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21

Establishment of Embryonic Stem Cells and Generation of Genetically Modified Rats

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1. Introduction

Authentic embryonic stem (ES) cells are derived from the inner cell mass (ICM) of preimplantation blastocysts in rodents. ES cells have been routinely derived since 1981 (Evans & Kaufman, 1981; Martin, 1981). They are capable of generating germline chimeras following injection into blastocysts. A very large number of knockin/knockout mice have been produced so far, leading to significant progress in both basic research and clinical investigation. However, recent reports indicate that the phenotypes of knockout mice sometimes do not correspond to human diseases (Rogers et al., 2008). Thus, the ES cells of other species, especially rats, have been desired for the generation of new animal models for human diseases.

In 2008, we successfully established rat ES cells with a chimeric contribution (Ueda et al., 2008). Soon after our report, authentic rat ES cells that could complete a germline transmission were established (Buehr et al., 2008; Li et al., 2008). These reports suggest that a removal of serum from a culture medium is necessary for maintaining the pluripotency of rat ES cells (Kawamata & Ochiya, 2010a). However, despite the assertion in these reports, we recently established high-quality rat ES cells by using a combination of 20% serum and signaling inhibitors. Furthermore, this culture condition enabled the ES cells to receive gene manipulation, leading to obtaining genetically modified rats via germline transmission. We also discovered an indispensable technique during a blastocyst injection process for the generation of germline chimeras (Kawamata & Ochiya, 2010b).

This new technology should provide valuable animal models for the study of human diseases by the induction of gene-targeting manipulations in the rat ES cells. In this chapter, we discuss the techniques for the establishment of rat ES cells compared to mouse ES cells and the creation of genetically modified rats.

2. Mouse ES cells

ES cells are derived from the inner cell mass (ICM) of blastocysts and are capable of unlimited, undifferentiated proliferation *in vitro*. Mouse ES cell lines were first established by culturing ICM (Evans & Kaufman, 1981; Martin, 1981) in the presence of serum and a feeder cell layer made of mouse embryonic fibroblast (MEF). Later, it was shown that the leukemia inhibitory factor (LIF) is the key cytokine secreted by feeders to support mouse ES cell self-renewal and that LIF was able to replace the requirement for feeders in propagation (Smith et al., 1988; Williams et al., 1988). These cells have a stable developmental potential to

form derivatives of all three embryonic germ layers even after prolonged culture (Thomson & Marshall, 1998) and have been used to study the mechanism of cell differentiation. Moreover, they are capable of generating germ-line chimeras following injection into the blastocyst (Bradley et al., 1984). Thus, the creation of targeted mutation in the mouse has been a valuable source of animal models of human disease.

In 2003, Ying et al. demonstrated that bone morphogenetic proteins (BMPs) could replace serum and act together with LIF to maintain mouse ES cell self-renewal (Ying et al., 2003). Furthermore, in 2008, they found that a combination of mitogen-activated protein kinase kinase (MEK) inhibitor PD0325901 and glycogen synthase kinase-3 (GSK3) inhibitor CHIR99021, termed 2i, could replace serum, MEF, and LIF in mouse ES cells (Ying et al., 2008). Under this condition, PD0325901 shields inductive differentiation stimuli including autocrine fibroblast growth factor-4 (FGF4). CHIR99021 enhances ES cell propagation, which might be due to its exerting a global modulation of the ES cell metabolic and biosynthetic capacity rather than having a direct anti-apoptotic action (Ying et al., 2008) (Fig. 1).

