Int. J. Dev. Biol. 57: 383-389 (2013)
doi: 10.1387/ijdb.130058ma

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Induction of intermediate mesoderm by retinoic acid receptor signaling from differentiating mouse embryonic stem cells

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ABSTRACT Renal lineages including kidney are derived from intermediate mesoderm, which are differentiated from a subset of caudal undifferentiated mesoderm. The inductive mechanisms of mammalian intermediate mesoderm and renal lineages are still poorly understood. Mouse embryonic stem cells (mESCs) can be a good *in vitro* model to reconstitute the developmental pathway of renal lineages and to analyze the mechanisms of the sequential differentiation. We examined the effects of Activin A and retinoic acid (RA) on the induction of intermediate mesoderm from mESCs under defined, serum-free, adherent, monolayer culture conditions. We measured the expression level of intermediate mesodermal marker genes and examined the developmental potential of the differentiated cells into kidney using an *ex vivo* transplantation assay. Adding Activin A followed by RA to mESC cultures induced the expression of marker genes and proteins for intermediate mesoderm, odd-skipped related 1 (Osr1) and Wilm's Tumor 1 (Wt1). These differentiated cells integrated into laminin-positive tubular cells and Pax2-positive renal cells in cultured embryonic kidney explants. We demonstrated that intermediate mesodermal marker expression was also induced by RA receptor (RAR) agonist, but not by retinoid X receptor (RXR) agonists. Furthermore, the expression of these markers was decreased by RAR antagonists. We directed the differentiation of mESCs into intermediate mesoderm using Activin A and RA and revealed the role of RAR signaling in this differentiation. These methods and findings will improve our understanding of renal lineage development and could contribute to the regenerative medicine of kidney.

KEY WORDS: *kidney, Activin A, odd-skipped related 1, Pax 2, Wilm's tumor 1*

Introduction

Mammalian pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981) and induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006) can differentiate into three germ layers. Number of studies demonstrated to differentiate into specific cell lineages from PSCs by cytokines and chemicals and provided useful insights into the molecular mechanisms of mammalian development *in vitro* and for future regenerative medicine. As to renal lineages, although several independent studies have been demonstrated to differentiate into these lineages from mouse ESCs (mESCs) (Bruce *et al.*, 2007; Vigneau *et al.*, 2007), directed differentiation methods into renal lineages have never been reported (Nishinakamura, 2008). Renal lineages are derived from intermediate mesoderm, which are differentiated from a subset of caudal primitive mesoderm (Dressler, 2009; Reidy and Rosenblum, 2009). To achieve directed differentiation into renal lineages, step-wise differentiation system through intermediate mesoderm is required.

We previously reported that treatment with Activin A and retinoic acid (RA) induces renal lineages from amphibian undifferentiated PSCs (Moriya *et al.*, 1993; Osafune *et al.*, 2002; Uochi and Asashima, 1996). As for the role of Activin A, it induces broad mesoendodermal derivatives from amphibian undifferentiated PSCs *in vitro* (Asashima *et al.*, 1990; Asashima *et al.*, 1991; Kaneko *et al.*,

Abbreviations used in this paper: iPSC, induced pluripotent stem cell; mESC, mouse embryonic stem cell; PSC, pluripotent stem cell; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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Accepted: 11 December 2012. *Final, author-corrected PDF published online:* 3 July 2013. *Edited by:* Makoto Asashima.

ISSN: Online 1696-3547, Print 0214-6282

2008; Okabayashi and Asashima, 2003). As for the role of RA, it does not display any inducing activity in the amphibian differentiation assays, but it can modify the action of Activin A on mesoderm pattern formation in concentration- and time- dependent manners (Ariizumi and Asashima, 2001; Fukui and Asashima, 1994). In mammalian development, number of studies demonstrated that Activin-related genes have important functions in mesoderm formation and differentiation using mice deficient in these genes (Goumans and Mummery, 2000; Schier and Shen, 2000). Mice deficient in RA-related genes impair various tissue development including renal lineages (Duester, 2008). From these findings, we hypothesized that the sequential addition of Activin A and RA could induce renal lineages from mammalian PSCs.

