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Evolution of Developmental Control Mechanisms

# Highly conserved functions of the *Brachyury* gene on morphogenetic movements: Insight from the early-diverging phylum Ctenophora

Atsuko Yamada [a,*], Mark Q. Martindale [b], Akimasa Fukui [c], Shin Tochinai [a]

[a] Department of Natural History Sciences, Faculty of Science, Hokkaido University, N10 W8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan
[b] Kewalo Marine Laboratory, Pacific Bioscience Research Center, University of Hawaii, 41 Ahui Street, Honolulu, HI 96813, USA
[c] Department of Biological Sciences, Graduate School of Science, Hokkaido University, N10 W8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan

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ABSTRACT

*Brachyury*, a member of the T-box transcription family identified in a diverse array of metazoans, was initially recognized for its function in mesoderm formation and notochord differentiation in vertebrates; however, its ancestral role has been suggested to be in control of morphogenetic movements. Here, we show that morpholino oligonucleotide knockdown of *Brachyury* (*MlBra*) in embryos of a ctenophore, one of the most ancient groups of animals, prevents the invagination of *MlBra* expressing stomodeal cells and is rescued with corresponding RNA injections. Injection of RNA encoding a dominant-interfering construct of *MlBra* causes identical phenotypes to that of RNA encoding a dominant-interfering form of *Xenopus Brachyury* (*Xbra*) in *Xenopus* embryos. Both injected embryos down-regulate *Xbra* downstream genes, *Xbra* itself and *Xwnt11* but not axial mesodermal markers, resulting in failure to complete gastrulation due to loss of convergent extension movements. Moreover, animal cap assay reveals that *MlBra* induces *Xwnt11* like *Xbra*. Overall results using *Xenopus* embryos show that these two genes are functionally interchangeable. These functional experiments demonstrate for the first time in a basal metazoan that the primitive role of *Brachyury* is to regulate morphogenetic movements, rather than to specify endomesodermal fates, and the role is conserved between non-bilaterian metazoans and vertebrates.



## Introduction

Predicting the body plan of the common ancestor at distinct nodes of metazoan evolution is one of the goals of Evo-Devo. Recent comparative molecular biology in basal metazoans (cnidarians, placozoans, ctenophores, and sponges) and bilaterians (e.g., protostomes and deuterostomes) has presented the opportunity to understand body plan evolution by comparing the functions of highly conserved genes during development (Steele, 2002; Meinhardt, 2004; Darling et al., 2005; Seipel and Schmid, 2005). *Brachyury* (or *T*) is the founding member of the T-box transcription factor family and one of the genes that have been extensively investigated functionally in diverse animal species. *Brachyury* was first identified genetically in mouse (Herrmann et al., 1990), and initial studies focused on its role in the mesoderm formation and notochord differentiation in various chordates. *Brachyury* mutants in mouse and zebrafish lack notochord and posterior mesoderm and display short tails (Dobrovolskaïa-Zavadskaïa, 1927; Chesley, 1935; Grüneberg, 1958; Halpern et al., 1993). In both amphioxus and all vertebrates so far investigated, *Brachyury* homologues are expressed transiently during gastrulation around the blastopore, in involuting mesoderm, and subsequently become restricted to the notochord (Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1994; Kispert et al., 1995; Holland et al., 1995; Terazawa and Satoh, 1997; Martin and Kimelman, 2008). *Brachyury* homologues in chick and *Xenopus* are induced by mesoderm-inducing factors such as activin A and basic FGF (Smith et al., 1991; Kispert et al., 1995), and *Xenopus Brachyury* (*Xbra*) causes ectopic mesoderm in the animal cap over expression assays (Cunliffe and Smith, 1992, 1994). Thus, the dual roles of *Brachyury* in early mesoderm formation and notochord differentiation were widely supported (Holland et al., 1995). However, functional analyses of *Brachyury* homologues identified from non-chordate phyla have questioned the ancestral role of *Brachyury* in mesoderm specification. In ecdysozoan insects, *Brachyury* homologues are all expressed in the posterior terminal region and play roles in the morphogenesis of the caudal hindgut and of the visceral mesoderm (Kispert et al., 1994; Singer et al., 1996; Kusch and Reuter, 1999; Shinmyo et al., 2006; Berns et al., 2008). In a non-chordate deuterostome sea urchin, *Brachyury* is functionally required for the morphogenetic movements associated with the blastopore and the forming ectodermal stomodeum (Gross and McClay, 2001). Similar patterns of expression of *Brachyury* around the blastopore and stomodeum is detected in

* Corresponding author. Present address: Graduate School of Information Science and Technology, Hokkaido University, N14 W9, Kita-ku, Sapporo, Hokkaido 060-0814, Japan. Fax: +81 11 706 6787.
*E-mail address:* atsukoy@ist.hokudai.ac.jp (A. Yamada).

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hemichordates and other echinoderms (Tagawa et al., 1998; Shoguchi et al., 1999), although their functions have not yet been analyzed directly. These data suggest that the ancestral role of *Brachyury* is in the morphogenetic movements of the blastopore, stomodeum and hindgut (Tagawa et al., 1998; Technau, 2001; Gross and McClay, 2001), although the expression in stomodeum and blastopore derivatives was lost in arthropods (Kispert et al., 1994; Singer et al., 1996; Shinmyo et al., 2006; Berns et al., 2008) and urochordates (Yasuo and Satoh, 1993; Corbo et al., 1997; Bassham and Postlethwait, 2000; Nishino et al., 2001). Consistent with this view, *Brachyury* has also been shown to effect morphogenesis in vertebrates. For example, mice mutations of *Brachyury* gene cause a disturbance of the primitive streak (Chesley, 1935; Grüneberg, 1958) and the chimeric analyses in mice demonstrate that the mutant cells of *Brachyury* accumulate in the primitive streak due to their inability to migrate (Beddington et al., 1992; Wilson et al., 1995; Wilson and Beddington, 1997). In *Xenopus*, inhibition of *Xbra* function prevents convergent extension movements during gastrulation (Conlon et al., 1996; Conlon and Smith, 1999; Kwan and Kirschner, 2003).

In addition to the functional analyses with vertebrates, a sea urchin, and insects, the expression of *Brachyury* orthologues have been identified also from non-model organisms. *Brachyury* is expressed in the blastopore and the mouth opening in chaetognaths whose phylogenetic status is uncertain (Takada et al., 2002) and associated with the mouth and anus in a polychaete and gastropod within the Lophotrochozoa (Arendt et al., 2001; Lartillot et al., 2002). In cnidarians, one of non-bilaterian animals, *Brachyury* is also expressed around the blastopore of late gastrula and early planula larvae (Scholz and Technau, 2003), at the posterior pole of early gastrulae where ingression is occurring in jellyfish (Spring et al., 2002) and is detected in the tissue surrounding the mouth of hydra (the hypostome) which corresponds to the blastopore of other animals according to the gastraea theory of Haeckel (Technau and Bode, 1999). These descriptive data all support the view that the ancestral function of *Brachyury* might be in morphogenetic movements associated with the blastopore, but no functional analyses have been performed in any of these animals.