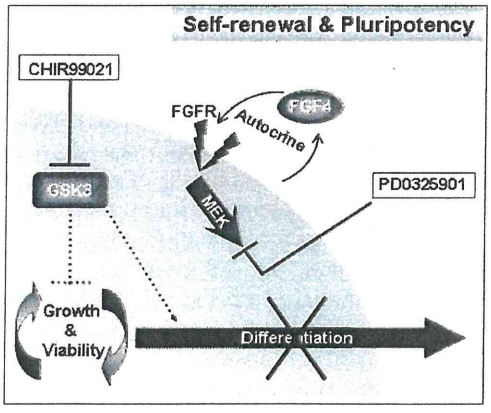

Fig. 1. Mechanism of maintenance of self-renewal and pluripotency

3. Rat

The laboratory rat was the first mammalian species domesticated for scientific research, and it has been used as an animal model in physiology, toxicology, nutrition, behavior, immunology, and neoplasia for over 150 years (Jacob, 1999). The physiology is easier to monitor in the rat, and, over time, a volume of data has developed that will take years to be replicated in the mouse. Moreover, in many cases, the physiology is more similar to the corresponding human condition. The size of the animal enhances its use as a disease model, not just because of the ability to perform surgical procedures but also because of the proportional size of important structures in organs that affects both the degree to which the organ is involved in an experimental lesion and the effects of the distance from the drug administration to specific anatomical areas (Iannaccone & Jacob, 2009).

3.1 Background of rat ES cells

After the first mouse ES cell lines were derived 29 years ago (Evans & Kaufman, 1981; Martin, 1981), many efforts were made to establish rat ES cells. Although a culture medium

of the mouse ES cells, composed of either serum and LIF or BMP and LIF, has been thought to be available in all species, several groups have failed to establish rat ES cells under similar conditions (Brenin et al., 1997; Buehr et al., 2003; Demers et al., 2007; Fandrich et al., 2002; Vassilieva et al., 2000). We have succeeded in cloning complete rat LIF cDNA and demonstrated that rat LIF has an effect on rat ES cells for the maintenance of a stem-cell phenotype (Takahama et al., 1998). However, their self-renewal potential was temporal in early passages, which may be due to the fact that a similar culture medium to that of mouse ES cells was used.

In 2008, we established new lines of rat ES cells with chimeric contribution by using a devised culture medium and passaging method (Ueda et al., 2008). However, our ES cells could not achieve germline transmission. While a general culture medium for mouse ES cells contains 15 or 20% FBS, our culture medium contains only 3% FBS. Details of the method for establishing rat ES cells are described below.

Methods; The rat ES-cell culture medium consisted of DMEM/F12 supplemented with 3% (vol/vol) FBS, 0.1 mM 2-mercaptoethanol, 1% nonessential amino acid, 2 mM L-glutamine, 1 mM sodium pyruvate, antibiotic antimycotic and a nucleoside solution. Frozen embryos obtained at 4.5 days post-coitum of Wistar rats were used for the establishment of ES cells. After removal of the zona pellucida by treating with tyrode's solution, seven to ten embryos were placed on a plate pre-seeded with mitotically inactivated MEFs by a treatment with mytomycin C. After 2 or 3 days, ICM-derived cells were dissociated into clumps mechanically or by exposure to 0.05% collagenase type IV before transfer onto new MEFs. The propagated cells were routinely passaged every 3-4 days up to 5 passages in the culture medium in the presence of rat LIF at 1000 U/ml and were then cultured in a medium supplemented with 0, 250, 500, or 1000 U/ml rat LIF.

These rat ES cells showed marker gene expression of ES cells, such as Oct4, Nanog, and SSEA1, and formed embryoid bodies (EBs) after the ES-cell colonies were dissociated by treatment with collagenase IV. Teratomas were formed by subcutaneous, intratesticular or intraperitoneal injection of rat ES cells into SCID mice. Finally, chimeric rats were generated from embryos in which the rat ES cells, cultured in the presence of rat LIF, were injected (Ueda et al., 2008; Kawamata & Ochiya, 2010a). However, germline transmission was not achieved in the chimeras.

Soon after this report, other groups succeeded in establishing authentic ES cells completing germline transmission (Buehr et al., 2008; Li et al., 2008; Hirabayashi et al., 2010). The common technique for maintaining the pluripotency of rat ES cells was to remove the content of fetal bovine serum (FBS) in the culture medium. The two groups suggested that failure in the establishment of authentic rat ES cells over the two past decades was due to the presence of serum (Buehr et al., 2008; Li et al., 2008). Indeed, serum may contain various kinds of nutrient factors as well as differentiation ones for rat ES cells (Kawamata & Ochiya, 2010a).

The two groups used the 2i, MEK and GSK inhibitors and LIF to overcome the difficulty in the generation of germline-competent rat ES cells (Buehr et al., 2008; Li et al., 2008; Hirabayashi et al., 2010). These studies suggest that cell-signaling inhibitors play a critical role in the maintenance of rat ES cells as well as rat iPS cells.