In this study, we aimed to differentiate mESCs into renal lineages using defined culture conditions and dissect the inductive signal pathways for intermediate mesoderm differentiation. We previously developed chemically-defined, serum-free culture system for mESCs (Furue *et al.*, 2005). This culture system enables us to analyze the direct effects of various cytokines including extracellular matrix (ECM) proteins (Hayashi *et al.*, 2007), Wnt proteins (Nakanishi *et al.*, 2009), bone morphogenetic proteins (BMP) (Hayashi *et al.*, 2010b), and fibroblast growth factor (FGF) (Aihara *et al.*, 2010). In this culture system, we used Activin A and RA as the candidates of inducer to differentiate mESCs into renal lineage and examined the expression levels of the intermediate mesodermal marker genes in these cells. We characterized that these differentiated cells can integrated into developing kidney explant cultures. Furthermore, we used RAR agonists and antagonists to examine the involvement of RAR signaling in intermediate mesodermal differentiation from mESCs.

Results

Induction of intermediate mesodermal gene expression by RA combined with Activin A

We first asked whether RA together with Activin A induced intermediate mesoderm from mESCs. Undifferentiated mESCs were seeded in the defined medium supplemented with 10 ng/ml of Activin A on laminin-coated dishes (2μg/cm^2) at 0th day. From 0th to 5th day, 100 nM of RA was added in the culture medium; i.e., RA and Activin A was treated for 6, 5, 4, 3, 2, 1 days. No addition of RA was used as a control condition. Culture medium was changed every day. The differentiated cells were collected on day 6 (Fig. 1A) and assayed by quantitative RT-PCR for the gene expression of intermediate mesoderm markers, odd-skipped related 1 (Osr1) and Wilm's tumor 1 (Wt1). Osr1 is expressed in intermediate mesoderm in developing mouse embryos (Wang *et al.*, 2005). Wt1 is also specifically expressed in intermediate mesoderm mouse embryos at embryonic day (E)9.5 (Armstrong *et al.*, 1993) and has an essential role in kidney development (Kreidberg *et al.*, 1993). We found that the expression of both Osr1 and Wt1 was up-regulated in the differentiated cells treated with RA under most conditions (except for Osr1 expression with RA treatment from 5th day) compared with undifferentiated mESCs and differentiated cells without RA (Fig. 1B, C). Osr1 expression was highest in the cells treated with RA from 3rd day. Wt1 expression were comparable in the cells treated with RA from 2nd and 3rd day (Fig. 1B). Thus, we treated the cells with RA from 3rd day in the following experiments. Next, we examined the expression level of Osr1 and Wt1 in the