Here, we apply morpholino antisense oligonucleotides (MO) to ctenophore embryos in order to test the role of *Brachyury* in basal metazoans. Ctenophores are biradially symmetrical animals along their major longitudinal body axis, the oral–aboral axis. Although some textbooks also describe ctenophores as diploblastic animals without derivatives of the mesodermal germ layer, ctenophores possess definitive contractile muscle cells and mesenchymal cells in the extracellular space between the ectoderm and endoderm that are derived from a distinct lineage of embryonic cells (Martindale and Henry, 1999) and could be thought of as mesodermal derivatives (Hernandez-Nicaise, 1991). Ctenophores have always been difficult to place phylogenetically, but molecular data put them as one of basal metazoan groups (Collins, 1998; Kim et al., 1999; Podar et al., 2001; Wallberg et al., 2004; Schierwater et al., 2009), potentially even the earliest branching animal group (Dunn et al., 2008; Hejnol et al., 2009). A different analyses using 128 different protein-coding genes proposed that ctenophores are the sister group to cnidarians and that the 'coelenterata' clade (Ctenophora and Cnidaria) is the sister group to the Bilateria (Philippe et al., 2009). These characters position ctenophores as an important organism to investigate early metazoan evolution and to predict the body plan of the metazoan ancestor, but data on the expression of their developmental genes are still sparse (Yamada and Martindale, 2002; Derelle and Manuel, 2007; Pang and Martindale, 2008; Jager et al., 2008). Recently, ctenophore *Brachyury* (*MlBra*) was isolated from *Mnemiopsis leidyi* and was shown to be expressed in ectodermal cells surrounding the site of gastrulation and in stomodeal/pharyngeal cells derived from the blastopore (Yamada et al., 2007). In this work, we injected a MO (MlBra-MO) designed to inhibit the functions of *MlBra* that specifically prevented stomodeal/pharyngeal precursor cells from invaginating. Additional experiments were conducted to compare the functional properties of *MlBra* with vertebrate *Brachyury*. A dominant-interfering form of *MlBra* (MlBra-EnR) was injected into *Xenopus* embryos and the resultant embryos failed to complete gastrulation similarly to those injected with Xbra-EnR, a dominant-interfering form of *Xbra*. *MlBra* encoding the full-length coding sequence mimicked the action of *Xbra* in that it induced the *Xbra* target gene *Xwnt11*, but not *chordin* and *goosecoid*. These results conclusively demonstrate that the ancestral role of *Brachyury* is involved in regulating morphogenetic movements, rather than cell type specification, and is conserved in animals as diverse as basal metazoans and vertebrates.

## Materials and methods

### *Animals*

Adult specimens of the lobate ctenophore *Mnemiopsis leidyi* were collected off the rock jetty at NOAA in Woods Hole, Massachusetts, USA, during the months of June or July. Self-fertile hermaphroditic animals were placed in the dark at night, and naturally fertilized eggs were collected approximately 8 h later. Fertilized eggs were reared in 0.45-μm membrane filtered seawater (FSW) at approximately 20 °C. At 20 °C, fertilized eggs completed normal gastrulation about 8 h and hatched about 16–19 h. Adult clawed frogs *Xenopus laevis* were purchased from suppliers and maintained in our laboratory. Eggs were obtained from female *Xenopus laevis* injected 8 h previously with 300 units of human chorionic gonadotrophin (Aska, Japan). Artificially fertilized eggs were maintained in Steinberg's solution (SBS; 58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.4–7.6) and dejellied with SBS containing 4.5% cysteine hydrochloride (pH 8.0) during the two-cell stage. The embryos were staged according to Nieuwkoop and Faber (1994).

### *Injection of MO and synthetic RNAs into ctenophore eggs*

To suppress translation of *MlBra* during ctenophore embryogenesis, we used antisense morpholino oligonucleotides (MO; Gene Tools) complementary to a 25-nucleotide sequence including the translational start site of *MlBra* (MlBra-MO: 5′-ACT GCG AAC AAA AGT TGG TAG ACA T-3′, antisense to a sequence spanning nucleotides 56–80 of *MlBra* cDNA (GenBank accession number: DQ988137). A Standard Control Oligo (Cont-MO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′) supplied from Gene Tools was also used as negative controls. For rescue experiments, we prepared two kinds of synthetic capped RNAs, rescue RNA and mis-pair RNA as below. By using the mMessage mMachine kit (Ambion), capped rescue RNA was *in vitro* transcribed from linearized pCS2 plasmids including a nucleotide sequence spanning nucleotides 56–91 of *MlBra* so that it was recognized by MlBra-MO. Similarly, capped mis-pair RNA which should be not recognized by MlBra-MO because it was incorporated five mismatch nucleotides into rescue RNA without changing the amino acids encoded was prepared.

rescue RNA: 5′-AUG UCU ACC AAC UUU UGU UCG CAG UUC CUG AAA CAG-3′
mis-pair RNA: 5′-AUG UCC ACU AAC UUC UGC UCT CAG UUC CUG AAA CAG-3′

MO and synthetic RNA was suspended in sterile distilled water and stored at −80 °C until use. Thawed aliquots of MO were heated to 65 °C for 10 min to ensure that they were completely dissolved and kept at 4 °C during use. Microinjections by the pressure were performed as previously described (Martindale and Henry, 1997b).

Uncleaved eggs after natural fertilization were manually removed vitelline membrane with fine tungsten needles or forceps in FSW and then injected with the solution containing only 1 mM of MO or 1 mM of MO and 0.7 μg/μl of RNA, together with rhodamine dextran in 40% glycerol to confirm whether the solution was correctly introduced into the eggs. The injected volume of the solution was controlled to be 0.52 fl by measuring the diameter of each droplet injected. Injected eggs were kept in gelatin-coated dishes filled with FSW and observed during their embryogenesis.

*Hematoxylin and eosin staining*

MlBra-MO injected embryos were fixed in 4% formaldehyde for 1 h and kept in MeOH at −20 °C until use. The specimens were dehydrated with ethanol and xylene, embedded in paraffin, sectioned at 6–8 μm with a microtome. After treatment with xylene to remove paraffin, the sections were hydrated with ethanol and stained with Delafield's hematoxylin and 1% eosin aqueous solution.

*Injection of dominant-interfering constructs of Brachyury into Xenopus embryos*

Constructs of MlBra-EnR and Xbra-EnR were made by cloning the region encoding amino acids 1–247 of *MlBra* and 1–228 of *Xbra* (GenBank accession number; M77243) into pCS2-EnR vectors (Addgene plasmid 11028), respectively. A construct containing only EnR domain was prepared by introducing a translation initiation site just upstream of EnR domain of pCS2-EnR vectors. Details on these constructs are available on requests. Capped RNAs were synthesized in the same way as rescue RNA, dissolved in sterile distilled water at a final concentration of 5 or 10 ng/μl, and used for microinjection by the pressure of nitrogen used to drive the injector. 50 or 100 pg of RNA was injected into the marginal zone of the two dorsal cells of a four-cell embryo that had been dejellied. Under some experiments, RNA was co-injected with 400 pg of *lacZ* RNA as a lineage tracer. Injected embryos were cultured in 5% Ficoll 400/1× SBS overnight and then in 0.1× SBS until the appropriate stage for each experiment.

*Histochemistry*

*Xenopus* embryos were fixed with MEMFA (0.1 M MOPS, 2.0 mM EGTA, 1.0 mM $MgSO_4$, 3.7% formaldehyde, pH7.4) for 1–2 h at room temperature and then kept in methanol at −20 °C. After fixed embryos were bleached by the treatment with 10% $H_2O_2$ in methanol under the light for 2–3 h, they were immunostained with a muscle-specific antibody (12/101; Kintner and Brockes, 1985) or a notochord-specific antibody (MZ15; Smith and Watt, 1985). Indirect immunohistochemical staining with antibodies was performed by standard methods with an alkaline phosphatase-conjugated goat secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate.

For X-gal or Red-gal staining, frog embryos were fixed in MEMFA for 20 or 60 min at room temperature, washed in 0.7× PBS (1×PBS: 137 mM NaCl, 8.0 mM $Na_2HPO_4$, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, pH7.3), and incubated in staining buffer (0.2 mg/ml X-gal or Red-gal, 3 mM $K_3[Fe(CN_6)]$, 3 mM $K_4[Fe(CN_6)]$, 1 mM $MgCl_2$, and 0.1% Tween-20 in 0.7× PBS) at 37 °C. After staining, specimens were rinsed in 0.7× PBS.

*Bisection of Xenopus embryos*

Embryos were fixed in MEMFA for 60 min at room temperature, washed with 0.7× PBS, divided sagittally into two halves using a disposal blade (Futaba; No. 19). Every pair of embryos was stained with staining buffer including X-gal as above.

