

# *Pax2* overexpression in embryoid bodies induces upregulation of *integrin $\alpha 8$* and *aquaporin-1*

Akihiro Nakane · Yoshiyuki Kojima · Yutaro Hayashi · Kenjiro Kohri · Shinji Masui · Ryuichi Nishinakamura

Received: 24 August 2008 / Accepted: 24 September 2008 / Published online: 27 November 2008 / Editor: J. Denry Sato  
© The Society for In Vitro Biology 2008

**Abstract** The transcription factor Pax2 is essential for kidney development in mice, and overexpression of Pax2 in chick embryos leads to ectopic formation of nephric structures. We have generated embryonic stem (ES) cell lines that repress Pax2 expression in a tetracycline-dependent manner. In the absence of tetracycline, embryoid bodies derived from these cell lines expressed Pax2 and subsequently *integrin  $\alpha 8$*  and *aquaporin-1* (*Aqp1*), both of which are possibly involved in kidney development. Considering the slow induction kinetics, our data suggest that Pax2 and additional factors that are induced in embryoid bodies synergistically regulate the two targets. The ES cell lines with inducible Pax2 expression will also be useful for dissecting genetic cascades functioning in a variety of organ development.

**Keywords** Embryonic stem cell · Pax2 · Integrin  $\alpha 8$  · Aquaporin-1

---

A. Nakane · R. Nishinakamura (✉)  
Division of Integrative Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan  
e-mail: ryuichi@gpo.kumamoto-u.ac.jp

A. Nakane · Y. Kojima · Y. Hayashi · K. Kohri  
Department of Nephro-urology, Graduate School of Medical Sciences, Nagoya City University, Nagoya City, Japan

S. Masui  
Division of Molecular Biology and Cell Engineering, Department of Regenerative Medicine, Research Institute, International Medical Center of Japan, Tokyo, Japan

R. Nishinakamura  
Global COE Cell Fate Regulation Research and Education Unit, Kumamoto University, Kumamoto, Japan

## Introduction

ES cells are continuously growing stem cells originally isolated from the inner masses of blastocysts and provide a potentially unlimited source for generating highly specialized cells and tissues in vitro. ES cells aggregate into embryoid bodies (EB), structures comprising three germ layers that interact with each other. By using embryoid body formation and other methods, ES cells can be differentiated into various cell lineages, for example, pancreatic cells (Lumelsky et al. 2001), hepatocytes (Kania et al. 2003; Yamamoto et al. 2003), motor neurons (Wichterle et al. 2002), and hematopoietic cells (Kyba et al. 2002; Daley 2003). However, few papers have described the induction of the kidney lineage from ES cells. Shuldiner et al. demonstrated that human ES cells cultured with growth factors, including hepatocyte growth factor and activin A, differentiated into cells expressing *Wt1* and *renin*. Kobayashi et al. showed that *Wnt4*-transformed mouse ES cells cultured with the same growth factors differentiated into cells expressing aquaporin (AQP)2 (Kobayashi et al. 2005). Kim et al. revealed that mouse embryoid bodies treated with a combination of activin A, retinoic acid, and bone morphogenetic protein 7 (BMP7) expressed markers of the kidney lineage and formed epithelial structures in vitro (Kim and Dressler 2005). However, the induction efficiency in these experiments is still low and needs to be improved.

The kidney develops in three stages: the pronephros, mesonephros, and metanephros. The nephric duct (Wolffian duct) and nephrogenic mesenchyme develop from the intermediate mesoderm, which is located between the lateral and preaxial mesoderm. The Wolffian duct and nephrogenic mesenchyme elongate caudally towards the cloaca, and the Wolffian duct converts the adjacent

mesenchyme into mesonephric tubules. Metanephric mesenchyme is formed in the caudal-most region of the nephrogenic mesenchyme. The metanephros is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. Upon induction by the ureteric bud, the metanephric mesenchyme differentiates into the epithelia of glomeruli and renal tubules.

