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Original Article

Xenopus glucose transporter 1 (xGLUT1) is required for gastrulation movement in *Xenopus laevis*

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ABSTRACT Glucose transporters (GLUTs) are transmembrane proteins that play an essential role in sugar uptake and energy supply. Thirteen GLUT genes have been described and GLUT1 is the most abundantly expressed member of the family in animal tissues. Deficiencies in human GLUT1 are associated with many diseases, such as metabolic abnormalities, congenital brain defects and oncogenesis. It was suggested recently that *Xenopus GLUT1 (xGLUT1)* is upregulated by Activin/Nodal signaling, although the developmental role of xGLUT1 remains unclear. Here, we investigated the expression pattern and function of xGLUT1 during *Xenopus* development. Whole-mount *in situ* hybridization analysis showed expression of *xGLUT1* in the mesodermal region of *Xenopus* embryos, especially in the dorsal blastopore lip at the gastrula stage. From the neurula stage, it was expressed in the neural plate, eye field, cement gland and somites. Loss-of-function analyses using morpholino antisense oligonucleotides against *xGLUT1* (xGLUT1MO) caused microcephaly and axis elongation error. This elongation defect of activin-treated animal caps occurred without downregulation of early mesodermal markers. Moreover, dorsal-marginal explant analysis revealed that cell movement was suppressed in dorsal marginal zones injected with xGLUT1MO. These findings implicate xGLUT1 as an important player during gastrulation cell movement in *Xenopus*.

KEY WORDS: *Xenopus, glucose transporter, gastrulation, cell movement*

Introduction

Glucose transporters (GLUTs) are crucial for the efficient movement of glucose across cellular plasma membranes. To date, 13 GLUT-like genes (GLUT1-12 and HMIT) have been identified in mammals, although GLUT6 was identified as a pseudogene and GLUT7 has been shown to be a cloning artifact (Burchell, 1998; Joost and Thorens, 2001). In spite of the high degree of sequence similarity, GLUT family members exhibit distinct intracellular localizations, affinities for sugar and responses to insulin. For example, GLUT1 is mainly expressed in brain and erythrocytes, while GLUT2 is predominantly found in liver and GLUT4 in muscle and adipocytes (Gould and Holman, 1993). GLUTs contain 12 membrane-spanning helices, which are separated by hydrophilic loops and are thought to constitute the substrate binding site (Walmsley *et al.,* 1998). GLUT1 is the most well characterized transporter of this family due to its availability in high amounts from human erythrocytes. It contains inward- (endofacial) and outward- (outfacial) facing sugar-binding sites and one potent mechanism for the kinetics of GLUT1 sugar transport proposes that the transporter exists in one of two conformations, exposing either the endofacial or the exofacial binding site with sugar translocation occurring as the transporter oscillates between the two conformations (Walmsley *et al.,* 1998). GLUT1 is an important player in glucose homeostasis in the brain of human infants (Virgintino *et al.,* 2000) and inherited heterozygous mutations of the *GLUT1* gene results in diseases such as seizures, microcephaly and ataxia in human development (Wang *et al.,* 2005). Recently, a link between glucose metabolism and apoptosis was reported (Danial *et al.,* 2003; Nutt *et al.,* 2005) and inhibition of GLUT1 induces apoptosis in fish neural development

Abbreviations used in this paper: GLUT, glucose transporter; MO, morpholino oligonucleotide; WISH, whole-mount in situ hybridization.

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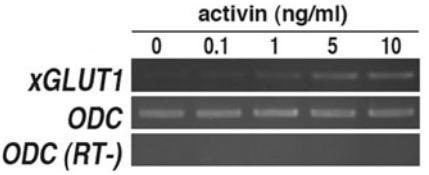
(Jensen *et al.,* 2006). Although these reports suggest that GLUT1 regulates cell survival, other developmental roles of GLUT1 remain to be elucidated.

Activin and nodal are both activators of activin receptors, which in turn phosphorylate Smad2 and trigger the nuclear translocation of Smad2/4 complexes. Recently, the *Xenopus GLUT1* homologue, *xGLUT1*, was identified as a candidate target gene for forkhead activin signal transducer-1 (FAST1) (Hayata *et al.,* unpublished data), a key mediator of Activin/Nodal signaling, where it acts as a co-factor for Smad2. Activin/Nodal signaling is also critical for the formation of mesoderm (Asashima *et al.,* 1990; Takahashi *et al.,* 2000).

In *Xenopus* embryo, mesoderm cells begin to express genes coding for factors that regulate the convergent extension movements of gastrulation, a critical morphogenetic process.–In mesodermal tissue, this process occurs via radial and mediolateral intercalation. At the mid-gastrula stage, the prospective notochordal and somitic mesodermal cells become bipolar in shape and their protrusions extend medially and laterally. Some components of the planar cell polarity pathway that controls epithelial cell polarity of sensory bristles and the eye in *Drosophila* are also involved in convergent extension movements in vertebrate (Adler and Lee, 2001; Adler and Taylor, 2001; McEwen and Peifer, 2000; Mlodzik, 1999; Shulman *et al.,* 1998; Strutt and Strutt, 1999). The non-canonical Wnt/PCP signaling pathway, which regulates planar cell polarity in invertebrates and vertebrates, also plays an essential role in controlling convergent extension movements during gastrulation. In *Xenopus* embryo, the T-box transcription factor xbrachyury (xbra) promotes the expression of *xWnt11*, which activates the Wnt/PCP pathway (Tada and Smith, 2000). Dominant-negative and loss-of-function analysis identified several regulators of this pathway, including xfz7 (Medina *et al.,* 2000), xdsh (Sokol, 1996; Wallingford *et al.,* 2000), Daam1 (Habas *et al.,* 2001), dapper (Cheyette *et al.,* 2002), xstbm (Goto and Keller, 2002) and xDal (Kobayashi *et al.,* 2005). Some of these have PDZ or PDZ-binding motifs, which are thought to be essential for Wnt/PCP signal transduction.