*Keller sandwiches of Xenopus embryos*

Keller sandwiches were prepared according to the method described previously (Keller and Danilchik, 1988). In brief, rectangular explants of dorsal mesendoderm and ectoderm were dissected from embryos at the stage 10.5 using an eyebrow knife and then transferred to a dish of Sater's modified blastocoel buffer (49.52 mM NaCl, 36.44 mM gluconic acid sodium salt, 5.0 mM $Na_2CO_3$, 4.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1 mg/ml BSA, approximately 7 mM HEPES, pH 8.1). Two rectangles of the same size were sandwiched together with their inner surfaces, but with the same direction along the animal–vegetal axis, under a coverslip until the sandwich had healed. The coverslip was removed, and the sandwiches were cultured in blastocoel buffer. Convergent extension was assessed when intact sibling embryos had reached the neural tube stage (about stage 20).

*Whole-mount in situ hybridization*

Digoxigenin (DIG)-labeled riboprobes were synthesized by using a DIG RNA-labelling kit (Roche, USA) and were digested into approximately 200-bp fragments by alkaline hydrolysis. Riboprobes for detection of *Xbra* mRNA were prepared from the 3′UTR of *Xbra* cDNA and the other probes were generated from the full-length cDNA. Whole-mount *in situ* hybridization was performed using the protocol described by Hemmati-Brivanlou et al. (1990) with some modification. Embryos were fixed with MEMFA for 20 min, stained with Red-gal by the above method, re-fixed with MEMFA for 100 min, and bleached in 10% $H_2O_2$ in methanol. Bleached specimens were washed with methanol and then kept in ethanol at −20 °C until use. Rehydration of specimens was followed by proteinase K treatment (10 μg/ml, 5 min), acetic anhydride treatment (0.25% in 0.1 M triethanolamine, 3 min), and postfixation (4% paraformaldehyde, 30 min). A prehybridization step was performed for 2 h at 60 °C in hybridization buffer (50% formamide, 5× SSC, 10 mM EDTA, 1× Denhardt's solution, 250 μg/ml yeast RNA, 0.1% CHAPS, 0.1% Tween-20) before hybridization with the DIG-labelled probe (0.5 μg/ml) at 60 °C for 24 h. Hybridized probe was immunodetected with alkaline phosphatase-conjugated anti-DIG antibody (Roche, Japan) and BCIP as the substrate in the presence of 2 mM levamisole.

*Animal cap assays*

For the animal cap assays, capped RNAs were synthesized from pCS2 plasmids containing wild-type *MlBra* or *Xbra* protein coding regions using the same method as rescue RNA (see above) and injected into the animal poles of each cell of four-cell stage embryos. Animal caps were dissected at stage 8 and cultured in 1× SBS until harvesting for RT-PCR analyses at stage 11. Total RNA was prepared from five animal caps for each assay with ISOGEN (Wako, Japan), and cDNA was synthesized with Superscript II RNaseH$^-$ (Invitrogen, USA). The primer sequences used for the RT-PCR and the numbers of PCR cycles were as follows: *chordin* 5′-AACTGCCAGGACTGGATGGT-3′, 5′-GGCAGGATTTAGAGTTGCTTC-3′ and 30 cycles; *goosecoid* 5′-CATCAGAGGAATCAGAAAATGCCC-3′, 5′-CCAATCAACTGTCAGAGTCCAGGTC-3′ and 33 cycles; *ODC* 5′-GTC AAT GAT GGA GTG TAT GGA TC-3′, 5′-TCC ATT CCG CTC TCC TGA GCA C-3′ and 30 cycles; *Sox17*β 5′-TATTCTGCGCAGAACCACC-3′, 5′-CCATCATGC-CATGTTCAGG-3′ and 33 cycles; *Xwnt11* 5′-CACTGGTGCTGCTATGT-CATG-3′, 5′-CAAGCAGATCAGACCAGTTGC-3′ and 30 cycles.

## Results

*Inhibition of MlBra function prevents invagination of stomodeum during ctenophore embryogenesis*

The expression pattern of the ctenophore *Brachyury*, *MlBra*, raises the possibility that *MlBra* is necessary for normal stomodeal

morphogenesis or alternatively for mesendodermal differentiation. To test this, antisense morpholino oligonucleotides (MlBra-MO) to prevent the function of *MlBra* by inhibiting its translation were used (Fig. 1 and Table 1a). Control embryos derived from fertilized eggs injected with commercially available Control MO (Cont-MO) before first cleavage, showed normal development through gastrulation and their ectodermal cells around the blastopore invaginated to form a normal stomodeum and pharynx, which were indistinguishable from uninjected controls (Fig. 1A, C, E, and I, *right column*). Conversely, ctenophore eggs injected with MlBra-MO developed normally up through gastrulation (Fig. 1B) but exhibited abnormalities during stomodeal and pharynx formation after gastrulation with ectodermal cells around the blastopore failing to invaginate (Fig. 1D, F, and I, *left column*). The phenotype induced by injecting MlBra-MO was shown to be specific by rescue experiments (Table 1b). Translation of synthetic mRNAs injected into ctenophore eggs has not been achieved so that we co-injected MlBra-MO with synthetic RNA (rescue RNA) encoding a 36-nucleotide region around the translation initiation site of *MlBra*. This rescue RNA should titrate the effective concentration of the inhibiting MlBra-MO and restore *MlBra* activity. The resultant embryos co-injected with MlBra-MO and rescue RNA showed normal development including stomodeal invagination (Fig. 1G and J, compare *left column* with *middle one*). In contrast, mis-pair RNA with introduced five nucleotide mismatches from the rescue RNA without changing the amino acid sequences encoded had no effect on the phenotype induced by MlBra-MO (Fig. 1H and J, *right column*). These experiments indicated that the action of MlBra-MO on stomodeal invagination was specific for the *MlBra* sequence. However, the possibility was not excluded that MlBra-MO has any non-specific effects on other genes in addition to its intended target *MlBra*.

**Table 1**
*MlBra* knockdown by injecting MlBra-MO.

| Injected samples | Number of specimens | Normal invagination (%) | Failure of invagination (%) | Significance |
|---|---|---|---|---|
| *a) Experiment 1* | | | | |
| MlBra-MO | 63 | 10 (15.9) | 53 (84.1) | $P<0.001$* |
| Cont-MO | 23 | 23 (100) | 0 (0) | |
| *b) Experiment 2* | | | | |
| MlBra-MO | 8 | 2 (25.0) | 6 (75.0) | |
| MlBra-MO + rescue RNA | 18 | 15 (83.3) | 3 (16.7) | $P<0.01$** |
| MlBra-MO + mis-pair RNA | 23 | 4 (17.4) | 19 (82.6) | $P<0.001$*** |

Probability was calculated by the Fisher's exact test when each result was compared to the result when Cont-MO was injected*, to the result when only MlBra-MO was injected in Experiment 2**, and to the result when MlBra-MO and rescue RNA were injected in Experiment 2***.