Gene targeting studies have revealed many genes that are involved in these processes of kidney development, including *Pax2*, *Lim1*, *Wt1*, *Sall1*, *Hoxa11*, *Pod1*, *Gdnf*, and *integrin  $\alpha 8$*  (Vainio and Lin 2002; Dressler 2006). We previously showed that *Sall1*, a zinc finger nuclear factor that is expressed in the metanephric mesenchyme, is essential for metanephros development and that mesenchyme expressing high level of *Sall1* contains multipotent progenitors for the epithelia of glomeruli and renal tubules (Osafune et al. 2007). By contrast, the transcription factor *Pax2*, which is also essential for metanephros development, is expressed both in the mesenchyme and the ureteric buds (Torres et al. 1995). In addition, *Pax2* is one of the most upstream regulators in the genetic cascade of kidney development. In the transient pro- and mesonephros of the mouse, *Pax2* is expressed in the mesenchymal primordium as well as in the epithelial components and controls the initial differentiation from intermediate mesoderm to Wolffian duct in cooperation with *Pax8*; thus, mutant mice lacking both of these genes lack pronephros formation (Bouchard et al. 2002). Interestingly, retroviral overexpression of *Pax2* in chick embryos leads to the ectopic formation of nephric structures in the intermediate mesoderm (Bouchard et al. 2002), suggesting that *Pax2* may initiate the kidney formation program in vivo. Therefore, we hypothesized that *Pax2* overexpression in ES cells may mimic the situation in vivo. In the present study, we generated ES cell lines harboring tetracycline-inducible *Pax2* (Tet-off system) and examined their differentiation potential by embryoid body formation.

## Materials and Methods

**Plasmid construction.** The *Pax2* fragment was PCR-amplified with TaKaRa LA Taq (TaKaRa BIO, Otsu, Japan) using E13.5 whole embryo cDNA as a template and the primer pair 5'-CGGAATTCATGGATATGCACTGCAAAGC and 5'-GCGCGGCCGCCTAGTGGCGGTCATAGGCAGC. The amplified DNA was digested with *EcoRI* and *NotI*, cloned into pBluescript KS II(-) (*pBlks-Pax2*), and sequenced. The exchange vector was created by inserting the 1.2-kbp *XhoI-NotI* fragment of *pBlks-Pax2* into the *XhoI* and *NotI* sites of *pPthC-Oct3/4* (Masui et al. 2005).

**Culture of ES cells and embryoid bodies.** MGZRTcH2 cells, in which gene expression can be controlled by the Tet-off system (Masui et al. 2005), were maintained on 0.1% gelatin-coated tissue culture plates in Glasgow minimal essential medium (Invitrogen, Carlsbad, CA), 10% fetal bovine serum, 0.1 mM  $\beta$ -mercaptoethanol, 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids solution (Invitrogen), 0.1 mg/ml G418, 0.05 mg/ml hygromycin B (Hyg) (Invitrogen), 0.01 mg/ml Zeocin (Invitrogen), and  $10^3$  units/ml mouse leukemia inhibitory factor (LIF; Chemicon, Pittsburgh, PA) at 37°C with 5% CO<sub>2</sub>. The ES cells were dissociated with 0.25% trypsin plus 1 mM EDTA (Invitrogen) and cultured in the same medium without LIF to induce embryoid body formation by the hanging drop culture method ( $5 \times 10^3$  ES cells/0.015 ml drop). The embryoid bodies in hanging drops were cultured for 5 d and then transferred to tissue culture plates coated with 0.1% gelatin in the same medium without LIF (designated as day 0). The adherent embryoid bodies were cultured for an additional 5 or 10 d (day 5 or 10).

**Generation of ES cells with tetracycline-inducible *Pax2*.** MGZRTcH2 cells were electroporated (250 V, 960  $\mu$ F) with 20  $\mu$ g of the *Pax2*-containing exchange vector and a Cre-expression plasmid (pCAGGS-Cre; Niwa et al. 1991) using a Gene Pulser (BioRad, Hercules, CA). The resultant ES cells were cultured in the presence of 1  $\mu$ g/ml tetracycline (Tet; Sigma, St. Louis, MO), without Hyg. After 2 d, the medium was changed to medium containing 1  $\mu$ g/ml Tet and 1.5  $\mu$ g/ml puromycin (Puro; Sigma) without Hyg to expand the Puro-resistant clones. Eight days after electroporation, Puro-resistant clones were picked up and the recombination was confirmed by Southern blot analysis using the gene images random-prime labeling and detect system (GE Healthcare, Slough, UK). Seven out of 11 picked clones were recombinants.

**Western blot analysis.** ES cells and embryoid bodies were lysed in buffer (20 mM HEPES, pH 7.8, 500 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1% NP-40, 1% protease inhibitor cocktail (Sigma)). Protein aliquots (50  $\mu$ g) were boiled for 3 min in sample buffer and electrophoresed on 0.1% SDS-7.5% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes at 60 V for 1 h, probed with a polyclonal antibody against mouse *Pax2* (Covance, Richmond, CA), and detected using horseradish peroxidase-conjugated goat anti-mouse IgG and the ECL plus system (GE Healthcare).

