The role of XTRAP-γ in Xenopus pronephros development

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ABSTRACT We isolated and characterized the Xenopus translocon-associated protein XTRAP-γ, one of four subunits of the translocon-associated protein complex. TRAP has been proposed to aid the translocation of nascent polypeptides into the lumen of the endoplasmic reticulum, but this has not been demonstrated until now. XTRAP-γ was specifically expressed in the pronephros tubules of Xenopus laevis from stage 25 during kidney development. Antisense morpholino oligonucleotide-mediated knockdown of XTRAP-γ suppressed tubulogenesis and decreased expression of the pronephros marker genes Pax-2 and Wnt-4. XTRAP-γ morpholinos also inhibited differentiation of the pronephros in activin/retnoi acid-treated animal caps. We conclude that XTRAP-γ plays an important role in the process of pronephros differentiation.

KEY WORDS: XTRAP-γ, Xenopus laevis, pronephros, Pax-2, Wnt-4

Introduction

During vertebrate development a succession of embryonic kidneys, known as the pronephros, mesonephros and metanephros, regulate fluid balance, osmolarity and disposal of metabolic waste products. The embryonic kidneys develop from the intermediate mesoderm, following a precise temporal and spatial sequence. Each successive kidney is formed as a result of an inductive interaction with the previous form. The pronephros, mesonephros and metanephros share a common basic functional unit, the nephron, but increase in complexity with respect to the number and organization of nephrons (Vize et al., 1995). The pronephros is the first to be established and is the simplest excretory organ in vertebrates. The simple tubule and excretory system of the pronephros provides an ideal model for studying kidney organogenesis. The precursors of the pronephros, derived from the intermediate mesoderm, are determined by develop-ment of the mesonephros and metanephros, including Lim-1, Pax-2, Pax-8, Wnt-4 and WT-1 (Chan et al., 2000, McLaughlin et al., 2000, Saunier et al., 2002, Seville et al., 2002). The regulatory networks of genes and proteins involved in pronephros development remain poorly defined and the initial events in kidney organogenesis are not yet fully understood. The aim of the present study was to use whole-mount in situ hybridization to identify novel genes expressed during pronephros development in Xenopus following loss-of-function experiments. Using this approach, we identified XTRAP-γ, which encodes the translocon-associated protein (XTRAP). XTRAP-γ is one of four subunits that makes up the translocon-associated protein complex. It is believed that this complex plays a role in the translocation of nascent polypeptides, but until now this had not been demonstrated. The translocation process is initiated by when the nascent polypeptide binds to a signal recognition particle (SRP) (Rapoport, 1992), which enables the complex to target a signal sequence receptor (Walter and Lingappa, 1986) on the ER membrane at specific

Abbreviations used in this paper: ER, endoplasmic reticulum; MO, morpholino; SRP, signal recognition peptide; XTRAP, Xenopus translocon-associated protein.

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The translocon core is composed of a Sec61p complex that is thought to form a protein-conducting channel through the ER membrane to allow transport of polypeptide into the ER lumen (Rapoport et al., 1996), (Beckmann et al., 1997). However, the localization of the translocon-associated protein and its interaction with Sec61p has not been fully defined.

The present study highlights the importance of TRAP in protein translocation during development. Although it was previously assumed that TRAP was ubiquitously expressed, the present study clearly shows that during Xenopus development XTRAP-γ is specifically expressed in the otic vesicles, pronephros tubules, eye and liver from stage 25 onward, coinciding with the time cells are condescending and differentiating into pronephric tubules. Therefore, we hypothesized that XTRAP-γ may play a role in pronephric tubulogenesis. In addition, the function of XTRAP-γ in pronephros development was further analyzed by loss-of-function experiments using morpholino antisense oligonucleotides injection. Pronephric tubulogenesis was inhibited by XTRAP-γ morpholinos and associated with a decrease in expression of the Pax-2 and Wnt-4 marker genes. These results suggest XTRAP-γ is essential for pronephros development.

