toward the top of the photographs. Either vegetal views of gastrula (A, C, and E) or dorsal views of neurula embryos (B, D, and F) are shown. Expression of *Islet* (A and B), *Lim* (C and D), and *Caudal* (E and F) in motor neuron precursors pointed by red arrowheads was suppressed in the injected side, whereas expression of *beta-tubulin* (G and H) was not. Scale bar indicates 50 μm.

expression of motor neuron markers, which suggests that *Islet* may regulate motor neuron differentiation during ascidian embryogenesis. The expression of motor neuron markers is sensitive to BMP, but not to Shh signaling, suggesting that distinct molecular mechanisms from those invertebrates may control the cell fate of ascidian motor neurons.

Possible mechanisms underlying patterning of the ascidian neural tube

Upon referral to a cell lineage map made by Nishida (1987), we confirmed that *Lim/Islet*-positive cells differentiate into neural cells in the lateral wall of the neural tube. In vertebrate embryos, the subtypes of neurons are populated along the D–V axis of the neural tube and each subtype is specified by transcription factors (Tanabe and Jessell, 1996). The ascidian homologues of *caudal*, *Pax3/7*, *HNF3*, and *Snail* are expressed with *Lim* and *Islet* in the neural plate at the gastrula stage (Fig. 9; Katsuyama et al., 1999; Shimauchi et al., 1997; Wada and Saiga, 1999; Wada et al., 1996). The expression pattern of these genes is well conserved, as it is similar to that of the vertebrate counterparts. This indicates that the D–V patterning of the ascidian neural tube including specification of the motor neuron lineage takes place at the gastrula stage.

Hedgehog activity is required for specification of motor neurons in the vertebrate spinal cord. However, that seems unlikely the case for ascidian neural tube. Overexpression of

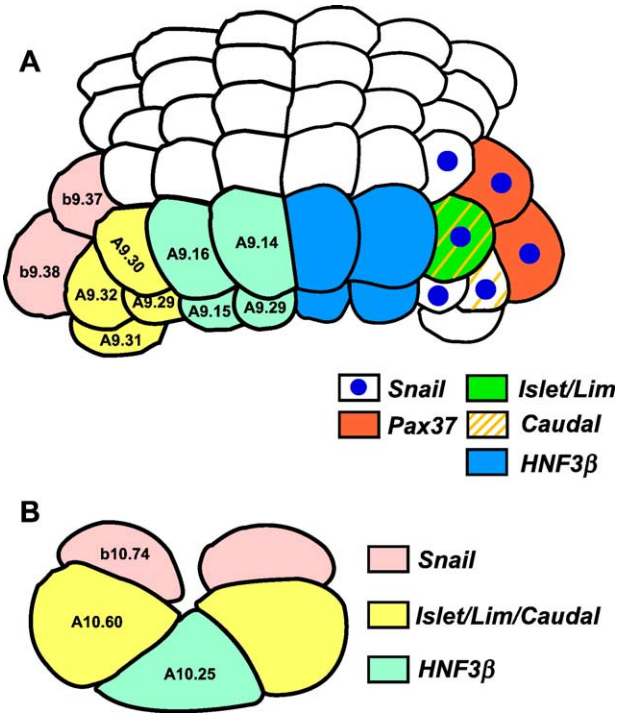

Fig. 9. (A) A schematic view of the ascidian neural plate at the late gastrula stage (neural plate stage); the blastomeres shown are in the 9th generation. The fate map and names of blastomeres that form the posterior neural tube are indicated on the left side. Gene expression pattern is indicated on the right side. The blastomeres that are colored pale red, yellow, and blue are fated to the dorsal, lateral, and ventral lines of the neural tube, respectively. (B) A schematic view of a cross-section of the neural tube forming region that generates motor neurons. The blastomeres shown are in the 10th generation. Subsequently, the dorsal most cells (b10.74) intercalate with each other to form a line, forming a tubular structure.