**Reverse transcription-PCR.** Total RNA was extracted using TRIZOL reagent (Invitrogen). One unit of DNase I (Roche, Mannheim, Germany) was added per microgram of RNA, and the mixture was incubated at 37°C for 30 min. Two

micrograms of total RNA were reverse transcribed using Stratascript RT (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Two negative control reactions were also performed, with water instead of RNA or without reverse transcriptase. The primer pairs spanning at least one intron were as follows: *Pax-2*, 5'-AGGGCATC TGCGA-TAATGAC-3', 5'-CTCGCGTTTCTTCTTCTCAC-3'; *Lim-1*, 5'-TGGACCGTTTCTTCTTGAAC-3', 5'-TGTTCTC TTTGGCGACACTG-3'; *Aqp1*, 5'-CCTCCAGGCACA GTCTTCTC-3', 5'-CAGTGGCCTCCTGACTCTTC-3'; *Pod1*, 5'-GGAACCTTCCAACGAGAGCAC-3', 5'-GTGGTC TTGAGCCTGGAGAA-3'; *Sall1*, 5'-CCCCATCCCTAT TAGCCATT-3', 5'-AGAGTACTGTTGCCCGCTGT-3'; *Wt1*, 5'-ACCCAGGCTGCAATAAGAGA-3' 5'-GCTGA AGGGCTTTTACTTG-3'; *Gdnf*, 5'-CCCGAAGATTATCC TGACCA-3', 5'-TAGCCCAAACCCAAGTCA-3'; *integrin  $\alpha 8$* , 5'-GGCGAAGTGCAGTCCTAAA-3', 5'-GAAGGAGACA TTCGGAGTGG-3'; and *Hoxa11*, 5'-GGATTTTGATGA GCGTGGTC-3', 5'-AGTAGCAGTGGGCCAGATTG-3'.

**Immunocytochemistry.** Embryoid bodies cultured for 10 d on chamber slides were fixed for 2 h with 4% paraformaldehyde at 4°C, washed with phosphate buffered saline and 0.1% Tween 20 (PBST), blocked for 2 h at room temperature with 2% goat serum in PBST, and incubated with rabbit anti-Pax2 (Covance) or mouse anti-AQP1 (Sigma) antibodies. After washing twice with PBST, fluorescence-conjugated secondary antibodies were added. Images were captured on a Nikon ES800 fluorescence microscope equipped with a SPOT digital camera. Staining without primary antibodies gave no signal.