**Results**

**Temporal and spatial expression of XTRAP-γ in Xenopus development**

The Xenopus TRAP subunit γ (XTRAP-γ) was isolated by screening a cDNA library constructed from the lateral mesoderm of Xenopus neurulae. XTRAP-γ was selected for further study as it was specifically expressed in Xenopus pronephros tubules. The TRAP complex, previously called the signal sequence receptor complex, consists of four integral membrane subunits (α, β, γ, δ) (Hartmann et al., 1993). The XTRAP-γ subunit was speculated to span the membrane four times and is thought to be responsible for interaction of the other subunits of the TRAP complex. The deduced amino acid sequence of XTRAP-γ (GenBank accession number AB058505) contains 153 amino acids, with 93% and 94% identity to human and rat TRAP-γ, respectively (Fig. 1A). The temporal expression of XTRAP-γ was analyzed by RT-PCR. Transcripts of XTRAP-γ were detected in unfertilized egg and XTRAP-γ expression increased from stage 12 until the tadpole stage. RT:- control reverse transcription without transcriptase.

**Table 1**

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<th>RESULTS OF XTRAP-γ MO AND 5-MIS-XTRAP-γ MO MICROINJECTION OR CO-INJECTION WITH 5-MIS-XTRAP-γ mRNA</th>
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Fig. 2. Whole-mount in situ hybridization analyses demonstrate the localization of XTRAP-γ transcripts. The expression of XTRAP-γ was weakly ubiquitous during all stages of development. (A-F) Localization of XTRAP-γ was observed from stage 12 onward. (D) Condensed XTRAP-γ signals were detected in the otic vesicles (arrowhead) and pronephric tubules (arrow) from stage 25 onward. (E) In the early tadpole stages, XTRAP-γ transcripts were localized in the eyes (arrow). (F) By stage 33/34, XTRAP-γ was expressed in liver (arrow). (G-I) Histology section of the Xenopus embryo from Fig. 2F. e, eye; ov, otic vesicle; p, pronephros; l, liver.

shown). XTRAP-γ signals were also strongly detected in the otic vesicles and pronephric tubules from stage 25 onward (Fig. 2D). In the early tadpole stages, XTRAP-γ transcripts were detected in the eyes (Fig. 2E) and by stage 33/34, XTRAP-γ was expressed in the liver (Fig. 2F). Stage 33/34 Xenopus embryo had specific localization of XTRAP-γ expression in the eye, otic vesicle, pronephros and liver (Fig. 2G, 2H, 2I).

Morpholino-mediated knockdown of XTRAP-γ causes failure of tubulogenesis during pronephros development

XTRAP-γ was over-expressed in Xenopus embryos to analyze the role of XTRAP-γ in Xenopus embryogenesis. This yielded no apparent phenotype (data not shown). To further investigate the role of XTRAP-γ, loss-of-function experiments using two XTRAP-γ antisense morpholinos were performed. The XTRAP-γ morpholino (XTRAP-γ MO) was used for translational inhibition of internal XTRAP-γ and the 5-mis-XTRAP-γ MO was designed to incorporate five mispairs relative to XTRAP-γ MO. HA-tagged XTRAP-γ mRNA was synthesized to demonstrate the effect and specificity of the XTRAP-γ MO. The HA-tagged XTRAP-γ was translated in vivo and its translation is blocked by XTRAP-γ MO. However, the 5-mis-XTRAP-γ MO did not affect the translation of HA-tagged XTRAP-γ (Fig. 3A).