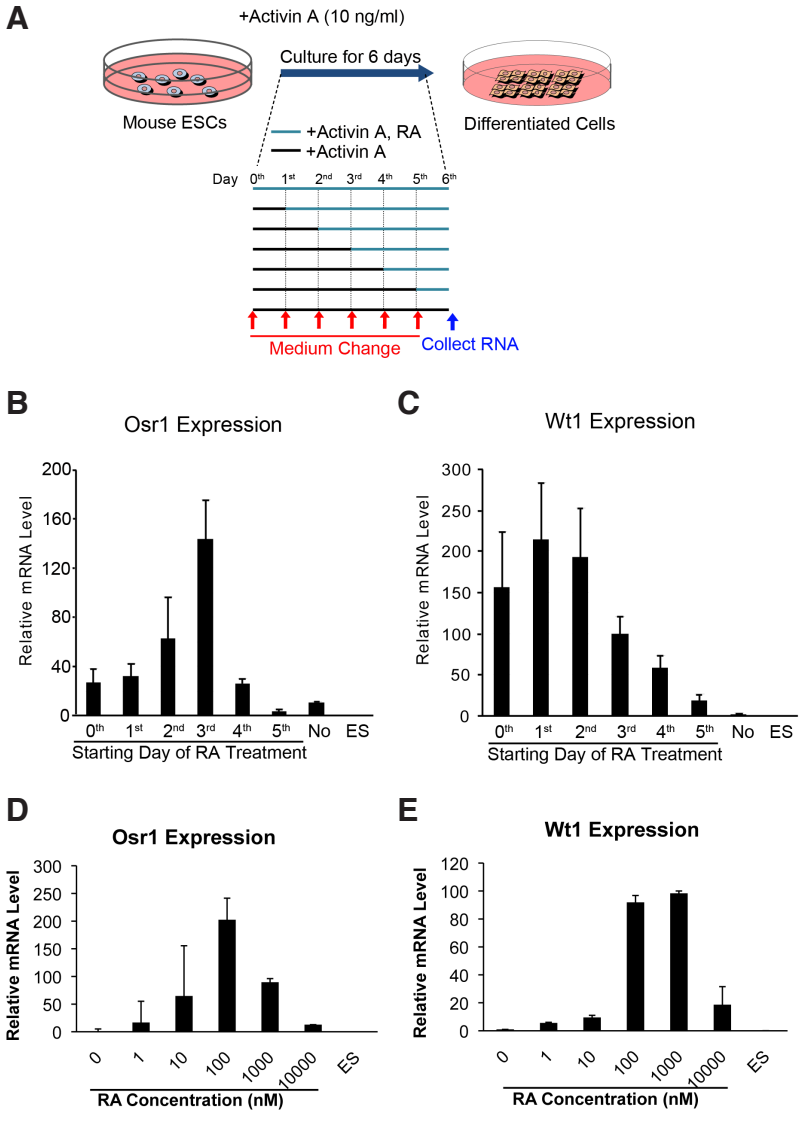

Fig. 1. Time- and concentration-dependent effects of retinoic acid (RA) on the induction of intermediate mesodermal marker gene expression in differentiated mouse embryonic stem cells (ESCs). (A) *Schematic representation of the experimental conditions.* **(B,C)** *Quantitative RT-PCR analysis of Osr1 and Wt1 mRNA levels in Activin A- and RA-induced mESCs cultured with 10ng/ml of Activin A (from 0th day) and 0.1 μM of RA (from each day as indicated in the graph) in ESF5 medium for 6 days.* **(D,E)** *Quantitative RT-PCR analysis of Osr1 and Wt1 mRNA levels in Activin A- and RA-induced mESCs cultured with 10ng/ml of Activin A (from 0th day) and various concentrations of RA (from 3rd day; concentration as indicated) in ESF5 medium for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The relative amount of the undifferentiated mESCs (ES) is indicated as 1.0. Values are mean m SEM* (n = 4).

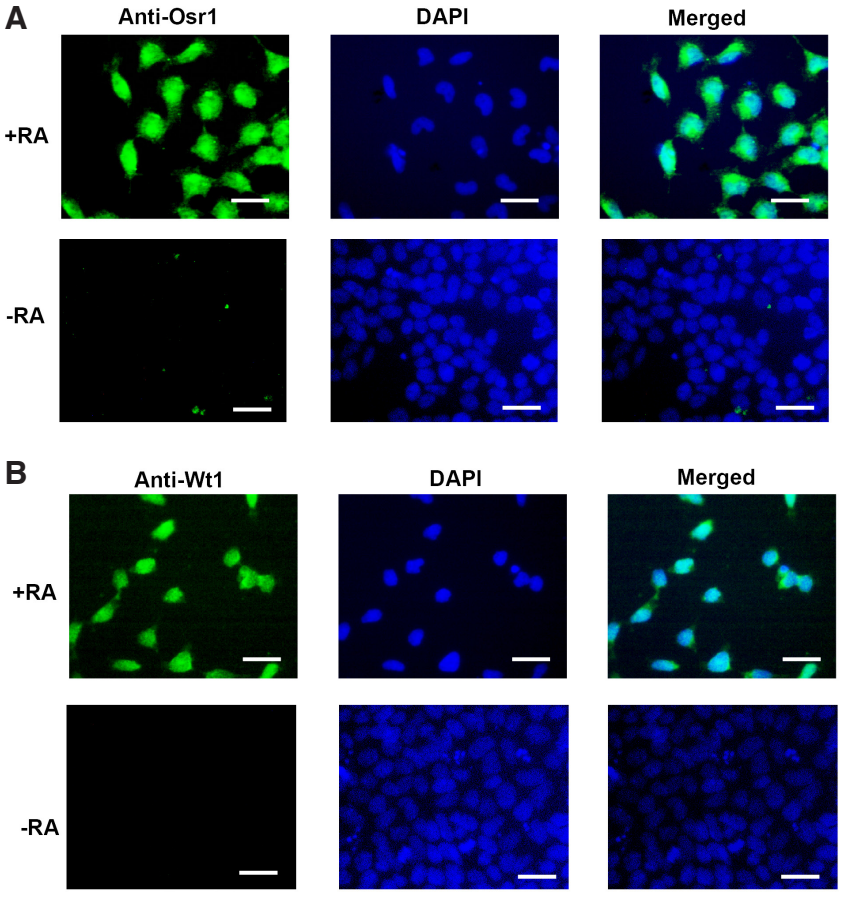

Fig. 2. Osr1 and Wt1 protein expression in differentiated mESCs treated with Activin A and retinoic acid (RA) by immunocytochemistry. *Immunostaining for Osr1 and Wt1 in cells cultured with or without 0.1 μM of RA (from 3rd day) for 6 days. Immunoreactivity of Osr1 and Wt1 was visualized with Alexafluor 488-conjugated second antibodies (green). Nuclei were stained with DAPI (blue). Scale bars indicate 40 μm.*

conditions with different RA concentrations by quantitative RT-PCR. The mRNA level of Osr1 was up-regulated dose-dependently in the RA-treated cells and was highest with 100 nM of RA treatment (Fig. 1D). The mRNA level of Wt1 was also up-regulated dose-dependently in the RA-treated cells and was highest with 100 or 1,000 nM of RA treatment (Fig. 1E). Thus, we used 100 nM of RA for all subsequent experiments. These results indicate that RA treatment of the appropriate timing and concentration increased Osr1 and Wt1 expression following Activin A treatment in differentiating mESCs.