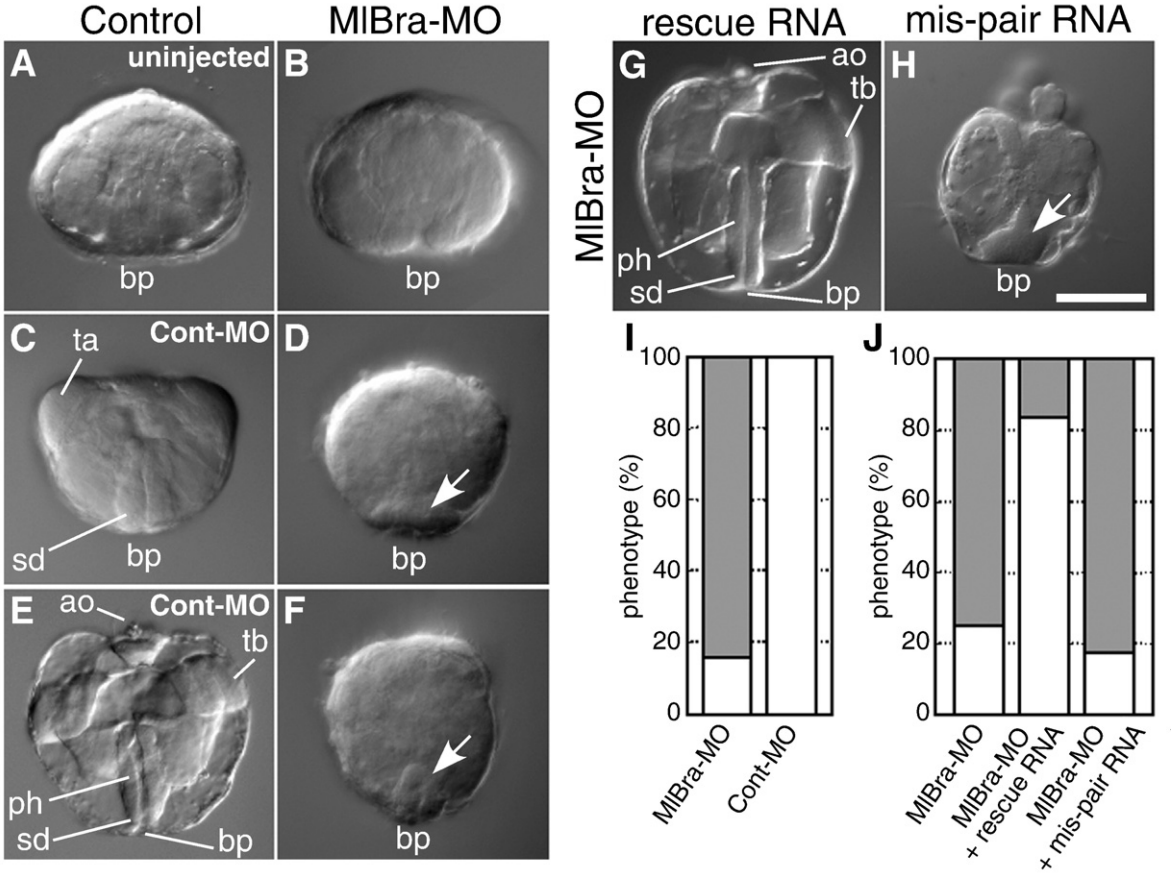


**Fig. 1.** *MlBra* knockdown by injecting MlBra-MO causes the failure of stomodeal invagination during ctenophore embryogenesis. (A–H) Morphology of the injected ctenophore embryos, lateral views with the aboral pole at top and the oral pole at bottom. Uninjected embryos (A), embryos injected with Cont-MO (C and E), and with MlBra-MO (B, D, and F) were observed under a microscope at the late gastrula (A and B), just after gastrulation (C and D) and before hatching (E and F). In embryos in which *MlBra* function was inhibited, ectodermal stomodeal cells accumulated around the blastopore and formed thickened mass (D and F, *arrows*). Stomodeal invagination was restored in embryos injected with MlBra-MO together with rescue RNA (G), but not mis-pair RNA (H, *arrow*). (I and J) Histograms showing the percentages of the phenotype induced by MlBra-MO. *White* and *grey* bars show normal invagination and failure of invagination, respectively. (I) In the first round of injections (*Experiment 1*), 84.1% of the embryos introduced MlBra-MO failed to invaginate their stomodeal cells (*left* column) while all embryos injected with Cont-MO underwent normal stomodeal morphogenesis (*right* column). (J) Co-injection of MlBra-MO and rescue RNA (*middle* column), but not mis-pair RNA (*right* column) restored their invagination in 83.3% of such embryos (*Experiment 2*). ao, apical organ; bp, blastopore; ph, pharynx; sd, stomodeum; ta, tentacle apparatus; tb, tentacular bulb. Scale bar, 100 μm.

In *MlBra* morphants, the thickened mass of cells around the blastopore appeared to be stomodeal cells because endodermal and mesodermal cells developed normally prior to stomodeum/pharynx formation (Fig. 1D and F and Fig. 2A and A′, *arrows*). Histological examination of the morphants also showed that stomodeal precursor cells accumulated around the blastopore (Fig. 2A and A′). In the case of uninjected embryos, the stomodeal cells were basically multilayered during the stomodeum invagination (Fig. 2B and B′) but became monolayered due to morphogenetic reorganization by the cyddipid larval stage (Fig. 2C and C′). These observations showed that MlBra-MO injection caused defects of intercalation of stomodeal cells. Therefore it was indicated that *MlBra* plays a role in the morphogenetic movements leading to stomodeum/pharynx formation rather than in the differentiation of ectodermal stomodeum or mesendodermal tissues.

### *The effects of MlBra-EnR injected into Xenopus embryos resemble those of Xbra-EnR*

The apparent functional conservation of *Brachyury* to regulate morphogenesis from a basal metazoan ctenophore to vertebrates prompted us to test whether MlBra protein has the same activity as vertebrate Brachyury. The DNA-binding domain of *MlBra* was fused to the transcriptional repression domain of the *Drosophila engrailed* gene thereby making the resulting hybrid construct (MlBra-EnR) a transcriptional repressor (Conlon et al., 1996; Gross and McClay, 2001). Synthetic RNA encoding MlBra-EnR was injected into the marginal zone of dorsal blastomeres of a *Xenopus* embryo at the four-cell stage (Fig. 3 and Table 2). Injected embryos developed normally until the early gastrula stage (Fig. 3A) but failed to complete gastrulation (Fig. 3E, *asterisk*) and to form posterior structures (Fig. 3I). This phenotype resembled that of embryos injected with RNA encoding Xbra-EnR (Fig. 3B, F, and J) (Conlon et al., 1996; Conlon and Smith, 1999). Unlike MlBra-EnR and Xbra-EnR, embryos injected with EnR RNA encoding the *engrailed* repressor domain alone were indistinguishable from uninjected controls (compared Fig. 3C, G, and K with D, H, and data not shown). We next characterized the posterior truncation of MlBra-EnR- and Xbra-EnR-injected embryos by whole-mount immunocytochemistry using the muscle-specific antibody 12/101 and the notochord-specific antibody MZ15 (Figs. 3I'–K' and I"–K", respectively). In the truncated tail phenotype induced by MlBra-EnR and Xbra-EnR injections, segmental muscle patterns were detected ($n=24/24$ and 21/21, respectively) as seen in embryos injected with EnR RNA (compared Fig. 3I′ and J′ with K′). Similarly, notochord differentiation was also observed in both embryos injected with RNA encoding MlBra-EnR ($n=17/18$) and Xbra-EnR ($n=14/14$) (Fig. 3I″ and J″ with K″) although they were partially inhibited (data not shown). These results indicated that the injection of MlBra-EnR RNA, as well as that of Xbra-EnR RNA into *Xenopus* embryos, blocks normal gastrula morphogenesis rather than mesodermal differentiation.