Whole-mount in situ hybridization revealed that XTRAP-γ expression is predominantly localized in the pronephric tubules from stage 25. To investigate the role of XTRAP-γ in normal pronephros tubulogenesis, XTRAP-γ MO was injected into one side of the lateral marginal zone of 32-cell-stage embryos. Both the C-2 and C-3 blastomeres of embryos were injected with morpholinos, corresponding to cells of the presumptive anterior somites (C-2) and posterior somites, pronephros and lateral plate (C-3), respectively (Dale and Slack, 1987). Only phenotypically normal embryos with β-galactosidase activity in the region of the pronephros were scored for the effects of morpholinos on pronephric development (Table 1). Formation of pronephric tubules was inhibited significantly on the side injected with XTRAP-γ MO. Tubule branches were shorter and reduced in number (Fig. 3B), compared with the non-injected-side (Fig. 3C). To determine whether the failure of pronephros tubulogenesis formation was due to secondary effects of morpholinos, one side of the embryos were injected with 5-mis-XTRAP-γ MO. Pronephros development was normal in 5-mis-XTRAP-γ MO injected embryos (Fig. 3D). The phenotype caused by injection of XTRAP-γ MO was rescued by co-injection with 5-mis-XTRAP-γ mRNA (Fig. 3E). These observations also suggest that depletion of XTRAP-γ causes direct suppression of tubulogenesis. It was also important to deter-

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Notes: ++ The expression pattern is enhanced; + Normal; - Repressed.

Table 2: XTRAP-γ MO AFFECTS THE EXPRESSION OF PRONEPHROS MARKER GENES
mine whether $XTRAP\gamma$ MO inhibited the formation of pronephric tubules by indirect methods, such as interfering with the development of other structures such as anterior somites, which are necessary for pronephros differentiation. Gross examination and histology (Fig. 3F, 3G) show that all structures except the pronephros tubules were normal.

**XTRAP\gamma is also required for pronephros development in activin/retinoic acid-treated explants**

Pronephros differentiation can be induced by activin/retinoic acid treatment (Nakamura et al., 1992), (Uochi et al., 1997). To further examine the effect of $XTRAP\gamma$ MO specifically on pronephros and to avoid any secondary effects of $XTRAP\gamma$ MO on pronephros development, $XTRAP\gamma$ MO was injected at the 2-cell stage and animal caps were isolated at stage 9 of development for analysis. After activin/retinoic acid treatment, examination of sections revealed that pronephros differentiation was markedly inhibited by $XTRAP\gamma$ MO (Fig. 4A). Electron microscopy was used to determine the effect of $XTRAP\gamma$ MO on the differentiation of pronephric and other cell types. This confirmed the absence of typical pronephric cells with microvilli and demonstrated condensation of mesodermal cells and a radial arrangement of cells with a basal membrane, characteristic of epithelium (Fig. 4C, D), compared to the normal structure described by Osafune (Osafune et al., 2002). Muscle differentiation was normal (Fig. 4E). These observations suggest that depletion of $XTRAP\gamma$ led to suppression of tubulogenesis.

**The depletion of XTRAP\gamma at the later stages of development had an effect on the expression of other pronephric genes**

To investigate whether $XTRAP\gamma$ affected the differentiation of pronephric tubules by inhibiting marker genes, the effect of depleting $XTRAP\gamma$ expression on other pronephros-specific genes was assessed. The expression of the pronephros-specific genes Lim-1, Pax-2, Pax-8 and Wnt-4 in $XTRAP\gamma$ MO injected embryos was analyzed by whole-mount in situ hybridization. Only phenotypically normal embryos with $\beta$-galactosidase activity in the region of the pronephros were scored (Table 2). The expression of Pax-2 and Wnt-4 was noticeably suppressed (Fig. 5E, I); expression of Lim-1 increased (Fig. 5A); and expression of Pax-8 was unchanged (Fig. 5M).

In stage 22 embryos, Pax-2 and Wnt-4 expression was not strongly affected by injection of $XTRAP\gamma$ MO (Fig. 5Q, S) or 5-mis-$XTRAP\gamma$ MO (Fig. 5R, T). As $XTRAP\gamma$ is not strongly expressed in pronephros until after stage 25, the pronephric tube markers tested were not affected by perturbation of $XTRAP\gamma$ expression at stage 22. After stage 25, the expression of pronephric genes was directly inhibited by $XTRAP\gamma$ MO. Pax-2 and Wnt-4 were co-injected with $XTRAP\gamma$ MO to rescue the $XTRAP\gamma$-depleted phenotype. Embryos injected with $XTRAP\gamma$ MO were compared with the co-injected and the non-injected control embryos. Pheno-