3.2 Maintenance of pluripotency and self-renewal by signal inhibitors

Recent reports suggest that small molecules, which inhibit GSK3, FGF4 through the MAPK pathway, TGFβ, or ROCK signaling, have effects on ES cells for the maintenance of pluripotency and self-renewal. GSK3 is a central node for the negative modulation of a range of anabolic processes and generally acts to suppress the cellular biosynthetic capacity

(Frame & Cohen, 2001). GSK3 is inhibited by phosphorylation downstream of growth factors that activate phosphatidyl inositol 3 kinase and Akt. GSK3 is also a key component of the β-catenin destruction complex, and pharmacological inhibition of GSK3 increases cytoplasmic and nuclear β-catenin, mimicking canonical Wnt signaling (Ding et al., 2000). The Wnt pathway was assumed to maintain self-renewal of ES cells because the main components of the canonical Wnt pathway were detected in undifferentiated human ES cells (Sato et al., 2003). Indeed, Wnt pathway activation by 6-bromoindirubin-3'-oxime (BIO), a specific pharmacological inhibitor of GSK3 (Meijer et al., 2003), maintained an undifferentiated phenotype in mouse and human ES cells and sustained expression of the pluripotent state-specific transcription factors Oct4, Rex1 and Nanog even in the absence of LIF and MEF (Sato et al., 2004). However, BIO is not highly selective and cross-reacts with cyclin-dependent kinases and other kinases, while CHIR99021 was defined as a more selective inhibitor of GSK3 (Bain et al., 2007; Murray et al., 2004; Zhen et al., 2007). Ying et al. found that the activity of mouse ES cells was reduced by BIO but not by CHIR99021 (Ying et al., 2008). In a report relating to the Wnt pathway, a high-throughput cell-based assay showed that a small molecule IQ-1 allowed for long-term expansion of mouse ES cells and inhibited spontaneous differentiation to prevent β–catenin from switching coactivator usage from CBP to p300 (Miyabayashi et al., 2007). These reports suggest that the addition of the GSK3 inhibitor or Wnt recombinants in the ES culture medium might be a useful method to continuously propagate undifferentiated ES cells.

FGF signaling is a conserved initiator of vertebrate neural development (Bertrand et al., 2003; Delaune et al., 2005; Launay et al., 1996; Streit et al., 2000; Wilson et al., 2000). Activation of FGF receptors (FGFRs) can initiate transduction via three major intracellular pathways: classical MAPk, phosphatidylinositol 3'-OH kinase (PI3K), and phospholipase C gamma (PLCγ), the last two of which can activate protein kinase C proteins (PKCs), which can in turn stimulate ERK1/2 signaling (Schonwasser et al., 1998). A high-throughput chemical screen with a library of 50,000 compounds revealed that the compound SC-1 dually inhibited RasGAP and ERK1, which propagate mouse ES cells in an undifferentiated, pluripotent state even in the absence of MEF, serum and LIF (Chen et al., 2006). Treatment of ES cells with the specific inhibitor for MEK, PD098059 (Burdon et al., 1999), ERK, PD184352 or FGFR, PD173074 and SU5402 also suppressed differentiation of ES cells (Kunath et al., 2007; Stavridis et al., 2007). Furthermore, the majority of Fgf null (Fgf-/-) ES cells (Wilder et al., 1997) or Erk2-/- ES cells were able to retain expression of Oct4 under a differentiation condition without LIF (Kunath et al., 2007). Since Fgf4 mRNA is expressed specifically in ES cells of various animals, FGF4 has been considered as a marker gene of ES cells. On the other hand, these reports suggest that an autoinductive stimulation of the MAPk by FGF4 enhances differentiation of ES cells, especially into neural cells. Thus, the MAPk inhibition might be a key method for suppressing differentiation of ES cells.

An addition of type 1 TGFβ receptor Alk5 (A-83-01) to the 2i plus LIF medium enabled the generation of rat-induced pluripotent stem (iPS) cells with chimeric contribution, although germline transmission was not accomplished (Li et al., 2009). Furthermore, a combination of MEK and the ALK5 inhibitors dramatically improved the efficiency of iPS cell generation from human fibroblasts (Lin et al., 2009). These reports indicate that the inhibition of TGFβ signaling also plays a key role in pluripotency.