In this study, we showed that *xGLUT1* is zygotically expressed in the mesodermal region in *Xenopus* and acts downstream of Activin/Nodal signaling. Loss-of-function analyses were performed to clarify the role of xGLUT1 in *Xenopus* development. Knockdown of xGLUT1 caused severe morphological abnormalities, including microcephaly, short axis and gastrulation error, but had no effect on the expression levels of mesodermal marker genes. In addition, time-lapse analysis of cell movement in cells lacking



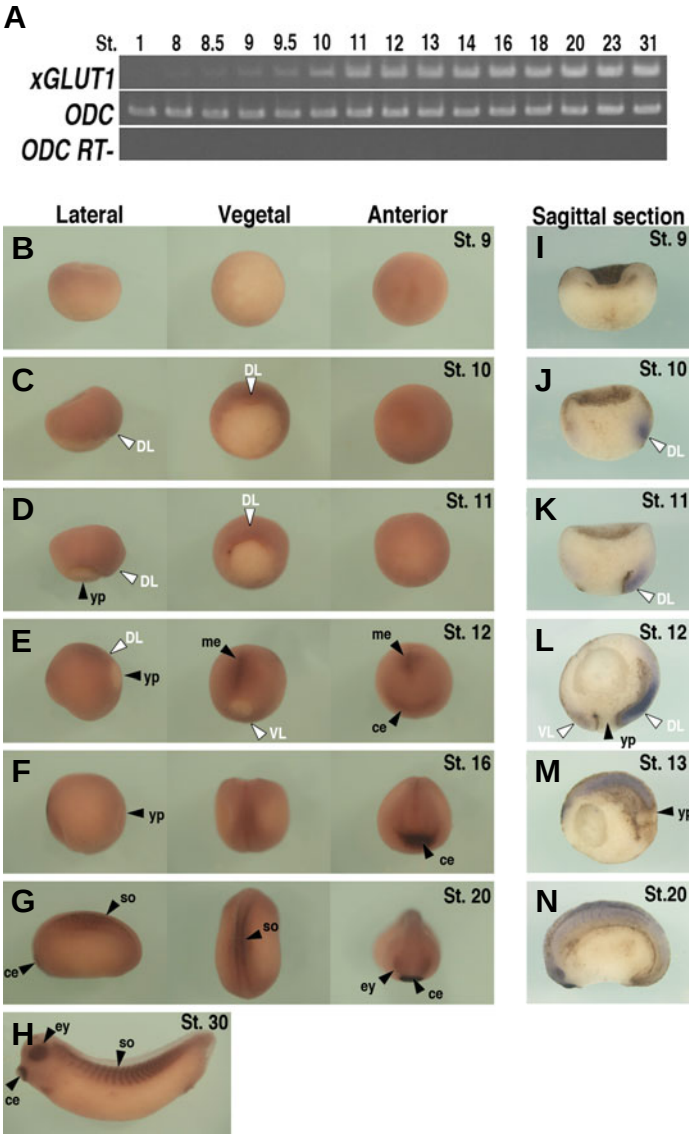


Fig. 1 (Left). *xGLUT1* expression in activin-treated animal caps. *Animal caps were treated with 0-10 ng/ml of activin for 5 hours. A low level of* xGLUT1 *expression was detected in animal caps treated with 1 ng/ml activin, whereas high levels of* xGLUT1 *expression could be seen in animal caps treated with 5 or 10 ng/ml activin.* ODC *was used as a loading control.*

Fig. 2 (Right). Expression patterns of *xGLUT1*. (A) *Temporal expression of* xGLUT1 *was analyzed by RT-PCR.* xGLUT1 *expression was not detected maternally. At the blastula stage, low levels of* xGLUT1 *expression were detected. Expression levels increased at stage 9.5, with high levels of expression being maintained by stage 31. Spatial patterns of* xGLUT1 *expression were analyzed by* in situ *hybridization.* **(B-N)** *Spatial expression patterns of* xGLUT1 *analyzed by whole mount* in situ *hybridization.* **(B-H)** *Outer view of albino embryos. Left column, lateral view; middle column, vegetal view; right column, anterior view.* **(I-N)** *Sagittal sections of wild-type embryos. Signal was detected as blue staining.* **(C,D,J,K)** *From late blastula stage,* xGLUT1 *mRNA expression was seen in the mesodermal region, especially in the dorsal lip (DL, white arrowhead).* **(E,L)** *At the late gastrula,* xGLUT1 *expression was extended to the ventral lip (VL, white arrowhead).* **(M)** *At stage 13, ventral expression had disappeared, but intense expression was maintained in the deeply involuted dorsal-mesodermal region.* **(F-H, N)** *From the neurula stage, expression was detected mainly in the cement gland, eye fields and somite. DL, dorsal lip; VL, ventral lip; yp, yolk plug; me, mesoderm; ce, cement gland; so, somite; ey, eye field.*

xGLUT1 revealed a loss of polarity as well as slow and random movements. Our results indicate that xGLUT1 plays an essential role in cell movement during gastrulation.

Results

Activin dependent expression of xGLUT1

The *xGLUT1* gene was identified as a candidate target gene of Activin/Nodal signalling factor, FAST1 (Hayata *et al.,* unpublished data). To evaluate the activin dependent enhancement of *xGLUT1* expression, we performed RT-PCR analysis using animal caps. *Ornithine decarboxylase (ODC)* was used as a loading control. No xGLUT1 expression was detected in animal caps in the absence of activin treatment. When animal caps were treated with 1 ng/ml of activin for 5 hours, *xGLUT1* expression was slightly induced and strong expression was observed following treatment with 10 ng/ml of activin (Fig. 1). Previous reports showed that 5-10 ng/ml of activin could induce mesodermal tissue in animal caps, thus our results suggested that *xGLUT1* expression is upregulated under mesoderm-inducing conditions.