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**Fig. 3. XTRAP\gamma MO affects pronephric tubulogenesis.** (A) $XTRAP\gamma$ MO specificity in vitro. Western-blotting analysis showed significant inhibition of $XTRAP\gamma$-HA (100 pg/embryo) protein synthesis by $XTRAP\gamma$ MO (40 ng/embryo). $XTRAP\gamma$-HA (100 pg/embryo) protein synthesis was not affected with 5-mis-$XTRAP\gamma$ MO (40 ng/embryo). (B-G) Whole-mount immunohistochemistry analysis on pronephros. (B) $XTRAP\gamma$ MO (40 ng), (D) 5-mis-$XTRAP\gamma$ MO (40 ng) and (E) $XTRAP\gamma$ MO (40 ng) plus 5-mis-$XTRAP\gamma$ mRNA (1 ng) were injected into one side of the lateral marginal zone of 16-cell-stage embryos. Embryos were fixed at stage 40. Development of the pronephros was analyzed by staining with the monoclonal antibody 3G8. (B) Pronephric tubule formation was inhibited in $XTRAP\gamma$ MO-injected embryos (arrow in B) compared with the non-injected-side (arrow in C) and the embryos injected 5-mis-$XTRAP\gamma$ MO (arrow in D). The phenotype of the pronephric tubules was rescued by co-injection of $XTRAP\gamma$ MO with 5-mis-$XTRAP\gamma$ mRNA (E). (F,G) Histological analysis of $XTRAP\gamma$ MO injected embryo and 5-mis-$XTRAP\gamma$ MO injected embryos. Pronephros tubules in $XTRAP\gamma$ MO-injected embryos showed severe developmental defects (arrow in F) compared with that of the 5-mis-$XTRAP\gamma$ MO-injected embryos (arrow in G).
Fig. 4. Induction experiments in XTRAP-γ MO-injected animal caps.
Two-cell stage embryos were injected with 40 ng/embryo XTRAP-γ MO. All animal caps were treated with activin/retinoic acid for 3 h. (A,B) For light microscopy, semi-thin sections were stained with 0.1% toluidine blue solution. (A) Pronephros differentiation was markedly inhibited by XTRAP-γ MO (arrow indicates muscle tissues) compared with (B) animal caps of the control (arrowhead indicates pronephros tissues). (C,D,E) For electron microscopy, ultra-thin sections were double stained with uranyl acetate and lead citrate. Only samples injected with XTRAP-γ MO are shown (C,D). The results show the condensation of mesodermal cells and a radial arrangement of the cells with the basal membrane (arrows), however the unique pronephric cells with microvillus was invisible. (E) Muscle differentiation was normal. Ld, lipid droplet; Yg, yolk granule. Scale bar of (A,B) is 100 μm; scale bar of (C,D) is 1 μm; scale bar of (E) is 5 μm.