## Results

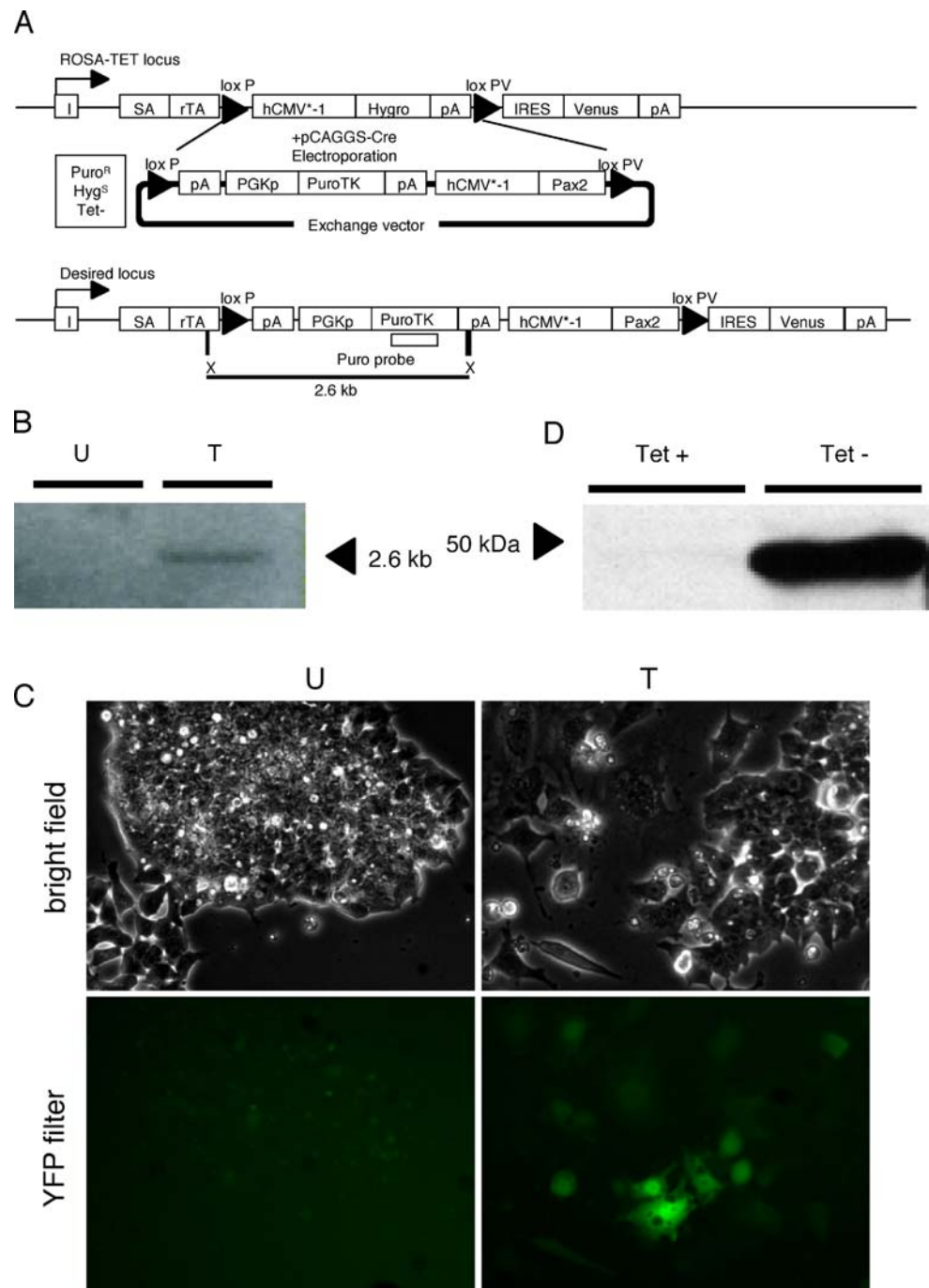
**Generation of ES cell lines with tetracycline-inducible Pax2.** The ROSA26 locus is widely used to drive ubiquitous expression of exogenous genes (Zambrowicz et al. 1997). MGZRTcH2 cells, an ES cell line, contain the ROSA26 locus in which the Tet-regulated transactivator (tTA) was inserted followed by loxP and lox PV sites, as shown in Fig. 1A. The exchange vector carrying the Tet-responsive CMV\*-1 promoter and Pax2 cDNA was introduced into this cell line along with the Cre-expression vector. The vector was efficiently incorporated into the ROSA26 locus upon Cre-mediated recombination, as confirmed by Southern blots (Fig. 1B), and Pax2 cDNA, as well as the downstream Venus, was placed under the control of the Tet activator. In this Tet-off system, tTA driven by the endogenous ROSA promoter binds to the CMV\*-1 promoter in the absence of Tet, thereby inducing expression of Pax2 cDNA-IRES-Venus. Indeed, Venus fluorescence was used to rapidly confirm the correct

recombinants (Fig. 1C), and Pax2 protein was detected only in the absence of Tet in the recombinants (Fig. 1D). Similar results were obtained when embryoid bodies were cultured without tetracycline (data not shown).

**Pax2 overexpression in embryoid bodies induced expression of Aqp1 and integrin  $\alpha 8$ .** To investigate the differentiation potential of ES cells toward the kidney lineage, the expression levels of nine genes that are essential for kidney development or functions, specifically Pax2, Lim1, Aqp1, Pod1, Sall1, Wt1, GDNF, integrin  $\alpha 8$ , and Hoxa11, were examined (Fig. 2A). RT-PCR analysis showed that nearly all genes were undetectable in the parental ES cells (MGZRTcH2). Sall1 was the exception, consistent with our previous report that Sall1 is expressed in ES cells (Sakaki-Yumoto et al. 2006). When cells were induced to form embryoid bodies for 5 d in hanging drops, followed by attachment to plastic dishes, the expression levels of Lim1 and Wt1 increased by day 5 after attachment, while that of Sall1 remained constant. As these genes are also expressed in nonrenal lineages, this does not imply differentiation toward a kidney fate. Pax2 was undetectable at all stages of differentiation.