Recently, Watanabe et al. found that a ROCK inhibitor, Y-27632, caused human ES cells to block apoptosis after dissociation into single cells by enzymatic treatment. Characteristically, human ES cells need to be subcultured by the bulk-passage method since single ES cells form

scant colonies. The propagated ES cells cultured by Y-27632 were positive for alkaline phosphatase (ALP), marker genes, such as E-cadherin, Oct4, and SSEA4, and the number of chromosomes was normally kept during a long-term culture (Watanabe et al., 2007). Although the mechanism that allows Y-27632 to form a human ES-cell colony with an undifferentiated state is unknown, the compound was recently used for a single-cell-passaging method.

3.3 Problems with current rat ES cells

Although the 2i plus LIF medium enables the establishment of authentic rat ES cells, the event of germline transmission is rarely achieved. A main reason for the failure is chromosomal instability in rat ES cells during long-term culture. It is known that a chromosomal abnormality is one of the major causes for the loss of germline competence of mouse ES cells (Liu et al., 1997). The germline competence seems to depend on a rat strain for donor ES cell-derivation and host blastocysts. Thus, trials to produce more potent cell lines and to find the optimal combination of rat strains for donor ES cells and host blastocysts remain to be addressed (Buehr et al., 2008; Li et al., 2008).
The rat ES cells cultured in the serum free-2i plus LIF medium are sensitive to the stimuli of genetic manipulation by electroporation and drug-selection, which lead to cell death. To overcome this problem, the temporal use of serum is necessary to protect rat ES cells from the death by such stimuli (Buehr et al., 2008; Li et al., 2008).

3.4 Importance to establish high-quality rat ES cells

To produce genetically modified rats, especially in knockout/knockin rats, it is necessary to establish high-quality rat ES cells that retain normal karyotype and pluripotency during long-term culture and should be strongly resistant to stimuli during the process of genetic manipulation. The use of serum is one way to overcome this problem because it generally enables culturing cells to be vigorous.

4. Establishment of rat ES cells

The use of serum is a way to overcome the problem of weakness in rat ES cells because it generally enables culturing cells to be vigorous. We addressed suitable combinations of signaling inhibitors based on a culture medium that included 20% (vol/vol) FBS, DMEM (including 110 mg/L sodium pyruvate and 200 mM GlutaMAX), 0.1 mM 2-mercaptoethanol, 1% nonessential amino acid stock, and 1 x antibiotic antimycotic. Mitomycin C-treated MEFs resistant to neomycin (Millipore) were used as feeders and maintained in DMEM / 10% FBS medium with 1 x antibiotic antimycotic.

4.1 An effect of Rho kinase inhibitor

We cultured Wistar rat blastocysts in a basic medium containing 20% FBS, which is generally used for mouse ES cell culture. Although the inner cell mass (ICM) outgrew and showed mouse ES cell-like morphology, an ES cell colony did not appear after dissociation and replating. An addition of the Rho kinase inhibitor Y27632 (10 μM) remarkably improved their outgrowth, leading to continuous expansion by performing a clump-passaging method. The clump included 5 to 20 cells. Once the colonies were dissociated into single cells, most of them immediately differentiated. The ES cell-like cells formed domed colonies and were positive for alkaline phosphatase activity and pluripotency markers such as *Oct4, Nanog* and *Sox2* mRNAs (Fig. 2).

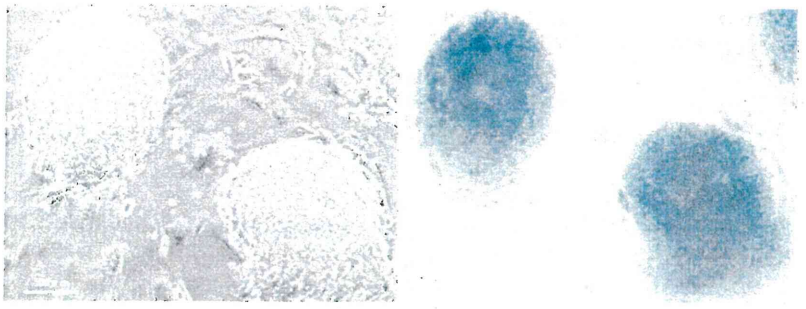

Fig. 2. Colonies cultured in a Y27632-containing medium. At passage 6, the colonies were stained blue, which means that they were positive for alkaline phosphatase activity (right). (scale bar, 100 μm)

Generating chimeric animals achieving germline transmission is the gold standard for documenting authentic ES cells. Thus, we tried to produce germline chimeras via microinjection with the rat ES cells into blastocysts. However, the cells did not contribute any tissues or organs. Thus, the cells established by an addition of Y-27632 alone were not authentic ES cells. Details of the blastocyst injection method are described below.