Fig. 5. Whole-mount in situ hybridization shows the effect on expression of pronephros marker genes XTRAP-γ MO injection. Embryos were injected with XTRAP-γ MO (40 ng) or 5-mis-XTRAP-γ MO (40 ng) and synthetic β-galactosidase mRNA (250 ng), then fixed at stage 30/31 (A-P) and stage 22/23 (Q-T). Expression of Lim-1 was increased in the side of embryos injected with XTRAP-γ MO (A), compared with the non-injected side (B). Expression of Pax-2 and Wnt-4 was inhibited by injection of XTRAP-γ MO (E,I), compared with non-injected side (F,J). For those embryos fixed at stage 22/23, expression of Pax-2 (Q) and Wnt-4 (S) in embryos fixed at stage 22/23 was not affected by injection of XTRAP-γ MO or the control 5-mis-XTRAP-γ MO (R,T). Expression of Pax-8 was not affected by XTRAP-γ MO (M,N). The expression of Lim-1 (C,D), Pax-2 (G,H), Wnt-4 (K,L) and Pax-8 (O,P) was unchanged in embryos injected with 5-mis-XTRAP-γ MO.
pronephric tubules were still visible. Injection of XTRAP-γ MO (40 ng) into one side of the lateral marginal zone of 16-cell-stage embryos. Embryos were fixed at stage 40. Development of the pronephros was analyzed by staining with the monoclonal antibody 3G8. (A) Proximal tubule formation was inhibited in the side of embryos injected with XTRAP-γ MO (arrow in A) compared to the non-injected side (arrow in B). (C) Proximal tubule formation was significantly rescued in embryos injected with XTRAP-γ MO (40 ng) plus Pax-2 (100 ng) (arrow in C) compared to XTRAP-γ MO-injected embryos (arrow in A). Although the dorsal branches of the pronephric tubules were shorter in the injected side than in the non-injected side (arrow in D), three dorsal branches of the pronephric tubules were still visible. Injection of E XTRAP-γ MO (40 ng) plus Wnt-4 (100 pg), or G XTRAP-γ MO (40 ng) plus Pax-2 (100 ng) and Wnt-4 (100 ng) almost completely rescued pronephric tubule formation, with relatively normal pronephric tubules being observed. 

Discussion

The aim of this study was to identify novel genes involved in pronephros development. We isolated, localized and analyzed the Xenopus translocon-associated protein-γ (XTRAP-γ), which is believed to aid in the translocation of nascent polypeptides into the ER lumen. The results of this study indicate that XTRAP-γ is necessary for pronephros development. We believe that XTRAP-γ may be involved in the translocation of proteins that are important for pronephros tubulogenesis in Xenopus laevis. TRAP was first identified as an integral glycoprotein of the ER membrane and was provisionally termed signal sequence receptor (SSR) (Wiedmann et al., 1987). SSR was subsequently localized to the translocation site and was therefore renamed “translocon-associated protein” (TRAP). The TRAP complex consists of four integral membrane subunits and the TRAPγ subunit is predicted to span the membrane four times and may be responsible for interacting with the other subunits of the complex (Hartmann et al., 1993). However, the localization of TRAP on the ER membrane and the role of XTRAP-γ in the translocation of nascent polypeptides had not yet been elucidated until now. Recently, the TRAP complex was shown to function in protein transport in a substrate-specific manner (Fons et al., 2003). As XTRAP-γ expression was found to localize to pronephros tubules, a series of analyses of pronephric tubulogenesis were undertaken. Using loss-of-function analyses, we identified a clear role for XTRAP-γ in pronephric tubulogenesis. Overexpression of XTRAP-γ yielded no apparent phenotype. One possible explanation for this is that the essential translocation machinery currently (the Sec61p complex, SR and TRAM) are able to mediate a basal level of translocation for many substrates (Gorlich and Rapoport, 1993),(Voigt et al., 1996), even if the translocation of some proteins required for pronephros development also require TRAP. If, however, the expression of only XTRAP-γ is increased, the translocation of these specific proteins cannot also be increased. Depletion of XTRAP-γ by antisense morpholino resulted in shortened proximal tubules and a shortening or absence of the distal tubule of the pronephros. Transverse sections of embryos also showed inhibition of pronephros tubule differentiation on the side injected with morpholinos, but other structure had differentiated normally. Electron microscopy of animal caps treated with activin and RA showed the condensation of mesodermal cells and a radial arrangement of the cells with the basal membrane, which was characteristic of epithelium. However, the characteristic pronephric cell with microvilli was not apparent and muscle cells differentiated normally. Together, these results demonstrated that XTRAP-γ MO inhibited pronephric tubulogenesis directly and not through secondary effects. Pronephros marker gene analysis revealed a decrease in the expression of Pax-2 and Wnt-4 and an increase in the expression of Lim-1 in the sides of embryos injected with XTRAP-γ MO compared to the non-injected sides. At stage 22, however, neither XTRAP-γ MO nor 5-min XTRAP-γ MO injected embryos were able to affect the expression of Pax-2 and Wnt-4. The process of pronephric tubule formation requires changes.