Next, we examined the protein expression of Osr1 and Wt1 in the differentiated cells cultured for 6 days with or without RA treatment in these culture conditions by immunocytochemistry. Many RA-treated cells were positively stained with Osr1- and Wt1-specific antibodies while untreated cells were negatively stained with these antibodies (Fig. 2A, B). These results indicated that RA treatment induced the protein expression of intermediate mesoderm in the differentiating mESCs.

Next, we examined the time course of the marker gene expression of undifferentiated mESCs, epiblast, and undifferentiated mesoderm during the induction into intermediate mesoderm. We determined the expression level of Oct4 as an undifferentiated mESC marker (Okamoto *et al.*, 1990; Scholer *et al.*, 1990), Fgf5 as an epiblast marker (Rathjen *et al.*, 1999), and Brachyury (T) as an undifferentiated mesodermal marker (Wilkinson *et al.*, 1990) by quantitative RT-PCR. Oct4 expression was down-regulated from 3rd day onward, suggesting that differentiation from pluripotent state occurred in these culture conditions (Fig. 3A). Fgf5 expression was transiently up-regulated from 2nd to 4th day of induction, suggesting that the cells differentiated via epiblast state (Fig. 3B). Brachyury expression was up-regulated throughout the induction with a peak at 3rd day of induction, suggesting that the cells also differentiated via primitive mesodermal state (Fig. 3C). These results confirmed that the addition of RA following Activin A in these culture conditions induces the step-wise mesodermal gene expression program from undifferentiated mESCs.

Differentiation potential of RA-induced cells using an ex vivo *transplantation system*

We asked whether the differentiated cells induced by RA following Activin A had the differentiation potential for kidney cells, we transplanted the cells into developing kidney *ex vivo.* RA-induced differentiated cells transduced with green fluorescent protein (GFP) by retroviral infection were injected into developing whole kidney isolated from mouse E12.5 and cultured for 5 days. Cryosections of the transplanted kidney were then immunostained for laminin and Pax2 protein expression. Laminin is expressed in the basement membrane of tubule structures in the developing kidney (Ekblom *et al.*, 1980). Pax2 is expressed in collecting duct, metanephric mesenchyme and tubules derived from metanephric mesenchyme (Torres *et al.*, 1995). Some of the GFP-expressing transplanted cells co-expressed laminin (Fig. 4A) or Pax2 (Fig. 4B) in the cryosections of cultured kidney explants. In addition, there were no teratoma-like structures observed in the explants (data not shown), suggesting mESCs were fully differentiated from the pluripotent stage. These results indicated that the transplanted cells were successfully integrated into the tubule structures and mesenchyme of a developing kidney and that the differentiated cells treated with RA following Activin A had the differentiation potential for kidney cells.

Induction of intermediate mesoderm from mESCs by retinoic derivatives

We asked how RA induced intermediate mesoderm from differentiating mESCs. RA generally regulates cell signaling and gene expression through binding to the RA receptors (RAR) or retinoid X receptors (RXR) (Altucci *et al.*, 2007). Since RA can act as a ligand for both RAR and RXR, it is difficult to distinguish between the receptor signaling pathways. To examine which receptor signaling pathway is involved in the induction of intermediate mesoderm from mESCs, we used a pan RAR agonist, TTNPB (Afonja *et al.*, 2002; Lu *et al.*, 2008), and a pan RXR agonist, PA024 (Honda *et al.*, 2005; Ishida *et al.*, 2003; Takahashi *et al.*, 2002), to mimic the effect of RA in our culture conditions. We detected the