### *MlBra-EnR inhibits the convergent extension movements during Xenopus gastrulation*

At least two types of cell movements are involved in gastrulation of *Xenopus*: migration and convergent extension (Gerhart and Keller, 1986). Conlon and Smith (1999) have shown that Xbra-EnR prevents the convergent extension movements during gastrulation. In order to examine whether MlBra-EnR inhibits cell movements during *Xenopus* gastrulation, we examined embryos co-injected MlBra-EnR RNA with *lacZ* RNA to visualize the behavior of the cells inheriting MlBra-EnR RNA. Both MlBra-EnR- and Xbra-EnR-injected embryos displayed the normal morphology at the early gastrula stage as detected by the activity of *lacZ* lineage tracer in the dorsal lip of blastopore (Fig. 4A–D, *blue*) from which notochord are derived according to *Xenopus* fate map (Moody, 1987). In addition, we noticed that all specimens formed the leading edge of mesendoderm (Fig. 4A–D, *arrowheads*) along the blastocoel roof toward the animal pole, showing that at least the initial step of the mesendodermal migration during gastrulation proceeded normally. However, at the late gastrula, the cells inheriting either MlBra-EnR RNA or Xbra-EnR RNA that were supposed to develop into the notochord, accumulated to the dorsal lip of the blastopore that failed to close properly (Fig. 4E and F, *blue*). By contrast, cells expressing *lacZ* RNA in control embryos are located beneath animal ectoderm along the future anterior-posterior axis that will form the notochord (Fig. 4G and H, *blue*). The accumulation of the

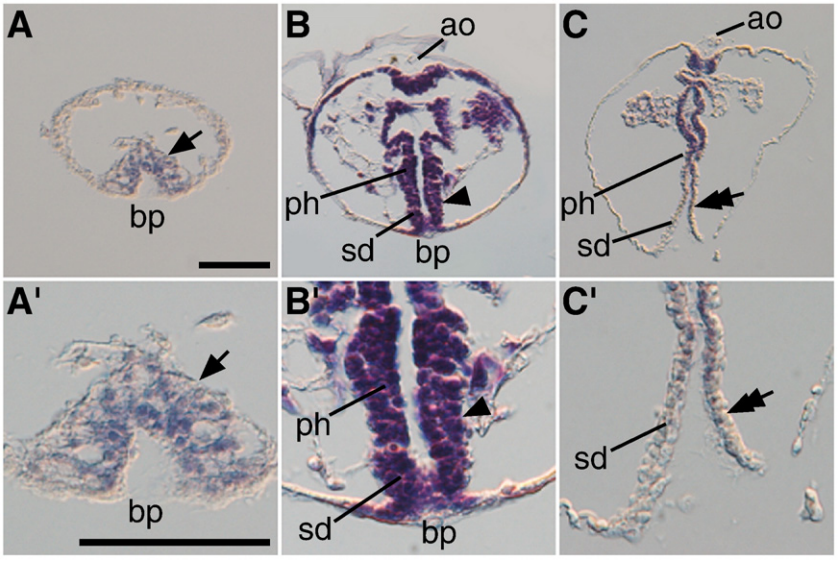


**Fig. 2.** Histological observation of MlBra-MO-injected embryos supports the role of *MlBra* in stomodeum invagination. Sections through MlBra-MO injected embryos (A and A′), uninjected embryos invaginating the stomodeum (B and B′), and uninjected cyddipid larva (C and C′). A′–C′ are magnified images of the area around the blastopore or the stomodeum in A–C, respectively. The developmental stage of MlBra-MO injected embryos (A and A′) was comparable in that of embryos invaginating the stomodeum (B and B′). The stomodeal cells in *MlBra* morphants accumulated around the blastopore (A and A′, *arrows*) while the stomodeum of uninjected embryos invaginated in multilayers (B and B′, *arrowheads*). The epithelial cells of stomodeum were basically monostratified at the larval stage in controls (C and C′, *double arrows*). In A, cells related to tentacle apparatus did not appear, but such cells were detected in other section. ao, apical organ; bp, blastopore; ph, pharynx; sd, stomodeum. Scale bar, 50 μm.

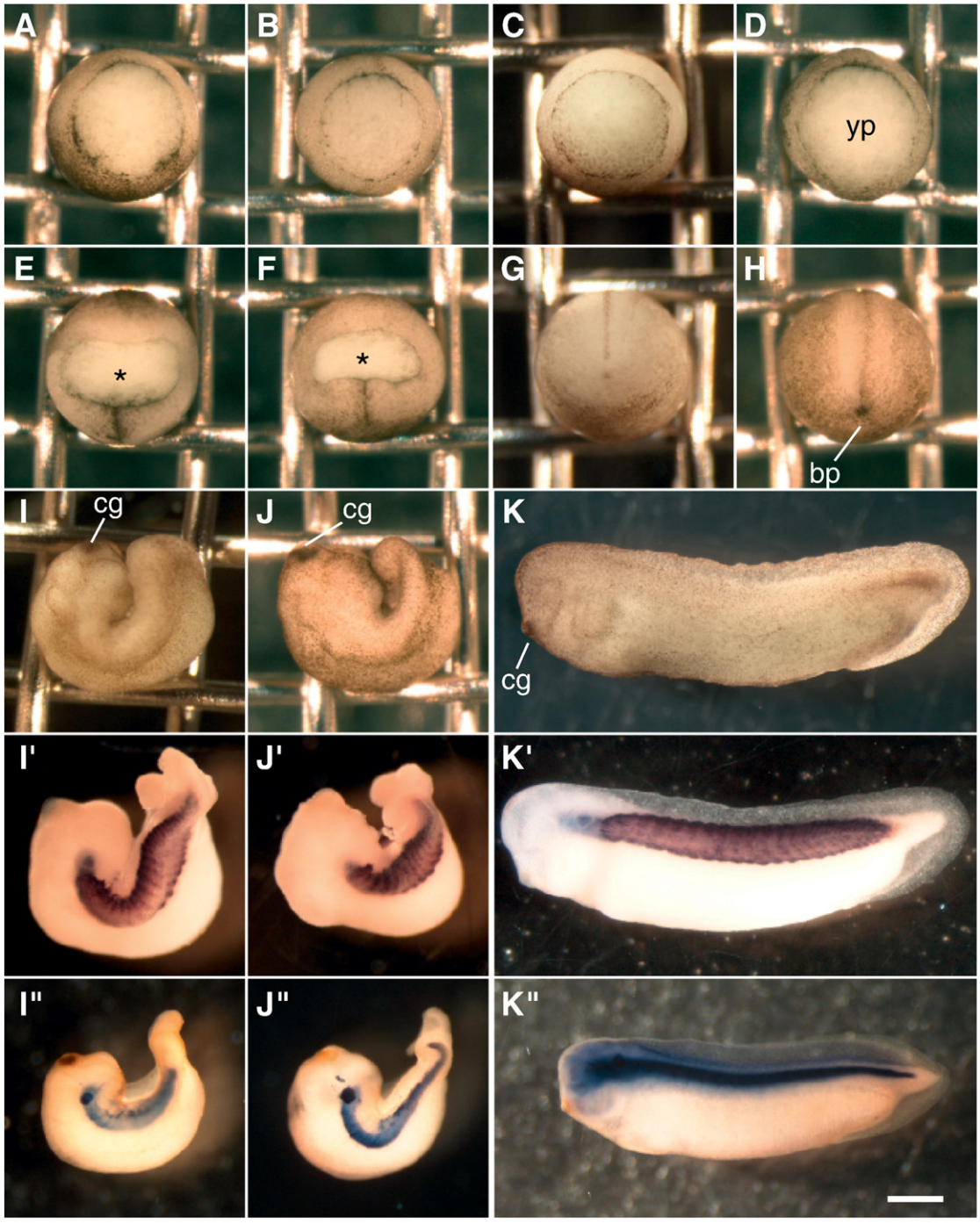


**Fig. 3.** Injection of RNA encoding MlBra-EnR into *Xenopus* embryos leads to the similar phenotype to those injected with RNA encoding Xbra-EnR. Embryos injected with RNA encoding MlBra-EnR (A, E, and I–I″), Xbra-EnR (B, F, and J–J″), and EnR (C, G, and K–K″) were compared to one another and to uninjected control embryos (D, H and data not shown). (A–D) Vegetal views of the early gastrulae (stage 10.5) and (E–H) those of the neural fold stage (stage 15) embryos were shown with dorsal to the up. Injection of MlBra-EnR and Xbra-EnR had no influence on the development of normal early gastrulae (compared A and B with C and D) but caused gastrulation defects (E and F, *asterisks*) while EnR injection was unaffected (G), compared to controls (H). (I–K, I′–K′, and I″–K″) Tailbud embryos derived from the injection were shown laterally. Anterior is to the left. Injections of MlBra-EnR and Xbra-EnR RNA led to posterior truncation (I, J) differently to controls (K) although muscle and notochord were present in such embryos shown by immunostaining with the muscle-specific antibody 12/101 (I′–K′) and the notochord-specific antibody MZ15 (I″–K″), respectively. bp, blastopore; cg, cement grand; yp, yolk plug. Scale bar, 500 μm.