Temporal and spatial expression of xGLUT1

To determine the temporal patterns of *xGLUT1* expression, we performed RT-PCR with cDNAs synthesized from embryos at various stages. Before gastrulation, *xGLUT1* expression was not detected, but was increased at stage 9.5 and maintained throughout the post-neurula stages (Fig. 2A).

Next, we performed *in situ* hybridization to determine the spatial expression pattern of *xGLUT1*. Before gastrulation, *xGLUT1* expression was not detected (Fig. 2B, I). At stage 10, *xGLUT1* expression was observed at low levels around the yolk plug and strong expression was detected in the dorsal lip (Fig. 2C, J). During gastrulation, the *xGLUT1* expression area extended marginally and narrowed (Fig. 2D, E). Cross sections of the midline revealed that *xGLUT1* expression was in the involuting dorsal mesodermal region and the ventral side of the blastopore during the gastrula stage (Fig. 2K and L). At the neurula stage, *xGLUT1* expression in the ventral lip had disappeared and transcript expression was detected mainly in the cement gland primordia and weakly in the anterior neural plate (Fig. 2F). From the tail bud stage, *xGLUT1* expression was observed in the cement grand, eye field, somite and part of the spinal cord (Fig. 2G, H).

xGLUT1MO *injection caused defects in gastrulation and neurulation*

To clarify the *in vivo* role of xGLUT1, we designed a morpholino antisense oligonucleotide against *xGLUT1* (xGLUT1MO) and examined the phenotypes of xGLUT1MO-injected embryos. At

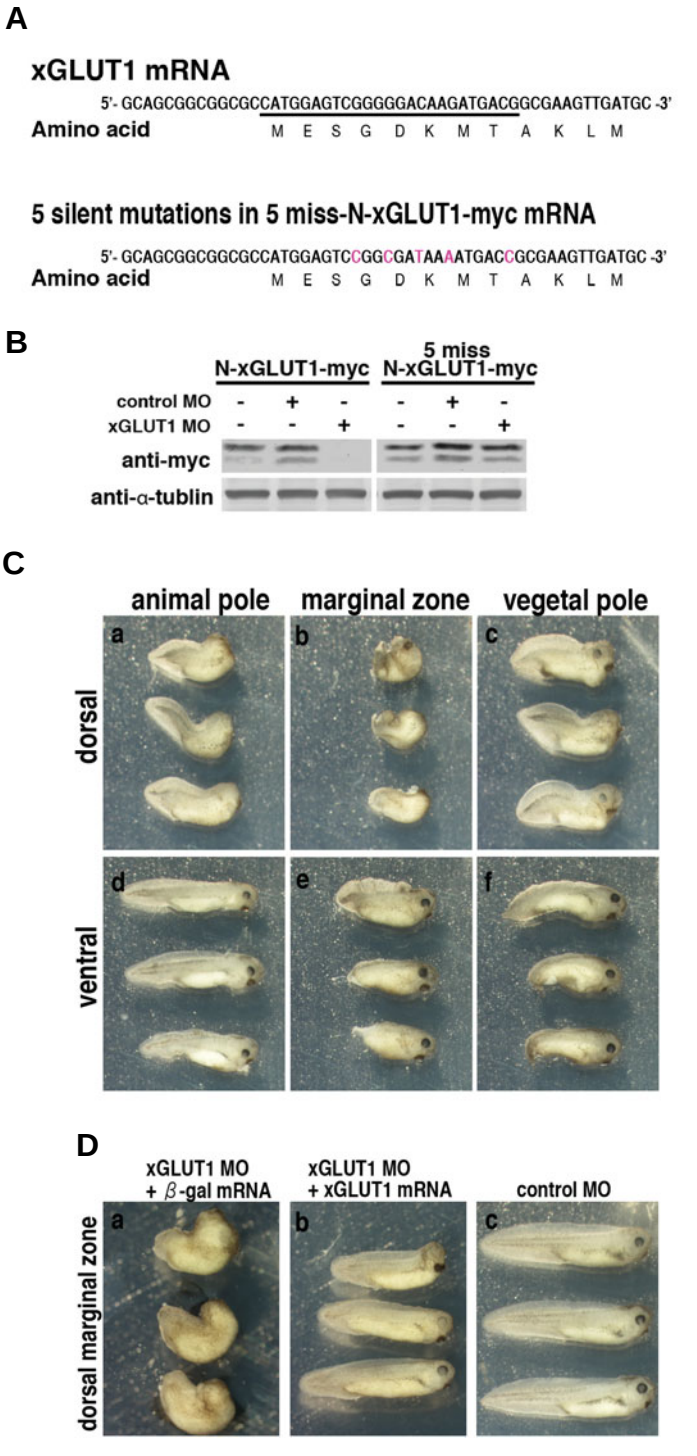


Fig. 3. *xGLUT1MO* caused gastrulation error and loss of head structure. (A) *Nucleotide sequence of* xGLUT1 *mRNA and* 5 miss-N-GLUT1-myc *mRNA. xGLUT1MO recognized the sequence indicated by the black line in the upper column. 5 silent mutations in* 5miss-N-xGLUT1-myc *mRNA are shown as pink letters in the lower column.* **(B)** *Western blot analysis of myc-tagged xGLUT1 fusion protein. Either 1 ng of* N-xGLUT1-myc *or* 5miss-N-xGLUT1-myc *mRNA was coinjected with 20 ng of xGLUT1MO or standard control MO into four blastomeres of a 4-cell stage embryo. Western blot analysis was performed with anti-myc antibody. Anti-α-tubulin antibody was used as a loading control. The translation of* N-xGLUT1-myc *mRNA was inhibited by the xGLUT1MO.* **(C)** *Phenotype of embryos injected with 20 ng of xGLUT1MO.* **(C.a-c)** *Two dorsal blastomeres were injected.* **(C.d-f)** *Two ventral blastomeres were injected. (a) xGLUT1MO injection into dorsal animal pole caused short axis and head disruption. (b) Dorsal marginal zone injection caused severe axis defect and neural tube closure defect. (c) Dorsal vegetal injection showed a weaker phenotype. (d-f) Ventral injection also showed an axis defect but the level of severity was weak.* **(D)** *Rescue of xGLUT1MO-derived defect by* xGLUT1 *mRNA. 10 ng of xGLUT1MO and 500 pg of* beta-galactosidase (β-gal) *mRNA* **(D.a)** *or* xGLUT1 *mRNA* **(D.b)** *was coinjected into two dorsal marginal zones of a 4-cell stage embryo. Obvious rescue was observed by addition of* xGLUT1 *mRNA.*