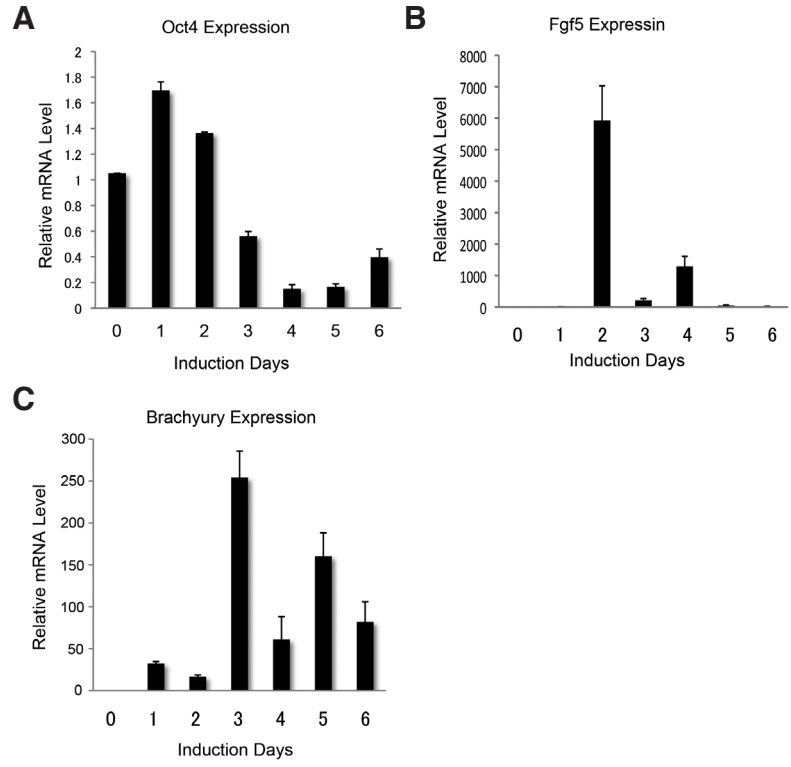

Fig. 3. The time course of differentiation marker expression in the differentiating mESCs treated with Activin A and retinoic acid (RA). *Quantitative RT-PCR analyses of mRNA levels of Oct4* **(A)**, *Fgf5* **(B)**, *and Brachyury* **(C)** *in the differentiated mESCs treated with Activin A and RA. Cells cultured with 10 ng/ml of Activin A (from 0th day) and 0.1 μM of RA (from 3rd day) were collected at 0th – 6th day. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of the undifferentiated mESCs (0th day) is indicated as 1.0. Values are mean SEM* (n = 4).

expression level of intermediate mesodermal markers, Osr1 and Wt1, in the differentiated mESCs treated with different concentrations of TTNPB or PA024 by quantitative RT-PCR. While PA024 at any concentration had no effect on the expression of either Osr1 or Wt1, TTNPB up-regulated both of their expression level dose-dependently (Fig. 5A, B). These results suggested that RAR signaling pathway was involved in the induction of intermediate mesoderm from mESCs.

To confirm the involvement of RAR signaling pathway in the induction of intermediate mesoderm from mESCs, we used an RAR antagonist, LE135 (Umemiya *et al.*, 1997), together with RA. We detected the expression level of intermediate mesodermal markers, Osr1 and Wt1 in the differentiated mESCs treated with or without LE135. LE135 decreased their expression level induced by RA dose-dependently (Fig. 6A, B). These results indicated that RAR signaling was crucial for the RA-induced differentiation of mESCs into intermediate mesoderm.

Discussion

In this study, we demonstrated that treating undifferentiated mESCs with RA following Activin A induced intermediate mesoderm. Under these defined culture conditions, both mRNA and proteins expressions of Osr1 and Wt1 were induced by RA addition. We also showed that the differentiated cells transplanted into developing kidney *ex vivo* were integrated into tubule structures marked by Laminin and renal epithelium marked by Pax2. Thus, these results suggested that the differentiated mESCs induced by RA and Activin A can differentiate into renal lineages. Previous studies using embryoid bodies, which were formed from mESC aggregates, showed that mESCs could differentiate into renal lineages (Bruce *et al.*, 2007; Vigneau *et al.*, 2007); however, directed differentiation into renal lineage has been proven difficult to achieve due to the methods used. Although embryoid body differentiation methods are useful and convenient for differentiation into many cell types (Desbaillets *et al.*, 2000; Keller, 1995), this method allows ESCs to differentiate in a random manner influenced by cell-cell contact and cell-autonomous secreted signals in the embryoid bodies. Moreover, these culture methods usually use serum or serum replacement, which include many unknown cytokines to affect cell differentiation. These influences are minimized in our culture method using serum- and feeder-free defined monolayer culture conditions, whereby known chemicals and cytokines are used to control cell fate decisions more precisely.