cells inheriting MlBra-EnR RNA and Xbra-EnR RNA was likely due to perturbation of convergent extension of presumptive notochord cells (Gerhart and Keller, 1986). To confirm this hypothesis, we examined the effects of MlBra-EnR on convergent extension by using Keller sandwiches (Keller and Danilchik, 1988). The explants were prepared by removing the entire dorsal marginal zone which consists of dorsal axial mesoderm, posterior neural ectoderm, and some anterior ectoderm from two different embryos. These two explants are cultured together with the deep cells facing one another. In control sandwich explants, convergent extension of mesodermal and neural portions transformed the initially rectangular explants into a stereotyped morphology with two domains of elongation in mesoderm and neuroectoderm (Fig. 4K, *arrows* and *double arrows*, respectively). When Keller sandwiches were made from embryos injected with RNA encoding MlBra-EnR or Xbra-EnR, explants failed to elongate (Fig. 4I and J). These results strongly suggested that the

**Table 2**
Injection of the dominant-interfering construct of *Xbra* and *MlBra* into *Xenopus* embryos.

| Injected RNA | Amount of injected RNA (pg) | Number of specimens | Phenotype at the stage 10.5 (early Gastrula) | Phenotype at the stage 15 (Neurula) | |
|---|---|---|---|---|---|
| | | | Normal (%) | Normal (%) | Gastrulation defects (%) |
| Xbra-EnR | 50 | 139 | 127 (91.4) | 1 (0.7) | 125 (89.9) |
| MlBra-EnR | 50 | 113 | 101 (89.4) | 1 (0.9) | 100 (88.5) |
| EnR | 50–100 | 87 | 86 (98.9) | 84 (96.6) | 2 (2.3) |

failure of gastrulation by the injection of MlBra-EnR RNA is due to the inhibition of the convergent extension.

*Injection of MlBra-EnR causes down-regulation of Xbra targets but not mesodermal genes*

We hypothesized that MlBra-EnR would inhibit the downstream targets of endogenous Xbra proteins. To examine this, the expression of *Xbra* itself was at first investigated (Fig. 5A–D and A′–D′) because the maintenance of *Brachyury* expression was known to require functional *Brachyury* in vertebrates (Herrmann, 1991; Schulte-Merker et al., 1994; Schulte-Merker and Smith, 1995) and the expression of ascidian *Brachyury* was shown to be autoregulated via the Brachyury-binding motif in its 5′ flanking region (Takahashi et al., 1999). At the middle gastrula (stage 11) just before the gastrulation defects appear, comparison between controls and Xbra-EnR-injected embryos was carried out by using riboprobes synthesized from the 3′ UTR of *Xbra* cDNA to detect the endogenous *Xbra* mRNA but not the injected Xbra-EnR RNA. *Xbra* expression in Xbra-EnR-injected embryos was strongly down-regulated at the dorsal lip of the blastopore where Xbra-EnR RNA was localized (compared Fig. 5B and B′ with C, C′, D, and D′), consistent with the previous study (Conlon et al.,1996). A similar down-regulation of *Xbra* expression was observed in the embryos injected with MlBra-EnR RNA (Fig. 5A and A'), demonstrating that the effects of MlBra-EnR were mediated by the inhibition of endogenous *Xbra* expression. Second, we investigated the expression of *Xwnt11*, which is known as a direct target of *Xbra* (Saka et al., 2000) and is involved in the convergent extension during gastrulation (Tada and Smith, 2000). Embryos injected with MlBra-EnR RNA as well as Xbra-EnR RNA decreased the expression of *Xwnt11* in the cells expressing the *lacZ* activity (Fig. 5E, E′, F and F′), compared to control embryos (Fig. 5G, G′, H and H′). Thus, MlBra-EnR inhibits *Xbra* expression, followed by *Xwnt11* repression. Finally, we examined whether the expressions of axial mesoderm genes, *chordin* and *pintallavis*, were regulated in embryos injected with RNA encoding MlBra-EnR or Xbra-EnR (Fig. 5I–L and M–P, respectively). The resultant embryos had no effect on their expressions at the mid gastrula stage, compared with controls (compared Fig. 5I, J with K, L and M, N with O, P, respectively).

*MlBra mimics Xbra activity in Xenopus animal caps*

It has been shown that *Xbra* functions as a transcriptional activator and that its activation domain lies within the C-terminal half of the protein, outside of the T-box DNA-binding domain (Conlon et al., 1996). In the diploblast *Hydra*, the C-terminal half of one *Brachyury* homologue *HyBra1* is a weaker transcriptional activator in *Xenopus* embryos than its *Xenopus* counterpart (Marcellini et al., 2003) and that of another homologue *HyBra2* has a different role than *HyBra1* (Bielen et al., 2007). Therefore, we were interested in whether the full-length coding sequence of *MlBra* had the same function as *Xbra*.

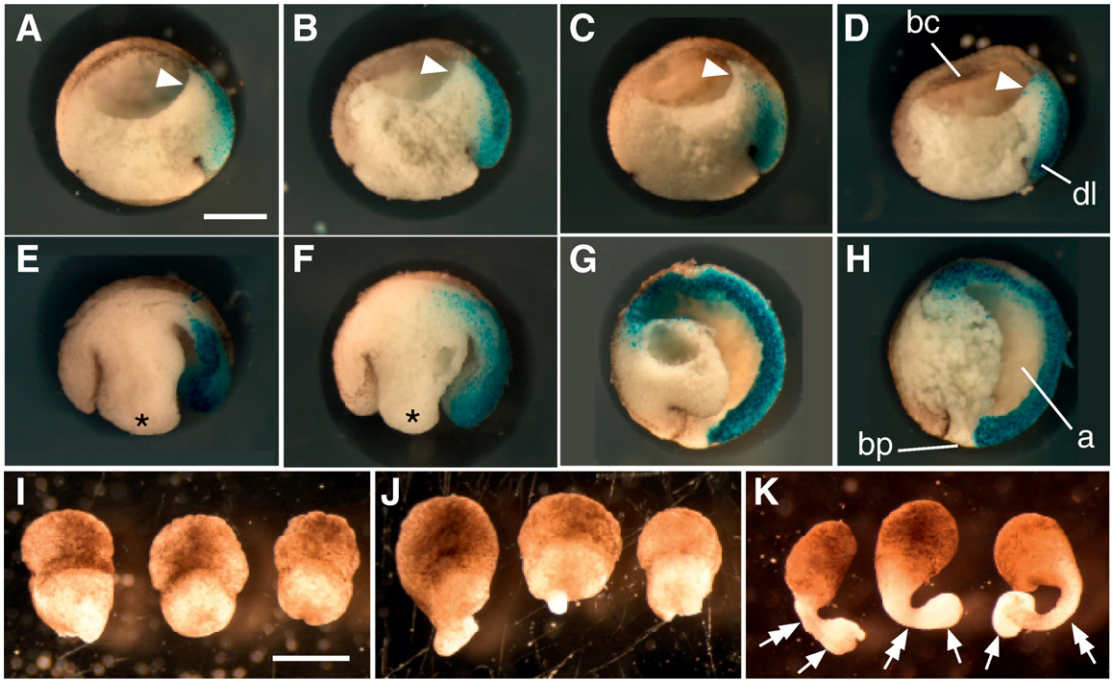


**Fig. 4.** Cells with introduced RNA encoding MlBra-EnR or Xbra-EnR fail to undergo convergent extension movements but not cell migration during gastrulation. (A–H) Bisected embryos to observe early cell migration at the middle gastrula (A–D) and at the late gastrula (E–H). Embryos were shown laterally with dorsal to the right and animal pole to the top. MlBra-EnR, Xbra-EnR, and EnR were injected with *lacZ* RNA into the dorsal marginal zone of 4-cell embryos (A and E, B and F, C and G, respectively). Only *lacZ* RNA was injected as controls (D and H). In the middle gastrulae, the leading edge of mesendoderm occurred normally (A–D, *arrowheads*), but embryos injected with MlBra-EnR RNA and Xbra-EnR RNA failed to close blastopore (E and F, *asterisks*) and the cells inheriting the injected RNA were blocked to move along the roof of blastocoel from vegetal side to animal pole and to future anterior side (E and F) in comparison with those in embryos injected with EnR RNA (G) and uninjected (H) as visualized by the *lacZ* lineage tracer (*blue*). (I–K) Keller explants to monitor convergent extension movements. The explants derived from embryos injected with MlBra-EnR RNA (I) and Xbra-EnR RNA (J) were inhibited to converge and extend their axial mesoderm and neuroectoderm. By contrast, control embryos underwent convergent extension of their axial mesoderm and neuroectoderm (K, *arrows* and *double arrows*, respectively). a, archenteron; bc, blastocoel; bp, blastopore; dl, dorsal lip. Scale bar, 500 μm.