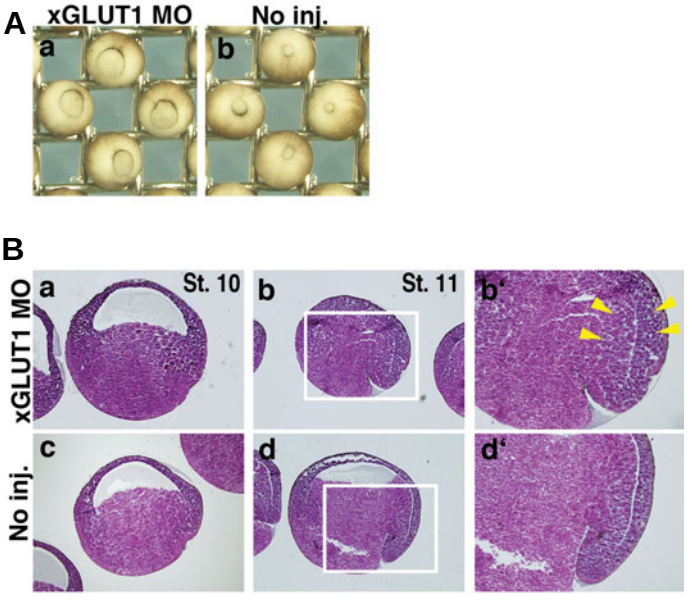


Fig. 4 (Left). *xGLUT1MO* caused gastrulation defect. (A) *Blastopore closure was delayed by xGLUT1MO injection compared with normal embryos.* **(A.a)** *10 ng of xGLUT1MO was injected into the dorsal marginal zone.* **(A.b)** *Control embryo.* **(B)** *Sagittal sections of the gastrula. (a,c) stage 10. (b,b',d,d') stage 11. (a-b') 10 ng of xGLUT1MO was injected into the dorsal marginal zone. (c-d') Control embryo. (b') and (d') are high magnification images of the white boxes in (b) and (d), respectively. In xGLUT1MO-injected embryos, the involuting marginal zone and inner surface of the deep layer remained thick (b', yellow arrowheads).*

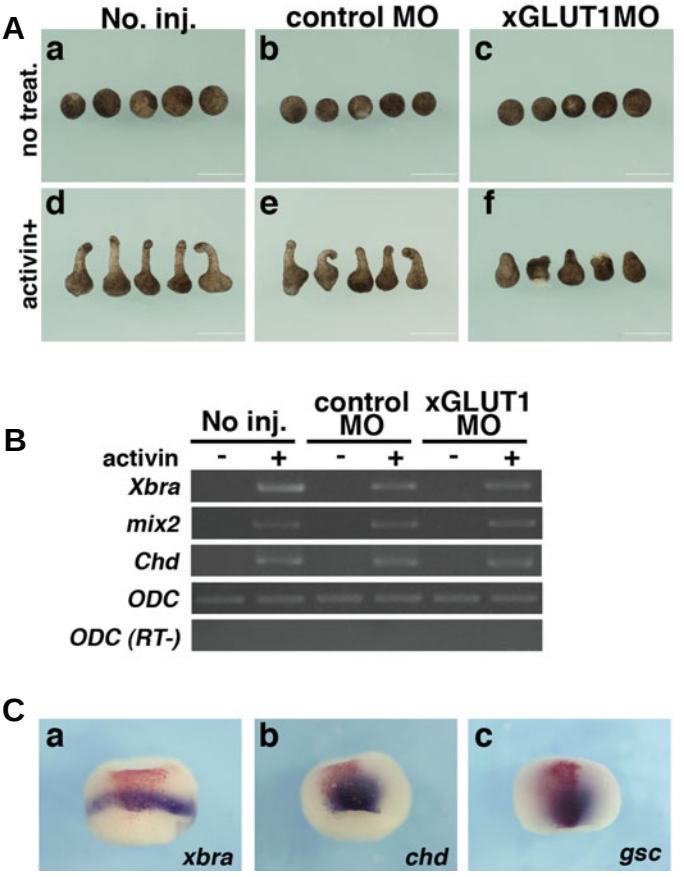


Fig. 5 (Right). Mesodermal genes were not influenced by inhibition of *xGLUT1*. (A) *xGLUT1MO inhibited elongation of animal caps. 20 ng of xGLUT1MO or control MO was injected into the animal pole of an 8-cell stage embryo. At late blastula, animal caps were dissected and treated with 10 ng/ml of activin and cultured for 10 hours. Without activin treatment, caps remained round* **(A.a-c)** *and activin-treated caps dissected from control embryos (d) or standard control MO-injected embryos (e) became elongated in response to activin. However, the elongation of xGLUT1MO-injected caps was inhibited* **(A.f)**. **(B)** *Expression of mesodermal marker genes in animal caps injected with xGLUT1MO. Animal caps were treated with activin for 3 hours and sampled for RT-PCR.* Xbra, mix2 and Chd *transcript levels were not influenced by xGLUT1MO injection.* ODC *was used as a loading control.* **(C)** *Whole-mount* in situ *hybridization (WISH) with late gastrula. 10 ng of xGLUT1MO and 100 pg of* β-gal *mRNA was coinjected into one side of the dorsal marginal zone of 4-cell stage embryos. Before WISH, the embryos were stained with Red-gal for cell lineage tracing. xGLUT1MO injection caused no change in* xbra *(a),* chd *(b), or* gsc *(c) expression.*