We have identified that RA is crucial for the induction of intermediate mesoderm using mESCs differentiation models. Generally, RA has pleiotropic effects in mESC differentiation and mammalian development (Clagett-Dame and DeLuca, 2002; Mark *et al.*, 2006). In undifferentiated mESCs, RA inhibits their self-renewal and promotes their neurogenesis (Glaser and Brustle, 2005; Rohwedel *et al.*, 1999). On the other hand, under our culture conditions, Activin A was used to induce mesendoderm from mESCs before RA is applied (Johansson and Wiles, 1995; Kubo *et al.*, 2004), confirmed by the up-regulation of early mesodermal marker, Brachyury. Furthermore, treatment with Activin A prior to RA was needed to up-regulated the expression of Osr1 and Wt1. Thus, our results

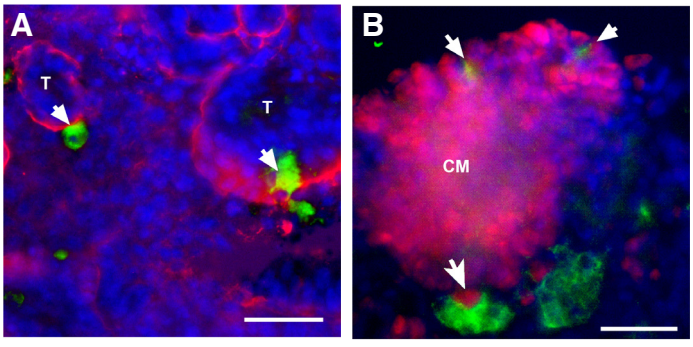

Fig. 4. The contribution of differentiated mESCS treated with Activin A and Retinoic acid (RA) to developing kidney *ex vivo*. *Immunohistchemistry for GFP, Pax2, and laminin expression in cryosections of cultured whole kidney containing GFP-positive transplanted cells. Differentiated, GFP-transduced mESCs cells cultured in ESF5 medium with 10 ng/ml Activin A (from 0th day) and 0.1 μM RA (from 3rd day) for 6 days were transplanted into cultured whole kidney collected from mouse embryos at E12.5. Immunoreactivity of GFP was visualized with Alexafluor 488-conjugated secondary antibodies (green). Immunoreactivity of laminin* **(A)** *and Pax2* **(B)** *was visualized with Alexafluor 594-conjugated second antibodies (red). Nuclei were stained with DAPI (blue). Arrows indicate cells double positive for GFP and differentiation markers. T, tubules; CM, condensed mesenchyme. Scale bars, 50 μm.*

suggested that RA directed intermediate mesoderm from primitive mesoderm. This step-wise, directed differentiation approach allows us to dissect the role of RA in mESC differentiation program and mammalian development.

We have also identified that RAR signaling pathway was involved in the differentiation into intermediate mesoderm. An RAR agonist specifically up-regulated the expression of intermediate mesodermal markers. Conversely, an RAR antagonist inhibited the RA-induced marker genes expression. These findings are consistent with the expression pattern of RAR and the phenotype of the RAR-deficient mice. RAR γ begins to express in the posterior region of the embryo at E8.0 (Ruberte *et al*., 1990). Mice deficient in RARα and RARβ2 genes or RARγ impair renal development (Lohnes *et al*., 1993; Mendelsohn *et al*., 1999). Thus, our findings established an *in vitro* model to demonstrate the role of RAR signaling pathway in intermediate mesodermal induction using a mESC differentiation system. Our previous study show that RXR is involved in heart development using mESC differentiation system (Honda *et al*., 2005), suggesting that downstream signaling pathways of RA may differ among the mesodermal tissue formation.

In conclusion, this study demonstrated that sequential addition of Activin A and RA induced intermediate mesoderm from mESCs. Together with our previous studies using amphibian pluripotent (animal cap) cells (Moriya *et al*., 1993; Osafune *et al*., 2002; Uochi and Asashima, 1996), our findings imply that the induction of renal lineages by Activin A and RA is conserved among vertebrates. Therefore, we also expect that these findings will be applied to human pluripotent stem cells (Takahashi *et al*., 2007; Thomson *et al*., 1998; Yu *et al*., 2007). Since stem cells in the mature human mature kidney remain elusive (Yokoo *et al*., 2008), the differentiation into renal lineages from human PSCs could be useful for the realization of regenerative medicine of the kidney and successful drug development for renal diseases.