We next examined whether Pax2 overexpression induces differentiation of ES cells toward the kidney lineage (Fig. 2B). In the presence of Tet, gene expression patterns were similar to those of the parental cells. In the absence of Tet, Pax2 was overexpressed both in ES cells and embryoid bodies, as expected. In ES cells, Pax2 overexpression did not lead to any phenotypic changes or to an increase in the expression levels of any genes examined, suggesting that Pax2 alone cannot trigger differentiation cues. During embryoid body formation, however, the expression level of integrin  $\alpha 8$  was increased by day 5 after attachment and that of Aqp1 was upregulated by day 10. The expression kinetics of Lim1, Wt1, and Sall1 remained unchanged. Although Pax2 is reported to be an upstream regulator of Gdnf (Brophy et al. 2001), Gdnf was undetectable throughout all the experiments. These results were reproducible using three independent clones (data not shown). We further examined expression changes at the protein level. Embryoid bodies from ES clones containing Tet-inducible Pax2 were cultured with or without tetracycline and stained with anti-AQP1 and anti-Pax2 antibodies (Fig. 3). Pax2 protein was detected only in the absence of Tet, consistent with the results shown in Fig. 1D. AQP1 protein was also upregulated in the absence of Tet, thus confirming the results shown in Fig. 2B. Commercially available anti-integrin  $\alpha 8$  antibodies did not give any signals, irrespective of the presence or absence of Tet; this was possibly due to antibody sensitivity, because we failed to detect signals using embryonic kidneys, which should express integrin  $\alpha 8$ . Thus, Pax2 can upregulate

**Figure 1.** Generation of ES cell lines with tetracycline-inducible Pax2. (A) The exchange vector containing *Pax2* cDNA was introduced, along with the Cre-expression plasmid, into MGZRTcH2 cells containing the ROSA-TET locus. The correctly targeted clones were resistant to puromycin. (B) Southern blot analysis of untargeted (U) and targeted (T) clones. The genomic DNA of puro-resistant clones was digested with *Xba*I and hybridized with the probe indicated in A. (C) Venus expression in untargeted (U) and targeted (T) clones. Cells were cultured in the absence of Tet and Venus fluorescence was detected only in targeted clones. (D) Western blot analysis of Tet-inducible Pax2 in the targeted ES clones. Pax2 protein was detected only in the absence of Tet.



the expression of *integrin  $\alpha 8$*  and *Aqp1* in embryoid bodies with slow kinetics, and additional factors that are induced in the embryoid bodies may be required for this phenomenon.

## Discussion

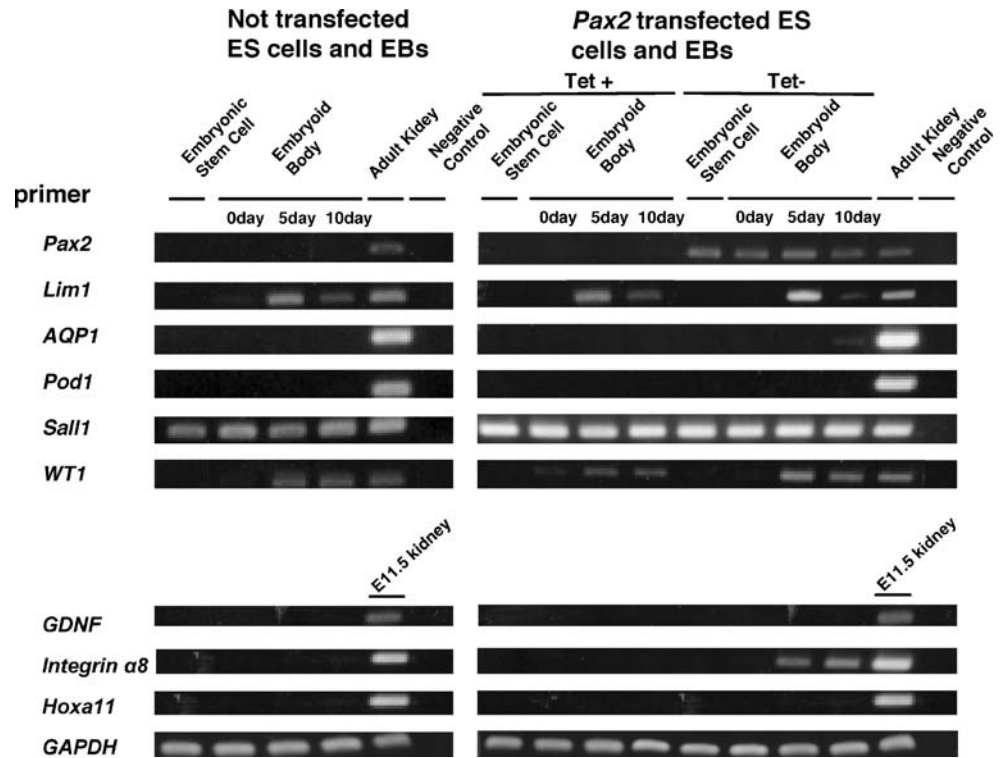
*Pax2* is one of the most upstream regulators in the genetic cascade of kidney development. By establishing tetracycline-inducible overexpression in ES cells, we showed that

*Pax2* can upregulate two genes that are possibly involved in kidney development (*integrin  $\alpha 8$*  and *Aqp1*) in embryoid bodies, but not in ES cells. Together with the slow induction kinetics (5–10 d), our data suggest that *Pax2* and additional factors that are induced in the embryoid bodies synergistically regulate the two targets.