first, we check the MO specificity. Western blot analysis revealed that xGLUT1MO reduced the translation of N-GLUT1-myc fusion protein, but not that of a 5miss-N-GLUT1-myc protein negative control (Fig. 3B). We then injected the xGLUT1MO into several parts of 4-cell stage embryos, which were subsequently cultured for 4 days at 16ºC. Injection of xGLUT1MO into the dorsoanimal region caused a short axis and disrupted head formation (Fig. 3C-a), while xGLUT1MO injection into the dorsal marginal zone induced short and bent axis formation and microcephaly (Fig. 3C-b). Vegetal injection with xGLUT1MO still caused short axis formation but the phenotype was weak (Fig. 3C-c) and xGLUT1MO injection into the marginal zone or vegetal region of the ventral blastomere caused a slight short-axis phenotype and the embryos seemed to lack ventroposterior structure (Fig. 3C-e, f). The defect caused by ventroanimal injection of the xGLUT1MO appeared the weakest (Fig. 3C-d).

To confirm that these phenotypes were actually derived from loss of xGLUT1 function, we coinjected xGLUT1MO and *xGLUT1* mRNA. This injection clearly rescued the axis abnormality, whereas when xGLUT1MO was coinjected with *beta-galactosidase (β-gal)* mRNA, a short and bent axis phenotype was still strongly observed (Fig. 3D).

xGLUT1MO *injection caused a gastrulation defect*

As shown in Fig. 3, xGLUT1MO injection into the dorsal blastomere caused axis defects. A previous study showed that such phenotypes are often apparent with abnormal gastrulation (Habas *et al.,* 2001). Indeed, the xGLUT1MO-injected embryos showed an obvious delay in blastopore closure in comparison to control embryos (Fig. 4A). To analyze this gastrulation defect in more detail, we prepared histological sections of the gastrulae stained with hematoxylin-eosin (Fig. 4B). In xGLUT1MO-injected embryos, invagination of the involuting mesoderm and inner surface of the deep layer (yellow arrowheads) was inhibited and there were markedly more intercellular spaces compared with normal embryos at stage 11 (Fig. 4B-b and b'), suggesting that xGLUT1MO caused a radial intercalation defect. At the late neurula stage, xGLUT1MO injection into the dorsal marginal zone also gave rise to a neural tube closure defect (data not shown). Together, these results indicated that xGLUT1 is required for

convergent extension movement.

Loss of xGLUT1 function did not affect mesodermal marker gene expression

Mesoderm formation is required for normal gastrulation movement. We therefore examined whether the xGLUT1MO-induced abnormal gastrulation movement was a result of disrupted mesoderm induction. First, the effect of xGLUT1MO injection on elongation of the animal caps was tested in response to activin treatment (Fig. 5A). Treatment with 10 ng/ml of activin causes animal cap elongation by inducing muscle cells, reflecting mesoderm induction (Smith *et al.,* 1990; Ariizumi and Asashima, 2001). In this study, animal caps dissected from normal blastulae and from control MO-injected embryos were elongated by activin treatment (Fig. 5A-d, e), but the elongation was clearly inhibited in xGLUT1MO-injected and activin-treated embryos (Fig. 5A-f). Next, we investigated the effect of xGLUT1MO on the expression of mesodermal marker genes. In animal caps dissected from normal embryos, mesodermal markers expressions were upregulated by activin treatment, while in the animal caps from xGLUT1MO-injected embyos, expressions of mesodermal markers, *xbra, mix2, chordin (chd)* and *goosecoid* (*gsc,* data not shown) were unchanged (Fig. 5B). Furthermore, *in situ* hybridization of the xGLUT1MO-injected embryos revealed no effect on the transcription of mesodermal genes, including *xbra, chd* and *gsc* (Fig. 5C). These experiments therefore suggested that xGLUT1 is not required for induction of mesoderm in *Xenopus*.

Effect of* xGLUT1MO *on gastrulation movement

Our experiments thus far indicated that the gastrulation defect caused by xGLUT1MO was not due to an inhibition of mesoderm induction. We next tested the influence of xGLUT1 on convergent extension movement in the dorsal marginal zone by dorsal sandwich explant analysis, which is an established way to examine cell movement during gastrulation (Keller, 1991; Saint-Jeannet *et al.,* 1994).

We dissected dorsal marginal zones from the gastrula previously injected with xGLUT1MO or standard control MO into the dorsal marginal zone at the 4-cell stage. Two dorsal marginal explants were put together to make dorsal sandwich explants, which were cultured for 12 hours. Elongation was observed in explants from normal embryos or those injected with the standard control MO (Fig. 6B-a and b), whereas it was clearly inhibited in the xGLUT1MO-injected embryo explants (Fig. 6B-c).

We further examined cell movement by examining dorsal open-faced explants (Fig. 7). When one dorsal marginal explant alone is observed on a fibronectin-coated dish, the marginal cells will show the latitudinal formation of notochord in spite of longitudinal elongation. In normal dorsal open-faced explants, the marginal cells will migrate exhibiting latitudinal polarity and form the 'dorsal ridge' bilaterally across the explants (Davidson *et al.,* 2004). Disorder of these movements in xGLUT1MO-injected explants would reflect abnormal dorsal cell movement, thereby implicating *xGLUT1* expression in this process. To test this possibility, we examined open-faced double-labelled explants as shown in Fig. 7A and green-fluorescein-labelled cells were chased by time-lapse imaging microscopy. After 24 hours, the explants developed as shown in Fig. 7B. Cell movement (white boxes) was shown at 3-h intervals (Fig. 7C) and 30-min trajectories of cell nuclei were overlaid on an image of earlier nuclei positions. Cells migrated respectively from the green points to the orange points. In the normal explants, unidirectional movements were observed. At 9 hours after observation the cells migrated along a bilateral polarity (Fig. 7C-f) and the cell shape was long and narrow (Fig. 7C-h). In contrast, the xGLUT1MO injected explants exhibited randomly moving cells without polarity (Fig. 7E-a, b, c) and the cell shape remained spherical (Fig. 7C-g). These movements were obviously different from those of normal embryos, suggesting strongly that xGLUT1 is required for marginal cell movement at gastrulation.