Types of embryos co-injected with either XTRAP-γ MO and Pax-2, XTRAP-γ MO and Wnt-4, or XTRAP-γ MO with Pax-2 and Wnt-4, were also significantly rescued (Fig. 6C, E, G; Table 3).

Table 3

Results of XTRAP-γ MO microinjection or co-injection with Pax-2 and/or Wnt-4

| XTRAP-γ MO + | + | + | + | - |
| Pax-2 - | + | - | + | - |
| Wnt-4 - | - | + | + | - |

Normal tubules 31 (55.4%) 37 (86.1%) 39 (81.3%) 40 (88.9%) 50 (100%) Total number 56 43 48 45 50
in cell shape and extensive cell rearrangements, which must be accompanied by changes in gene expression patterns. Lim-1 is one of the earliest marker genes of pronephros: its expression is first detected during late gastrulation at stage 12/13 (Taira et al., 1994). This corresponds precisely with the occurrence of pronephric tubule lineage specification (Brennan et al., 1998). Expression of Wnt-4 was detected as early as stage 19, where it was weakly expressed throughout the pronephric mesoderm. Wnt-4 expression increased in the developing pronephric tubule until the tailbud stages (Ungar et al., 1995). Pax-2 was expressed at very low levels in the pronephric mesoderm at stage 22. However, its expression strengthened progressively throughout the tailbud stages (Heller and Brandli, 1997). The onset of expression of XTRAP-γ occurred at stage 25, so the pronephric tubule markers tested were not affected by perturbation of XTRAP-γ expression at stage 22. At later stages, progression of pronephric tubule differentiation was affected by depletion of XTRAP-γ gene function. Therefore, we proposed that the defective of pronephric tubules in XTRAP-γ MO-injected embryos was caused by a decrease in the expression of Pax-2 and Wnt-4 at a later stage. In Xenopus pronephros development, Lim-1 expression decreased at the later stages and was confined to the distal tips by the late tadpole stage. However, depletion of XTRAP-γ inhibited presumptive pronephric cells from differentiating into tubules and kept these cells at an earlier stage of tubulogenesis, so that Lim-1 expression was stronger in the injected side compared to the non-injected side. Based on these results, we propose that XTRAP-γ is necessary for pronephric tubulogenesis and that it plays a role in the differentiation of pronephros tubules by affecting the expression of marker genes Pax-2 and Wnt-4 in the pronephros. Recently, the TRAP complex was demonstrated to function in protein transport in a substrate-specific manner (Fons et al., 2003). We determined that XTRAP-γ may promote the translocation of unknown factors that are responsible for the regulation of Lin-1, Pax-2 and Wnt-4 and for the differentiation of pronephric tubules. Further analyses are required to reveal the role of XTRAP-γ in the translocation of nascent polypeptides necessary for pronephros differentiation. Although the molecular mechanisms for TRAP regulation remain unclear, this study has demonstrated a role for XTRAP-γ in vertebrate embryogenesis in pronephros development of Xenopus laevis.

Materials and Methods

Eggs and embryos

Eggs were obtained by the injecting of human chorionic gonadotropin into Xenopus laevis (Gestron: Denka Seiyaku, Japan). Staging of embryos was performed according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). The jelly coat was removed with Steinberg’s solution containing 3% cysteine hydrochloride (pH 7.8).

Construction of the cDNA library and screening

A cDNA library from the lateral mesoderm of Xenopus neurulae was constructed as reported by Sato et al (Sato et al., 2000). Subtraction and whole-mount in situ hybridization was used to identify clones expressed in the pronephric region. Using this process, XTRAP-γ was selected for further analysis.

Morpholino nucleotide and microinjection

Morpholino oligonucleotides were obtained from Gene Tools LLC. The sequence of XTRAP-γ antisense morpholinos were: XTRAP-γ MO, 5'-GCTGTTGGCCAGCTTTCCAGGCACT-3'; 5-mis-XTRAP-γ MO, 5'-GCATGTGCTGCTATCGCAGGAT-3'. Genbank searches failed to detect significant homologues of the morpholinos in the Xenopus genome. Microinjection was carried out according to the method described by Chan et al (Chan et al, 2000).