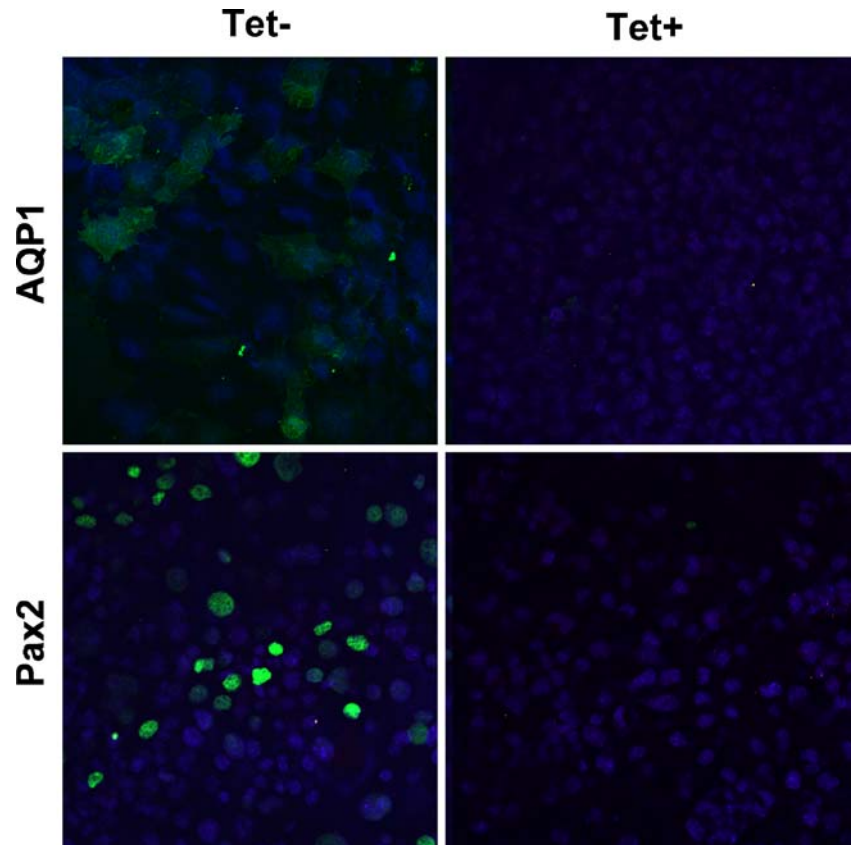
Many integrins have been shown to be important for cell–matrix interactions during developmental processes. *Integrin  $\alpha 8$*  is expressed in the metanephric mesenchyme surrounding the branching ureter tips, and mice deficient for this gene display kidney agenesis or dysgenesis (Muller



**Figure 2.** *Pax2* overexpression in embryoid bodies induces expression of *Aqp1* and *integrin  $\alpha 8$* . (A) RT-PCR analysis of the parental ES cell line (MGZRTcH2) and EB. Embryonic kidneys were used as a positive control. (B) RT-PCR analysis of clones containing Tet-inducible *Pax2*. In the presence of Tet (Tet+), gene expression patterns were similar to those in parental cells. In the absence of Tet (Tet-), *Aqp1* and *integrin  $\alpha 8$*  were upregulated when cells were induced to form embryoid bodies. Embryoid bodies were formed for 5 d in hanging drops, subsequently attached to the plastic dishes, and cultured for the indicated number of days.



**Figure 3.** *Pax2* overexpression in embryoid bodies led to upregulation of AQP1 protein. Embryoid bodies derived from ES clones containing Tet-inducible *Pax2* were cultured for 10 d after attachment to the slide chambers, with or without tetracycline, and stained with anti-AQP1 and anti-*Pax2* antibodies.



et al. 1997). This is due to an impaired interaction of the mesenchyme with the ureteric epithelium that expresses the ligand for this integrin, nephronectin (Brandenberger et al. 2001; Linton et al. 2007). Although our data suggest that *Pax2* upregulates *integrin  $\alpha 8$*  in embryoid bodies, it is unknown whether *Pax2* acts upstream of *integrin  $\alpha 8$*  in vivo. Expression analysis using *Pax2* mutant mice will help elucidate the answer to this question. Recently, it was shown that Hox11 paralogous proteins cooperate with Eya1 and Pax2 to activate *Six2* and *Gdnf*, two genes that are essential for kidney development. Hox11, Eya1, and Pax2 proteins physically associate, bind to a metanephric mesenchyme-specific enhancer region, and synergistically activate reporter expression (Gong et al. 2007). Although *Six2* and *Gdnf* expression was not induced by *Pax2* in our experiments (Fig. 2B and data not shown), it would be interesting to examine whether *integrin  $\alpha 8$*  is also regulated by a Hox11/Eya1/Pax2 protein complex in vivo.