neither Shh nor constitutively active PKA altered or blocked the expression of *Islet* and *Lim*. Likewise, Shh overexpression had no effect on the expression of *TuNa2*. The expression pattern of two hedgehog genes, *Cihh1* and *Cihh2*, during embryogenesis has recently been studied in *C. intestinalis* (Takatori et al., 2002). *Cihh1* exhibits maternal transcripts and shows no specific pattern of its expression throughout embryogenesis. *Cihh2* is expressed in the ventral side of the neural tube in tailbud embryos, thus spatial expression pattern is conserved with vertebrate *Shh*. However, *Cihh2* expression starts from the tailbud stage, a timing of which is later than the start of *Islet* and *Lim* expression in the motor neuron lineage. Thus, there is no evidence that suggests involvement of hedgehog signaling in ascidian motor neuron differentiation. On the other hand, we found that *HrBMPb* upregulates the expression of motor neuron markers. This is consistent with findings that the expression of *HrBMPb* in the motor neuron lineage predates that of *Islet* and *Lim*, and disappears around at the neurula stage (Miya et al., 1997). The overexpression of dominant-negative BMP receptor decreased *Islet* and *Lim* expression. Our results suggest that local BMP signaling plays a role in the specification of motor neuron.

Our findings that the expression of motor neuron markers is sensitive to BMP, but not to Shh signaling, were unexpected, but this might suggest that molecular mechanisms that are different from those of the vertebrate neural tube operate in differentiation of urochordate motor neurons. One possible explanation for this unique mechanism of motor neuron differentiation is that ascidians skip the inductive step with their more determinant early embryogenesis. In this case, BMP gives local cue for starting gene expression. An alternative explanation is Hedgehog signaling joined the molecular mechanism of motor neuron differentiation after divergence of ascidians from the evolutionary branch of vertebrates.

In the ascidian neural tube, the *Caudal* gene is expressed specifically in the lateral lines of the embryo (Fig. 2E; Katsuyama et al., 1999), and *Caudal*-expressing blastomeres in the gastrula neural plate include those expressing *Lim* and *Islet*, as summarized in Fig. 9A. The blockade of *Caudal* gene function by antisense oligonucleotide or overexpression of a dominant-negative molecule suppresses the expression of *Islet* and *Lim* in the motor neuron lineage (Katsuyama et al., 1999). In this report, we showed that *Caudal* expression is sensitive to BMP. It suggests that the function of *Caudal* may be one of the factors that mediate BMP-dependent specification of motor neuron lineage in ascidian.

Differentiation of Moto-C

In all the embryos that we examined, Moto-A/B was observed at the same level to the anterior top of the tail mesoderm, indicating that Moto-A/B precursor arises in an invariant pattern. On the other hand, Moto-C differentiates at two levels along the A–P axis. In some embryos, Moto-C was located just posteriorly adjacent to Moto-A/B, whereas in others it was shifted by one cell posteriorly. Furthermore, the exact position of Moto-C was asymmetric in some cases. These observations suggest a mechanism in which a cell was sorted to become Moto-C among two cells lining along the A–P axis at the neurula stage, and the other cell does not become neuron. In vertebrate neuronal differentiation, at the final cell division of the neural precursors in the ventricular zone, one of the daughter cells commits to become a mature neuron and the other cell gets suppressed from differentiating by Notch signaling (Zhong et al., 1996). A similar lateral inhibition mechanism may be involved in the random positioning of Moto-C precursors along the A–P axis. Probably two A9.29-derived sister cells are equipotent to become Moto-C. Once a cell is determined to become Moto-C, then the neuronal differentiation of the other cell may be suppressed. In this context, it is intriguing that an ascidian homologue of *Notch* is expressed in the motor neuron-forming area of the neural tube (Y.K. unpublished data).