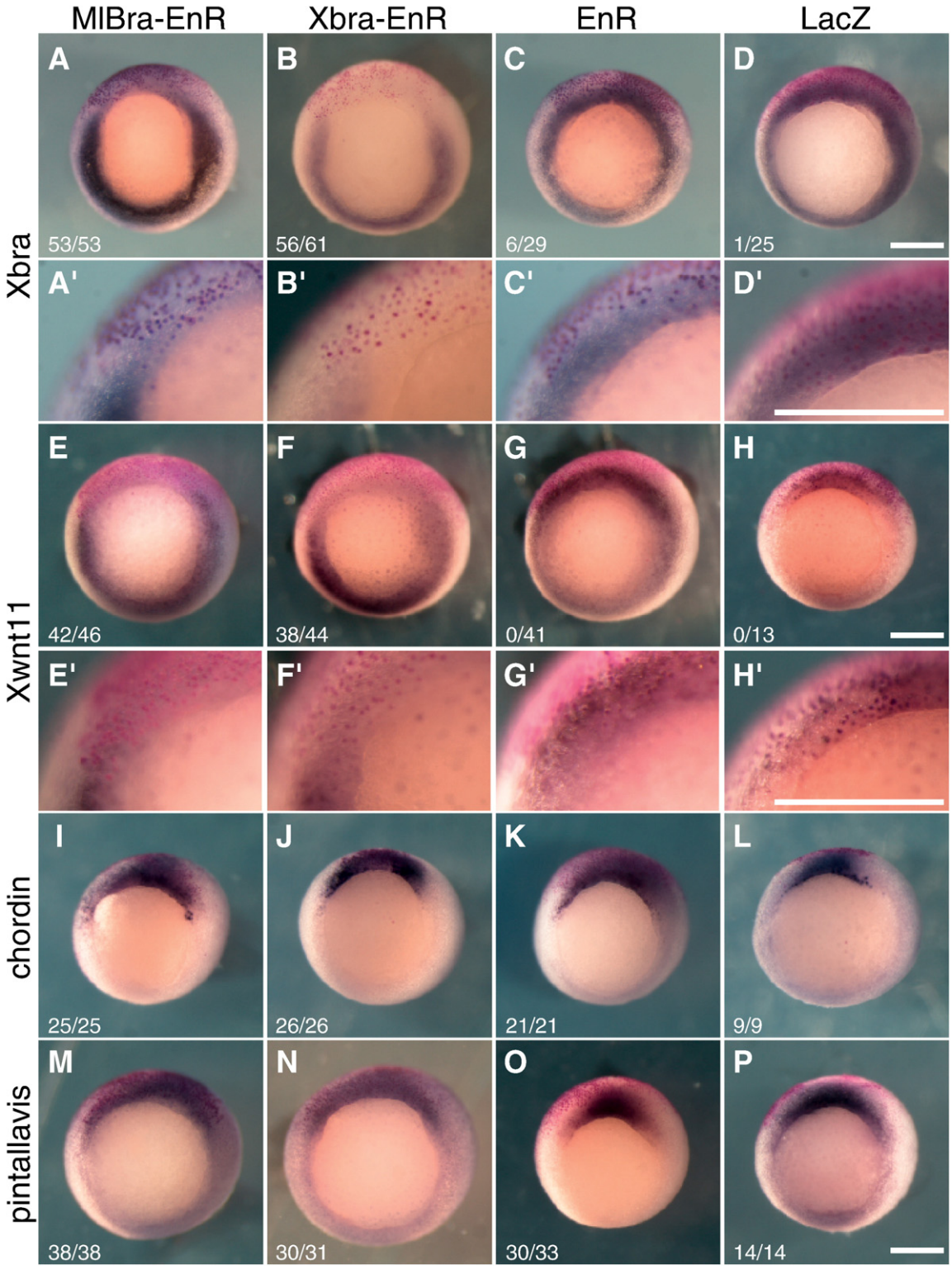


**Fig. 5.** *Xenopus* embryos injected with RNA encoding MlBra-EnR and Xbra-EnR show the similar responses at a molecular level. Vegetal views of the injected embryos hybridized using probes for *Xbra* (A–D and A'–D'), *Xwnt11* (E–H and E'–H'), *chordin* (I–L), and *pintallavis* (M–P) at the middle gastrula (stage 11) with dorsal to the top. Hybridization signals were detected in *purple*. *LacZ* RNA was co-injected into all embryos and its activity was stained in *pink*. Embryos were injected with RNAs encoding MlBra-EnR (A, A′, E, E′, I and M), Xbra-EnR (B, B′, F, F′, J and N), and EnR (C, C′, G, G′, K and O), together with *lacZ* RNA. As injection controls, *lacZ* alone were injected (D, D′, H, H′, L and P). The regions inheriting the injected RNA were enlarged from photographs A–D and E–H to A′–D′ and E′–H′, respectively. The expressions of *Xbra* and *Xwnt11* were down-regulated in cells expressing *lacZ* activity, that is, including RNA encoding MlBra-EnR (A, A′ and E, E′, respectively) and Xbra-EnR (B, B′ and F, F′, respectively), but not in embryos introduced RNA encoding EnR alone (C, C′ and G, G′, respectively) and in controls (D, D′ and H, H′, respectively). Frequency of the abnormal phenotype was shown in each panel (A–D and E–H). By contrast, the expressions of *chordin* and *pintallavis* were little affected in any embryos, irrespectively of the kinds of injected RNA (I–L and M–P, respectively). Frequency of the similar expression pattern to controls was shown in each panel (I–L and M–P). Scale bar, 500 μm.

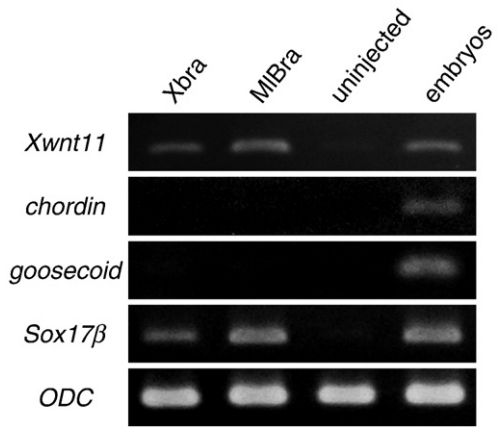


**Fig. 6.** RT-PCR analysis of animal caps injected with *MlBra* RNA or *Xbra* RNA. *MlBra* and *Xbra* induced expression of *Xwnt11* and *Sox17*β in animal caps, but not *chordin* and *goosecoid*. Expression of ornithine decarboxylase gene (*ODC*) was used as an internal control. Without reverse transcriptase, any PCR products were not amplified (data not shown).