Aqp1 is a water channel expressed in epithelial cells of the proximal tubules and in the descending limb of Henle, but not in other nephron segments (Nielsen and Agre 1995; Agre and Nielsen 1996). *Aqp1* is also expressed in red blood cells, lung, eyes, brains, and capillary endothelial cells (Agre et al. 1993). Several reports analyzing the proximal promoter of *Aqp1* suggest that *Aqp1* is regulated by thyroid transcription factor-1 and hypertonicity (Umenishi and Schrier 2003; Kim et al. 2007). Although it is not known whether Pax2-binding sites exist in the *Aqp1* promoter, our data raise the possibility of a unique regulation of this water channel.

Pax2 overexpression did not induce kidney lineage differentiation or upregulate other genes, including *Gdnf*, which is known to be a downstream target of *Pax2* in vivo (Brophy et al. 2001). Even an increase in the expression levels of *integrin  $\alpha 8$*  and *Aqp1* does not imply kidney differentiation, as these two genes are not restricted to the kidney. Thus, *Pax2* alone is not sufficient to initiate the kidney development program in ES cells or embryoid bodies. Alternatively, conventional embryoid body formation may not be optimal for such a program. Kim et al. reported that the addition of activin A, retinoic acid, and BMP7 to embryoid bodies increased the expression of many genes involved in kidney development (Kim and Dressler 2005). It will be worth testing whether this condition plus Pax2 overexpression further enhances kidney induction from ES cells. Nonetheless, our rapid method for the generation of tetracycline-inducible ES cell lines will be useful for dissecting the genetic cascades functioning in the early stages of development, as well as for screening candidate genes that direct ES cells toward the desired lineages, which may be applicable to future cell therapies.

**Acknowledgments** We thank H. Niwa for providing MGZRTcH2 cells. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology and by the Ministry of the Health, Labor and Welfare of Japan.

## References

- Agre P.; Nielsen S. The aquaporin family of water channels in kidney. *Nephrology*. 17: 409–415; 1996.
- Agre P.; Preston G. M.; Smith B. L.; Jung J. S.; Raina S.; Moon C.; Guggino W. B.; Nielsen S. Aquaporin CHIP: the archetypal molecular water channel. *Am. J. Physiol. Renal Fluid Electrolyte Physiol*. 265: F463–F476; 1993.
- Bouchard M.; Souabni A.; Mandler M.; Neubüser Nephric lineage specification by Pax2 and Pax8. *Genes Dev*. 16: 2958–2970; 2002. doi:10.1101/gad.240102.
- Brandenberger R.; Schmidt A.; Linton J.; Wang D.; Backus C.; Müller U.; Reichardt L. F. Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin  $\alpha 8 \beta 1$  in the embryonic kidney. *J. Cell Biol.* 154: 447–458; 2001. doi:10.1083/jcb.200103069.
- Brophy P. D.; Ostrom L.; Lang K. M.; Dressler G. R. Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* 128: 4747–4756; 2001.
- Daley G. Q. From embryos to embryoid bodies: Generating blood from embryonic stem cells. *Ann. N. Y. Acad. Sci.* 996: 122–131; 2003.
- Dressler G. R. The cellular basis of kidney development. *Annu. Rev. Cell Dev. Biol.* 22: 509–529; 2006. doi:10.1146/annurev.cell.bio.22.010305.104340.
- Gong K. Q.; Yallowitz A. R.; Sun H.; Dressler G. R.; Wellik D. M. A Hox–Eya–Pax complex regulates early kidney developmental gene expression. *Mol. Cell. Biol.* 27: 7661–7668; 2007. doi:10.1128/MCB.00465-07.
- Kania G.; Blyszczuk P.; Czyz J.; Navarrete-Santos A.; Wobus A. M. Differentiation of mouse embryonic stem cells into pancreatic and hepatic cells. *Methods Enzymol.* 365: 287–303; 2003. doi:10.1016/S0076-6879(03)65021-4.
- Kim D.; Dressler G. R. Nephrogenic factors promote differentiation of mouse embryonic stem cells into renal epithelia. *J. Am. Soc. Nephrol.* 16: 3527–3534; 2005. doi:10.1681/ASN.2005050544.
- Kim J. G.; Son Y. J.; Yun C. H.; Kim Y. L.; Nam-Goong I. S.; Park J. H.; Park S. K.; Ojeda S. R.; D'Elia A. V.; Damate G.; Lee B. J. Thyroid transcription factor-1 facilitates cerebrospinal fluid formation by regulating aquaporin-1 synthesis in the brain. *J. Biol. Chem.* 282: 14923–14931; 2007. doi:10.1074/jbc.M701411200.
- Kobayashi T.; Tanaka H.; Kuwana H.; Inoshita S.; Teraoka H.; Sasaki S.; Terada Y. Wnt4-transformed mouse embryonic stem cells differentiate into renal tubular cells. *Biochem. Biophys. Res. Commun.* 336: 585–595; 2005. doi:10.1016/j.bbrc.2005.08.136.
- Kyba M.; Perlingeiro R. C. R.; Daley G. Q. HoxB4 confers definitive lymphoid–myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 109: 29–37; 2002. doi:10.1016/S0092-8674(02)00680-3.
- Linton J. M.; Martin C. R.; Reichardt L. F. The ECM protein nephronectin promotes kidney development via integrin  $\alpha 8 \beta 1$ -mediated stimulation of Gdnf expression. *Development* 134: 2501–2509; 2007. doi:10.1242/dev.005033.
- Lumelsky N.; Blondel O.; Laeng P.; Velasco I.; Ravin R.; McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292: 1389–1394; 2001. doi:10.1126/science.1058866.