Roles of LIM type homeobox genes in determining motor neuron characteristics in ascidian embryos

Overexpression of *Islet* in the ectodermal lineage under *synaptotagmin* promoter caused ectopic expression of *Lim* and *TuNa2*. Since *Lim* is also capable of causing ectopic expression of *TuNa2* in the ectodermal lineage (Okada et al., 2002), it is possible that ectopic expression of *TuNa2* by *Islet* is mediated by induction of *Lim* expression. We could not succeed in performing loss of function experiments to know the function of endogenous molecule in motor neuron differentiation by means of dominant-negative molecules generated by deletion of homeodomain. Injection of antisense oligonucleotide to *Islet* suppressed *TuNa2* expression accompanying suppression of gastrulation, thus we cannot know if the effect of antisense oligo is specific to expression of motor neuron marker or not. Although further experiments are required to define roles of *Islet* gene in ascidian motor neuron differentiation, our experiment suggests a possibility that *Islet* is essential for motor neuron differentiation. Considering the fact that mRNA microinjection of wild-type *Islet* also gave the effect that is not specific to motor neuron differentiation, *Islet* may have significant functions in other aspect of ascidian embryogenesis. Actually prominent expression of *Islet* is in the mesoderm, the expression in motor neuron is weak, and maternal transcripts were also detected.

Islet shows a distinct temporal expression pattern from that of *Lim*. In the BMP-overexpressed embryo, the expression of *Islet*, but not *Lim*, was enhanced at the tailbud stage, whereas both genes were upregulated at the gastrula and neurula stages. The pattern of ectopic expression of *Lim* induced by BMP or *Islet* gives insight into a molecular mechanism of the normal expression

pattern of *Lim* and *Islet* where these genes are coexpressed in motor neuron lineage in early stage, but expressions of these two genes become exclusive to each other in later stage. *Lim* and *Islet* share common functional motifs in their protein structure, which implies that they share common cofactors that bind to Lim and Islet proteins as well. Perhaps, both BMP and *Islet* play a role in the initiation of *Lim* expression. Only at later stages higher level of *Islet* expression may downregulate *Lim* expression by competing such cofactors by which a distinct expression pattern is generated among three motor neurons. Then what is the significance of the expression of two LIM-HD proteins in motor neuron precursors in ascidian larvae? In the ascidian larvae, only Moto-B traverses its axon to the ventral muscle, whereas other motor neurons extend axons dorsally (Okada et al., 2002). In *Drosophila* and mouse embryos, the target of motor neuron axons changes when Lim3/Lhx3,4 is either disrupted or ectopically expressed (Sharma et al., 1998; Thor et al., 1999). The sequence of the ascidian *Lim* gene shows a higher similarity toward Lim3/Lhx3,4 subgroup than others. Thus, it is possible that *Lim* and *Islet* are involved in the diversification of axonal phenotypes in ascidian embryos. In young tadpoles, the precursors of Moto-A and Moto-C express *Lim* and *Islet*, respectively, whereas Moto-B expresses neither. The absence of LIM homeobox gene expression in Moto-B may be responsible for the ventral innervation pattern that is unique to Moto-B. Alternatively, there may exist other LIM homeobox genes that are expressed in the Moto-B lineage, but are yet to be examined. Both the *Islet* and *Lim3* mutant of *Drosophila* and the *Lim3 (Lhx3/4)* mutant of mouse yield abnormal pathfinding of motor neurons. However, the molecular mechanism for such a change in the pathfinding is unclear. Blockade of neuronal activity with ion channel inhibitors during the stage of axonal growth impairs the outgrowth and target selection of the axons in some developing vertebrate nervous systems (Catalano and Shatz, 1998; McFarlane and Pollock, 2000). Therefore, regulation of axonal growth may require neural activity based on the ion channel functions, of which expression is regulated by LIM-type homeobox genes in the motor neuron lineage. To test this hypothesis, the function of *Islet* target genes including ion channels needs to be identified. A further characterization of ascidian genes, of which vertebrate homologues are involved in motor neuron differentiation, will help us to understand the molecular mechanisms of neural tube specification and how they are conserved or modified during evolution.

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