Discussion

In this study, we investigated the developmental role of xGLUT1 in early *Xenopus* development. We showed *xGLUT1* expression in the dorsal lip region of mesoderm at the late blastula stage, in a similar manner to Nodal/Activin downstream genes *Chd*, *gsc* and *mix2*. Taken together with the evidence that activin A treatment promotes the expression of *xGLUT1* in animal caps, we suggest that the expression of *xGLUT1* is induced by Activin/Nodal signaling at the blastula stage in *Xenopus* development. Loss-of-function analysis using xGLUT1MO suggested that xGLUT1 is required for gastrulation movement. This result is consistent with the expression pattern of *xGLUT1* in dorsal mesoderm, where the convergent extension movement is actively occurring. As shown in Fig.3C, injection with xGLUT1MO into the dorsal marginal zone caused more severely abnormal phenotypes such as short axis and neural tube closure defect compared with injection into animal poles. From these observations, we propose that xGLUT1 has a stronger effect on the

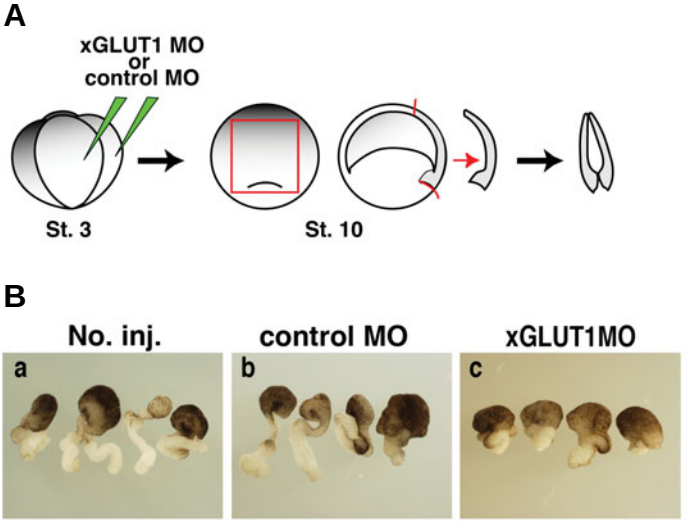


Fig. 6. Dorsal sandwich explant analysis on *xGLUT1MO*-injected embryos. (A) *Schematic diagrams of making dorsal sandwich explants. Four-cell stage embryos were injected with 40 ng of xGLUT1MO or control MO into the dorsal marginal zone of two dorsal blastomeres. At stage 10, the dorsal marginal zones were dissected and two explants were put together to make a sandwich, which was cultured for 12 hours.* **(B)** *Dorsal sandwich explants from normal embryo (a) or standard control MO-injected embryo (b) showed elongation, whereas in explants injected with xGLUT1MO, elongation was inhibited (c).*

convergent extension movement in mesoderm cells than in ectoderm cells. In *Xenopus*, gastrulation involves important morphological movements requiring the precise execution of physiological events such as mesoderm induction, cell cycle regulation and Wnt/PCP signal transduction (Saka and Smith, 2001; Smith *et al.,* 1990; Wallingford *et al.,* 2002). A key question therefore is how xGLUT1 functions in this cell movement? We demonstrated here that xGLUT1MO caused no remarkable changes in expression of mesodermal markers (Fig. 5) and overexpression of *xGLUT1* induced neither expression of mesodermal markers nor ectopic axis formation (data not shown). From these results, we speculated that xGLUT1 regulates cell movement in gastrulation independently from mesoderm induction. A previous study showed that inhibition of GLUT1 by antisense *GLUT1* mRNA causes G1 arrest in cultured cell (Noguchi *et al.,* 2000). It is therefore possible that xGLUT1 regulates cell movement via control of cell cycle.

Inhibition of GLUT1 was also shown recently to induce apoptotic cell death in the presumptive neural region during embryonic brain development in zebrafish (Jensen *et al.,* 2006). In spite of these findings, neither gain- nor loss- of xGLUT1 induced remarkable apoptosis of mesoderm cells in this study (data not shown). These conflicting results might be due to differences in the area injected. As this study predominantly used xGLUT1-MO injection into dorsal marginal zones, defects in neural development would be unlikely to have been detected. In the mesoderm, xGLUT1 might contribute not to cell survival but to cell movement, with its energy supplied via glucose uptake. The sugar uptake ability of xGLUT1 was not analyzed here, however, xGLUT1 possesses the relevant amino acid motif (R-X-G-R-R) conserved among GLUT-family members that determines the membrane topology required for the sugar transport activity of GLUT1 (Sato and Mueckler, 1999). Classical experiments in amphibian showed that glycolysis starts before gastrulation begins and that glycogen distribution in the dorsal blastopore lip is higher than in the ventral regions at the late blastula and gastrula stages (Miranda, 1977; Raddatz and Lovtrup-Rein, 1986; Saxen and Toivonen, 1962). These findings suggest that energy for gastrulation movement is supplied by glycolysis, implying that xGLUT1 might be required for this glycolysis via its glucose uptake activity.