- Masui S.; Shimosato D.; Toyooka Y.; Yagi R.; Takahashi K.; Niwa H. An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. *Nucleic Acids Res.* 33: e43; 2005. doi:10.1093/nar/gni043.
- Muller U.; Wang D.; Denda S.; Meneses J. J.; Pedersen R. A.; Reichardt L. F. Integrin  $\alpha 8 \beta 1$  is critically important for epithelial–mesenchymal interactions during kidney morphogenesis. *Cell* 88: 603–613; 1997. doi:10.1016/S0092-8674(00)81903-0.
- Nielsen S.; Agre P. The aquaporin family of water channels in kidney. *Kidney Int.* 48: 1057–1068; 1995. doi:10.1038/ki.1995.389.
- Niwa H.; Yamamura K.; Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108: 193–199; 1991. doi:10.1016/0378-1119(91)90434-D.
- Osafune K.; Takasato M.; Kispert A.; Asashima M.; Nishinakamura R. Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. *Development* 133: 151–161; 2007. doi:10.1242/dev.02174.
- Sakaki-Yumoto M.; Kobayashi C.; Sato A.; Fujimura S.; Matsumoto Y.; Takasato M.; Kodama T.; Aburatani H.; Asashima M.; Yoshida N.; Nishinakamura R. The murine homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in anorectal, heart, brain and kidney development. *Development* 233: 3005–3013; 2006. doi:10.1242/dev.02457.
- Torres M.; Gomez-Pardo E.; Dressler G. R.; Gruss P. Pax-2 controls multiple steps of urogenital development. *Development* 121: 4057–4065; 1995.
- Umenishi F.; Schrier R. W. Hypertonicity-induced aquaporin-1 (AQP1) expression is mediated by the activation of MAPK pathways and hypertonicity-responsive element in the AQP1 gene. *J. Biol. Chem.* 278: 15765–15770; 2003. doi:10.1074/jbc.M209980200.
- Vainio S.; Lin Y. Coordinating early kidney development: lessons from gene targeting. *Nat. Rev. Genet.* 3: 533–543; 2002. doi:10.1038/nrg842.
- Wichterle H.; Lieberam I.; Porter J. A.; Jessel T. M. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110: 287–303; 2002. doi:10.1016/S0092-8674(02)00835-8.
- Yamamoto H.; Quinn G.; Asari A.; Yamanokuchi H.; Teratani T.; Terada M.; Ochiya T. Differentiation of embryonic stem cells into hepatocytes: Biological functions and therapeutic application. *Hepatology* 37: 983–993; 2003. doi:10.1053/jhep.2003.50202.
- Zambrowicz B. P.; Imamoto A.; Fiering S.; Herzenberg L. A.; Kerr W. G.; Soriano P. Disruption of overlapping transcripts in the ROSA  $\beta$ geo 26 gene trap strain leads to widespread expression of  $\beta$ -galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.* 94: 3789–3794; 1997. doi:10.1073/pnas.94.8.3789.