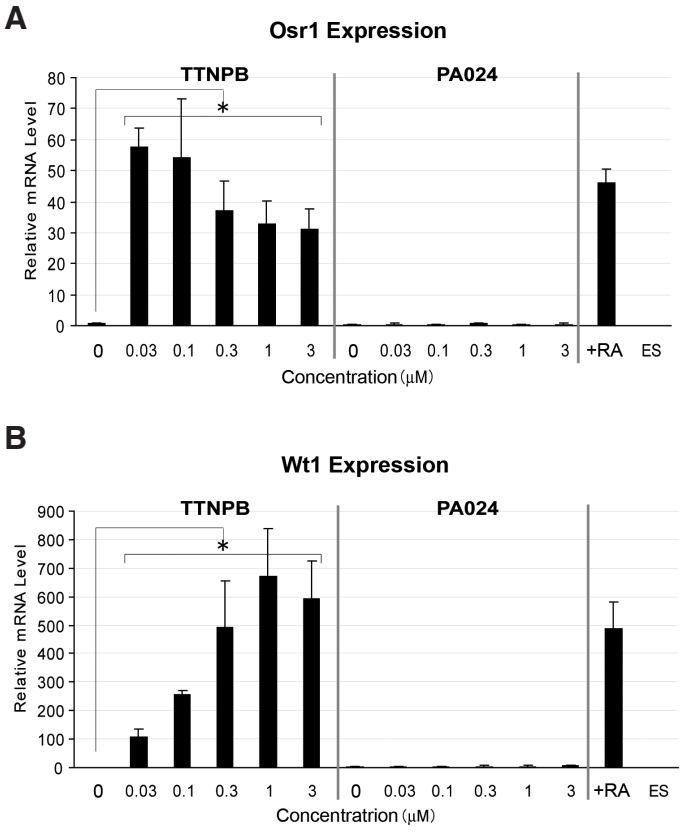

Fig. 5. The effect of retinoic acid receptor (RAR) and retinoid X receptor (RXR) agonists on intermediate mesodermal marker expression. *Quantitative RT-PCR analyses of* Osr1 **(A)** *and* Wt1 **(B)** *mRNA levels in the differentiated mESCs, treated with Activin A and retinoic derivatives. The cells were cultured with 10 ng/m Activin A (from 0th day) and various concentrations of an RAR agonist, TTNPB, or an RXR agonist, PA024 (from 3rd day) for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of differentiated mESCs without RA is indicated as 1.0. The values are mean SEM (n = 4).*

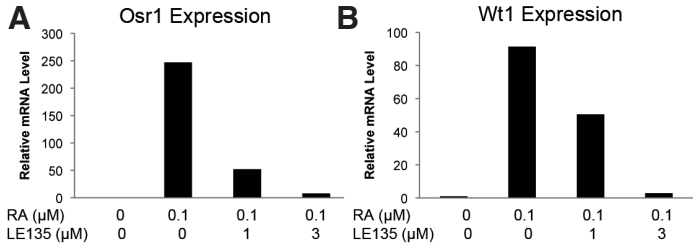

Fig. 6. The effect of a retinoic acid receptor (RAR) antagonist on intermediate mesodermal marker expression. *Quantitative RT-PCR analyses of* Osr1 **(A)** *and* Wt1 **(B)** *mRNA levels in the differentiated mESCs, treated with Activin A and RA with or without an RAR antagonist, LE135. The cells cultured with 10 ng/ml of Activin A (from 0th day) and various concentrations of LE135 with 0.1 μM of RA (from 3rd day) for 6 days. The gene expression of each sample was normalized against the* Gapdh *mRNA level. The amount of differentiated mESCs without RA is indicated as 1.0. Values are mean.*

Materials and Methods

Cell culture

The mESC D3 (ATCC, CRL1934) line was routinely cultured as described previously (Furue *et al*., 2005; Hayashi *et al*., 2007). Briefly, the mESCs were cultured in 75-cm^2 plastic flasks (Corning), coated with 15 μg/ml of type I collagen (Nitta gelatin), in a humidified atmosphere of 5% CO_2 at 37°C in a defined ESF7 medium. The ESF7 consisted of ESF basal medium (Cell Science & Technology Institute) supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite, 9.4 μg/ml oleic acid conjugated with 2 mg/ml fatty acid-free bovine serum albumin (FAF-BSA; Sigma), and 10 ng/ml of leukemia inhibitory factor (LIF; Chemicon). For the differentiation experiments, mESCs were seeded at a density of 1 × 10^4 cells/cm^2 in ESF5 medium, which comprised ESF basal medium supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-ethanolamine, 20 nM sodium selenite added with 0.5 mg/ml FAF-BSA, and 10 ng/ml human recombinant Activin A (Ajinomoto pharmaceuticals), on 2 μg/cm^2 laminin-coated (Sigma) dishes. The medium was changed every day. After the indicated days of culture under differentiating conditions, retinoid derivatives, or 0.02% dimethyl sulfoxide (DMSO, Sigma) as a solvent control were added in the medium. The preparation of these chemical solutions was described previously (Honda *et al*., 2005). Briefly, the RA (Sigma), RAR agonist (TTNPB; Sigma), RXR agonist (PA024, gifted from Dr. Kagechika (Takahashi *et al*., 2002)), and RAR antagonist (LE135, gifted from Dr. Kagechika (Takahashi *et al*., 2002)) were dissolved in DMSO to a stock concentration of 1 mM. The reagents were then filtered through a 0.22-μm membrane filter (Millex-LG, Millipore), and aliquots were stored at –20 °C. The concentrations of retinoid derivatives in each experiment are indicated in the Results section. Stock retinoid derivatives solution was diluted with DMSO (Sigma).