An alternative possibility is that xGLUT1 regulates convergent extension movement via a novel function that is independent of glucose uptake. For example, normal Wnt/PCP signal

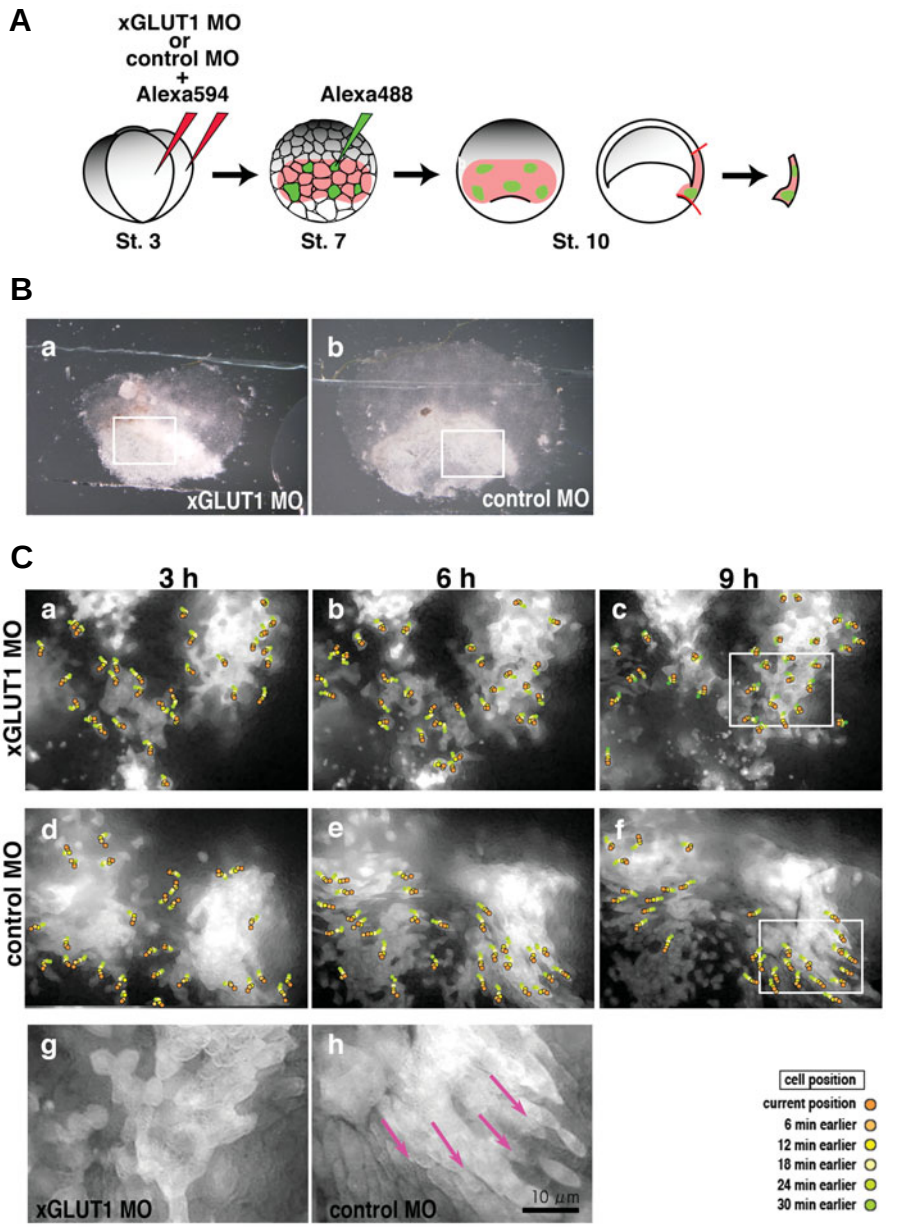


Fig. 7. *xGLUT1MO* inhibited cell movement in dorsal open-faced explants. (A) *Schematic diagram showing construction of the dorsal open-faced explants. To observe cell behavior, scattered double-labelled embryos were prepared. At the 4-cell stage, 20 ng of xGLUT1MO or control MO was coinjected with 1 ng of Alexa594 (red) into two dorsal blastomeres. At stage 7, 50 pg of Alexa488 (green) was injected into several dorsal blastomeres for making scattered labeled cells. At stage 10, the dorsal marginal region was dissected with any involuted endodermal and mesodermal cells shaved off and observed by time-lapse imaging.* **(B)** *Dorsal open-faced explants 24 hours after dissection. (a) xGLUT1MO-injected embryo explants. (b) Standard control MO-injected embryo explants.* **(C)** *Cell movement observed in white box of Fig. 7B.* **(a-c)** *Explant from xGLUT1MO-injected embryo.* **(d-f)** *Explant from standard control MO-injected embryo.* **(g,h)** *Magnified images of areas indicated by white boxes in (e) and (h). Frames from a time-lapse imaging sequence of a single explant expressing Alexa488-green fluorescence at 3-h intervals. 30-min trajectories of cell nuclei were overlaid on an image of earlier nuclei positions. Orange indicates the current position, while yellow-orange, yellow, light yellow, light green and green indicates the position of 6, 12, 18, 24 and 30 min earlier, respectively. In control MO-injected explants, cell shape was long and narrow and the cells showed polarized movement (pink arrows), whereas cell shape in the xGLUT1MO-injected explants was round and lacked polarity.*

transduction might be required for interaction of xGLUT1 with Wnt/PCP pathway mediators, such as Dvl. The GLUT1 C-terminal binding protein (GLUT1CBP) contains a PDZ domain that is required for its interaction with GLUT1 (Bunn *et al.,* 1999) and xGLUT1 also contains the four C-terminal amino acids that are required for GLUT1CBP binding. The PDZ domain also exists in Dvl and is essential for the interaction of Dvl with another Wnt/PCP pathway mediator, Daam1 (Habas *et al.,* 2001). From these findings, it is possible that xDvl interacts with the C-terminal region of xGLUT1 via the PDZ domain and that the interaction is required for normal Wnt/PCP signaling.

In this study, we showed that xGLUT1 is involved with convergent extension movement during gastrulation, though it remains unclear whether the regulation of cell movement occurs via glucose transport or other novel mechanisms. Detailed studies to investigate how xGLUT1 regulates the convergent and extension movement should be undertaken in the future. To date, interest in GLUT1 has focused mainly on its glucose uptake ability. It is likely that future studies will reveal novel roles for this protein in fields such as embryology. Until now, more than 10 GLUT-family members have been identified and functional analysis of other GLUT-family members may also provide interesting findings for developmental biology.