In vitro translation of XTRAP-γ and Western blotting

The XTRAP-γ ORF was amplified by PCR and cloned into pCS2-HA (Nitta et al., 2004) to generate the XTRAP-γ-HA fusion protein. XTRAP-γ proteins with HA tags were detected using the 3F10 monoclonal antibody (Santa Cruz). The 5-mis-XTRAP-γ mRNA was constructed according to Yabe et al (Yabe et al., 2003). For the secretion assay, the mRNAs were injected into defolliculated oocytes and Western blotting was performed as described previously (Kaneko et al., 2003).

Preparation of test solution

The test solution contained 10 ng/ml activin A (kindly provided by Dr. Yuzuru Eto, Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan), 104 mol/l retinoic acid and 0.1% BSA in Steinberg’s solution.

Animal cap assay

The vitellin membrane of the blastula embryos was removed with fine forceps and the presumptive ectoderm was isolated using tungsten needles. Both presumptive mesodermal and endodermal cells were removed from the isolated ectoderm. Ectoderm sheets were immediately transferred into Steinberg’s solution and cultured at 20°C in a 24-well plate (Sumilon MS-8024; Sumitomo Bakelite, Tokyo, Japan). Explants were fixed after a four-day incubation for histological examination.

Light and electron microscopy

Ectoderm sheet explants were fixed in prefixed solution (3% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate buffer at pH 7.4) overnight at room temperature. After several buffer washes, the specimens were post-fixed in 1% OsO4 and 0.1 M cacodylate buffer at pH 7.4 for 2 h, then washed, dehydrated with alcohol and acetone and embedded in epoxy resin. Semi-thin sections (approximately 1 µm) were prepared for light microscopy and stained with 0.1% toluidine blue solution. For electron microscopy, ultra-thin sections (80-90 nm) were stained with uranyl acetate and lead citrate, then examined under an electron microscope (JEM-1010 or JEM-2000CX; JEOL, Tokyo, Japan).

RT-PCR

Total RNA isolation and RT-PCR were performed as described previously (Chan et al., 2000). The following primer sets were used: XTRAP-γ sense: 5'-AGGACAAGGCAAGTGTAGGC-3', antisense: 5'-TGAGGAGATCATTGCAGG-3'; ODC (ornithine decarboxylase) sense: 5'-TCCATGTAGGAGGTATGATGATC-3', antisense: 5'-TCCATTTGCTCTCCTGAGC-3'.

Whole mount in situ hybridization

Single probe in situ hybridization was performed according to Harland (Harland, 1991). Antisense digoxigenin (DIG)-labeled RNA probes were transcribed from full-length XTRAP-γ, Lim-1, Pax-2, Wnt-2 and Wnt-4 constructs. DNA was linearized and transcribed with RNA polymerase to generate antisense transcripts. A series of albino embryos encompassing a range of developmental stages were hybridized with the labeled probes. The color reaction was performed using NBT/BCIP.

Immunohistochemistry and histological examination

Histological analyses were performed as described previously (Chan et al, 2000). For immunohistochemistry, specimens were fixed in MEMFA for 1.5 h at room temperature, assayed for β-galactosidase activity, refixed for 30 min, washed in PBS and then dehydrated in methanol overnight. Embryos were rehydrated in PBS and then incubated in primary antibody, a pronephric tubule specific antibody 3GB, at 4°C overnight. Binding of an alkaline phosphatase-conjugated secondary
antibody, Goat anti-mouse IgG (BIOSOURCE), to 3G8 was visualized using NBT/BCIP. After staining, specimens were fixed in MEMFA and dehydrated in methanol overnight. Wild-type embryos were bleached in 10% H2O2 for 6 h. Specimens were transferred to ethanol and xylene and then embedded in paraffin. Embedded specimens were sectioned at 10 μm.

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