RT-PCR

RT-PCR was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2007). Briefly, total RNA was extracted from the cultured cells using the total RNA extraction kit (Agilent) and reverse transcribed using Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed with SYBR Green PCR Master Mix according to the supplier's directions (Applied Biosystems) using an ABI Step One Plus sequence detector (Applied Biosystems). Relative mRNA expression of each sample was normalized against Gapdh expression. The primer sequences are as follows: Gapdh (FW: 5'-ACCCAGAAGACTGTGGATGG-3', RV: 5'- CACATTGGGGGTAGGAACAC-3'), Brachyury (FW: 5'-TACCCCAGCCCCTATGCTCA-3', RV: 5'-GGCACTCCGAGGCTAGACCA-3'), Fgf5 (FW: 5'-GCTGTGTCTCAGGGGATTGT-3', RV: 5'-CACTCTCGGCCTGTCTTTTC-3'), Oct4 (FW: 5'-TTCTGCGGAGGGATGGCATA-3', RV: 5'-TTTCCACTCGTGCTCCTGCC-3'), Osr1 (FW: 5'- GACCGCGGCGGAACAAGATA-3', RV: 5'- CACTGTGGGCAGGCCATTCA-3'), Wt1 (FW: 5'-GCAACCACGGCACAGGGTAT-3', RV: 5'- GGGGCCACTCCAGATACACG-3'). All the results are expressed as the mean values with standard error ($n = 4$).

Immunostaining

Immunocytochemistry was performed as described previously (Furue *et al.*, 2005; Hayashi *et al.*, 2010a; Hayashi *et al.*, 2007). Briefly, mESCs were fixed in 4% (w/v) paraformaldehyde before being permealized with 0.1% Triton X-100, and then reacted with primary antibodies. The bound primary antibodies were visualized with AlexaFluor 488-conjugated anti-rabbit, anti-mouse, or anti-goat IgG or AlexaFluor 594-conjugated donkey anti-mouse, anti-rabbit, or anti-goat IgG (Invitrogen). The following primary antibodies were used: anti-Osr1 antibody (sc-68392 from Santa Cruz Biotechnology; 1:100), anti-Wt1 antibody (sc-192 from Santa Cruz Biotechnology; 1:100). anti-Pax2 antibody (covance; 10 μg/ml), anti-laminin antibody (Sigma; 8 μg/ml), and anti-GFP antibody (Abcam; 1:200).

***Transplantation of differentiated mESCs into developing kidney* ex vivo**

The developing kidneys of E12.5 embryos were collected from pregnant mice (C57BL/6JJcl, CLEA Japan) and cultured in DMEM + 10% FCS on the culture filter. Differentiating mESCs were infected with retrovirus (concentrated) carrying GFP on 5th day of induction. Retrovirus was concentrated from the culture medium of Plat-E cells (Cell Biolab) transfected with pMXs-GFP for 48 hours. At 6th day of induction, GFP-transduced, differentiated mESCs (10,000 cells / explant) were injected into cultured whole kidney by microinjection using mouth pipettes. After 5 days of *ex vivo* culture, the cultured kidney were fixed with 4% PFA in 4°C for 30 min. Fixed samples were cryopreserved in OCT compound using the sucrose substitution method. Cryopreserved sections were cut at 13 μm using a cryostat-microtome and used for immunostaining.

Acknowledgements

We would like to thank H. Kagechika for generously providing retinoic derivatives. This study was supported by the following funding: Grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to M.A.; Grants from Wako pure chemical industries, Ltd. to M.A.; Grants-in-aid for scientific research from the Japan Society for the Promotion of Science to Y.H. and M. T.

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