Materials and Methods

Eggs and embryo

Eggs of *Xenopus laevis* were obtained by injecting 300 IU of human chorionic gonadotropin (Gestron, Denka Seiyaku) into *Xenopus laevis* females. Fertilized embryos were dejellied by 4.5% cysteine hydrochloride solution (pH 7.8) and cultured in 10% Steinberg's solution. Developmental stages were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956).

Cloning of xGLUT1 *and construction of xGLUT1-myc fusion protein*

The cDNA clone coding for xGLUT1, XL208m06, was obtained from Xdb3 in the National Institute for Basic Biology (http://xenopus.nibb.ac.jp/). To obtain xGLUT1-myc fusion protein, the N-terminus of xGLUT1 fragment was amplified by PCR using the primers,

5'-GTCACTTTGTGTTTCTGTAGCAG-3' and

5'-CCACAGGGACTCGTTCCCC-3' and cloned into pCS2-myc vector. This construct was named *N-xGLUT1-myc*. Mutants of *N-xGLUT1-myc,* 5miss-N-xGLUT1-myc, containing 5 silent mutations in the morpholino oligonucleotide-binding region were created by PCR.

RT-PCR

Total RNA was extracted from whole *Xenopus* embryos or animal caps using ISOGEN (Nippon Gene). First-strand cDNAs were synthesized from 1 μg of the total RNA with oligo (dT) primers, using the reverse transcription enzyme, Superscript^{TM}II (Invitrogen). PCR was performed with the following primers:

xODC
5' GTCAATGATGGAGTGTATGGATC 3' and
5' TCCATTCCGCTCTCCTGAGCAC 3'
xGLUT-1
5'-TGGCTCTACTGGAGTCTGTC-3' and
5'-ACATATTGGAAGCCCATGCC-3'
Chd
5'-AACTGCCAGGACTGGATGGT-3' and
5'-GGCAGGATTTAGAGTTGCTTC-3'
gsc
5'-GACAGTTGCACGTACAGACG-3' and
5'-CTGGTTAAGCAACTGCAGC-3'
Xbra
5'- AGCCTGTCTGTCAATGCTCC -3' and
5'-ACTGAGACACTGGTGTGATGG-3'
mix2
5'-GCCTCCAAAAGAATACCAACTG-3' and
5'-ATTTTGTTGGTCTGGAAGCTGT-3'

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed according to a previous method (Harland, 1991). Digoxigenin (DIG)-labeled antisense mRNA probes were transcribed from pBS-*xGULT1* (*xGULT1* inserted in pBluescript II (-)) using RNA polymerase, following by linearization with appropriate restriction enzymes. Albino embryos were fixed with MEMFA. For cross section observation, fixed wild-type embryos were cut with a surgical knife after embedding in 3% low-melting agarose gel solution (3% low melting agarose, 1 M PBS, 0.3 M sucrose.) The embryos were hybridized with DIG-labeled probes, followed by anti-DIG antibody reaction. Embryos were stained using NBT/BCIP (Roche).

Inhibition of xGLUT1 function by morpholino oligonucleotides

xGLUT1MO (5'-CGTCATCTTGTCCCCCGACTCCATG-3') and standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA -3') were purchased (GeneTools, USA). MOs were suspended in sterile water and then injected into *Xenopus* eggs according to the mRNA injection method. Injected eggs were cultured in 10% Steinberg's solution at 16^{O}C.

xGLUT1-MO *specificity determined by western blotting*

mRNAs were transcribed using N-GLUT1-myc-pCS2 or 5miss-N-GLUT1-myc-pCS2 as templates and injected into the animal pole of 4-cell stage embryos. Injected eggs were lysed in 200 μl SDS-PAGE buffer at stage 9. The mixtures were subjected to SDS-PAGE and probed with the anti c-myc antibody (catalog number: SC789; Santa Cruz Biotechnology).

Microinjection

Capped mRNAs were produced using mMessage mMachine kit (Ambion). Fertilized *Xenopus* eggs were dejellied with 100% Steinberg's solution containing 4.5% L-cysteine hydrochloride monohydrate and cultured in 100% Steinberg's solution. Four- to eight-cell stage embryos were injected with the capped mRNAs and/or MOs into the indicated region in 5% Ficoll. After injection, embryos were cultured in 10% Steinberg's solution and lysed in ISOGEN (Nippon Gene) for RNA extraction.

Animal cap elongation assay

Animal cap assay was performed as previously reported (Tanegashima *et al.,* 2004). Eggs injected with the indicated mRNA(s) or MO into animal poles were cultured in 5% Ficoll. Animal caps dissected from stage-8 embryos were treated with 10 ng/ml activin for 5 hours and washed with 10% Steinberg's solution. Some animal caps were then sampled for RT-PCR analysis and the remainder were cultured overnight in 10% Steinberg's solution at 16^{O}C and then examined.

Dorsal sandwich explants and dorsal open-faced explants assay

The dorsal-marginal zone was dissected from the embryos previously injected with xGLUT1MO at stage 10 and endodermal and outer epithelia cells were shaved off using an eyebrow hair knife. For dorsal sandwich explant analysis, 2 dorsal explants were put together according to a method previously described (Keller, 1991). For dorsal open-faced explant analysis, the deep surfaces of the explants were placed on fibronectin-coated cover glasses, cultured and imaged as described previously (Davidson *et al.,* 2004; Goto and Keller, 2002). Low-light fluorescence imaging and time-lapse recording of cell behavior were carried out for 24 hours with a digital camera (Hamamatsu, Japan), an inverted compound microscopy IX81SF1F-2 (Olympus, Japan) and a Metamorph imaging system (Universal Imaging Corp., USA).

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