To test this, we compared the effect of overexpression of *MlBra* in the *Xenopus* animal caps with that of *Xbra* (Fig. 6). Injection of 100 pg of *Xbra* RNA induced *Xwnt11* strongly and an endodermal gene *Sox17*β weakly but had little effect on an axial mesodermal gene *chordin* and an anterior mesodermal gene *goosecoid*, which were induced by a different T-box gene *VegT* (Horb and Thomsen, 1997; Conlon et al., 2001). Similar inductions of *Xwnt11* and Sox17β were detected in the explants injected with 100 pg of *MlBra* RNA, although *MlBra* induced Sox17β more strongly than *Xbra*. In contrast, *chordin* and *goosecoid* were hardly detectable in *MlBra*-injected explants.

## Discussion

### *Function of MlBra*

The expression of ctenophore *MlBra* around the blastopore and in the invaginating stomodeum implies that *MlBra* might be involved in gastrulation and/or morphogenesis of the stomodeum/pharynx, but its role has not been confirmed by functional analyses (Yamada et al., 2007). In this work, we demonstrated that *MlBra* plays an important role in ectodermal cell movements during stomodeum invagination although the possibility cannot be excluded that it is also involved in the stomodeal differentiation because we have no stomodeal markers (Fig. 1 and 2). However, the thickened mass of putative stomodeal/pharyngeal cells around the blastopore in embryos in which *MlBra* function was inhibited were quite distinct from lateral epidermal cells, or endodermal and mesodermal cells that had invaginated earlier in gastrulation. In addition, in preliminary experiments that MlBra-MO was injected into one of two blastomeres at the two-cell stage, we noted in several of such embryos that the ectodermal cells in the injected side invaginated comparably to intact embryos (Supplemental Fig. 1). This observation could be interpreted as MlBra-MO-injected cells being rescued by their wild-type neighbors by providing necessary signals that allows the injected cells to invaginate into the stomodeum/pharynx or that the uninjected cells could mechanically pull the injected cells into the invaginating stomodeum/pharynx. Recovery of cell behavior of injected cells was also reported in sea urchin embryos in which *Brachyury* functions were inhibited in one blastomere during the two-cell stage and indicated the possible nonautonomy in *Brachyury* downstream activity (Gross and McClay, 2001). In either case, we suggest that the major role of *MlBra* in ctenophore development is to regulate the morphogenetic movements during stomodeal and pharynx formation.

### *The functional similarities of MlBra and Xbra*

We showed that the effects of MlBra-EnR in *Xenopus* embryos were remarkably similar to those of Xbra-EnR at both morphological and molecular levels (Figs. 3, 4 and 5). These results suggest the possibility that MlBra-EnR could prevent the convergent extension during *Xenopus* gastrulation via the same genetic pathway as Xbra-EnR, that is, by inhibiting the maintenance of endogenous *Xbra* expression and blocking *Xwnt11* function. Moreover, synthetic RNA encoding full-length *MlBra*-coding sequence was able to induce the *Xbra* downstream gene *Xwnt11* but not Spemann's organizer genes, *chordin* and *goosecoid* (Fig. 6), thus mimicking the endogenous *Xbra* activity. Taken together, it is likely that *MlBra*, especially its T-box DNA-binding domain, conserves the same ability to activate the common molecular components underlying the convergent extension as that of *Xbra* during metazoan evolution.

We noted that the injection of *MlBra* led to the stronger expression of an endodermal gene *Sox17*β than *Xbra* (Fig. 6). This might indicate that *MlBra* has high activity for endoderm induction. Although *Xbra* itself induces no endoderm in animal caps, it has been known that *Brachyury* orthologues from ascidians and *Drosophila* also possess endoderm-inducing activity conferred by its short N-terminal domain and T-box domain (Marcellini et al., 2003). These two domains of *MlBra* might confer somewhat derived properties on its protein. Alternatively, there is the possibility that the T-box domain of *MlBra* could contact target genes of the other T-box family gene *VegT*, which functions in not only mesoderm formation but also endoderm specification. VegT protein has previously been shown to share very similar target DNA sequences with Xbra (Conlon et al., 2001), indicating that *MlBra* might act on *VegT* target genes. However, this does not seem to be the case, as *VegT* induces expressions of *chordin* and *goosecoid* (Conlon et al., 2001) while in our assay neither *MlBra* nor *Xbra* did not (Fig. 6). Furthermore, expression of VegT-EnR inhibits the formation of the dorsal blastopore lip of the organizer when injected on the dorsal side (Horb and Thomsen, 1997) although MlBra-EnR and Xbra-EnR both did not (Fig. 3A and B).

*Xbra* is expressed throughout the mesoderm in a circumblastoporal ring at the gastrula stage (Smith et al., 1991). In our preliminary experiments, we injected with RNA encoding Xbra-EnR or MlBra-EnR into various marginal zones of *Xenopus* four-cell embryos. As a result, ventral and lateral injections led to the down-regulation of *Xbra* expression in ventral and in lateral, respectively (data not shown). These data indicate that the autoregulation of *Xbra* expression occurs not only in dorsal mesoderm but also in ventral and lateral mesoderm. Thus, *Xbra* likely controls various degrees of convergent extension required throughout the mesoderm to ensure proper blastopore closure via radial-lateral convergent extension movements.

### *Conserved roles of Brachyury during metazoan evolution*

The roles of *Brachyury* on the morphogenetic movements around the blastopore are well established in vertebrates (Chesley, 1935; Grüneberg, 1958; Beddington et al., 1992; Halpern et al., 1993; Wilson et al., 1995; Conlon et al., 1996; Wilson and Beddington, 1997; Conlon and Smith, 1999, Kwan and Kirschner, 2003) and a sea urchin (Gross and McClay, 2001). In addition, *Brachyury* functions are reported in posterior gut formations of insects (Kispert et al., 1994; Singer et al., 1996; Shinmyo et al., 2006), in stomodeal formation of hemichordates and sea urchin (Tagawa et al., 1998; Gross and McClay, 2001), in convergent extension movements during ascidian notochord formation (Hotta et al., 2007), and in murine allantois elongation (Inman and Downs, 2006). In relation to these data, our studies in the ctenophore not only revealed that *MlBra* is necessary for the invagination of stomodeal cells around the blastopore (Figs. 1 and 2), but also predicted that *MlBra* is involved in the formation of tentacular bulbs and apical organ (Yamada et al., 2007). In the latter two domains,

the morphogenetic movements such as invagination are also present (Martindale and Henry, 1997a). As shown in Fig. 1F, these two domains were not observed in most of MlBra-MO injected embryos although thickened epithelial cells related to tentacle apparatus were detectable in the histological observation (data not shown). However, we could not distinguish whether MlBra-MO injection directly inhibited the formation of these two domains or whether they did not form secondarily due to the failure of stomodeal invagination. Lineage specific perturbation of *MlBra* functions will be required to uncover all roles of *MlBra*. Taken together, these data clearly demonstrate that a primitive role of *Brachyury* is to activate transcription of genes required for the morphogenetic movements such as invagination and convergent extension and that the role has been highly conserved during metazoan evolution. Morphogenetic movements accompany dynamic change in cell shape, and therefore it is expected that genes playing roles in cell adhesion and cytoskeleton are activated by *Brachyury*. One of such genes might be a component of the Wnt/planar cell polarity (PCP) pathway. Members of the Wnt/PCP signal transduction pathways, *prickle* and *Xwnt11* are known to work in convergent extension during ascidian notochord formation and *Xenopus* gastrulation, respectively (Conlon and Smith, 1999; Hotta et al., 2007). The involvement of members of Wnt/PCP pathway in ctenophore stomodeal invagination is therefore an intriguing question although these genes have not identified from ctenophores yet.

Moreover, another suggestion from the present work is that interference with *Brachyury* functions might cause apoptosis. We noted that the ctenophore embryos injected with MlBra-MO tend to fall apart, which might represent the induction of apoptosis. Apoptosis has also been reported in embryos in which *Brachyury* functions were prevented from fly, frog and mouse (Yanagisawa et al., 1981; Singer et al., 1996; Conlon and Smith, 1999). It would be interesting to predict that the secondary conserved role of *Brachyury* is to be involved in regulating apoptosis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.12.019.

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