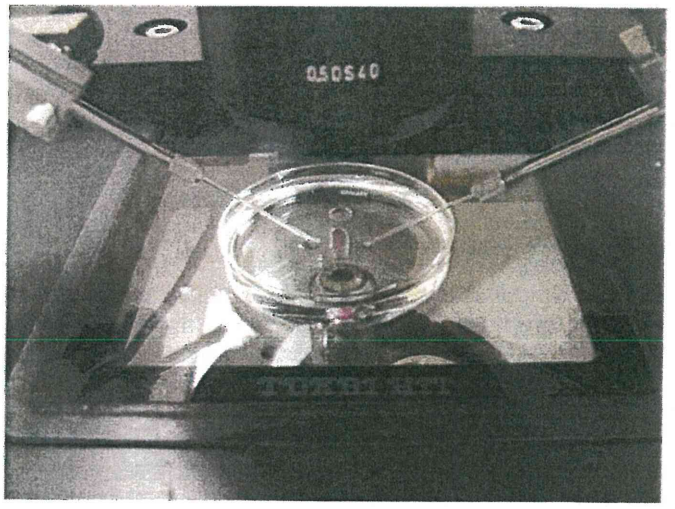

Fig. 3. Device for blastocyst injection with ES cells

Methods; The blastocysts from E4.5 timed-pregnant rats were placed into 500 l of an injection medium, a basal ES cell medium without antibiotic antimycotic, and then were incubated for 2-3 hr. The well-expanded blastocysts were used for microinjection. For ES cell preparation, 10 to 20 domed or floated colonies were picked up by hand-made capillary and treated with Accutase droplet for 5 min at 37 °C, followed by splitting into single cells in a droplet of the injection medium. The cells were transferred in 500 µl of the injection medium and incubated for 30-60 min at room temperature. After centrifugation, ES cells were transferred into a droplet of the injection medium in mineral oil (SIGMA). Ten to 15 ES cells were injected into each blastocyst (Fig. 3) and incubated at 37 °C for 3-5 hr in the injection

medium to allow the embryos to recover. Ten to 20 embryos were then transferred into the uterine horn of each E3.5 pseudopregnant female rat.

4.2 Four signaling inhibitors are necessary to establish rat ES cells

We tried various combinations of signaling inhibitors to establish authentic ES cells. Finally, we found that the use of a combination of 4 inhibitors, 10 μM Y-27632, 1 μM PD0325901, 0.5 μM A-83-01, and 3 μM CHIR99021 (termed YPAC) allowed the establishment of authentic rat ES cells. PD0325901, A-83-01, and CHIR99021 are the signaling inhibitors for MEK, Tgfβ, and GSK, respectively.

Cell proliferation of ICM outgrowth was quite rapid under the YPAC condition. The ICMs were picked up using a hand-made needle, followed by extraction of mRNA for the analysis of gene expression. The expression levels of ES cell-marker genes, Oct4, Nanog, Sox2, and Rex1, in ICM cells with YPAC were over 100-fold higher than those without YPAC. Under the YPAC condition, blastocyst outgrowths were observed in 51 samples for all the tested embryos regardless of the strains. The blastocyst strains were derived from Wistar (albino), Long-Evans Agouti (LEA, agouti), or a hybrid of Wistar and LEA (agouti).

A total of Six ES cell lines were established from the blastocysts derived from the three strains. Details of the method are described below.

Methods: After approximately 7 days, the blastocyst outgrowths were cut into pieces and replated under the same YPAC conditions. Emerging ES cell colonies were then dissociated using Accutase and expanded. Established ES cell lines were routinely maintained under MEF-YPAC conditions and passaged every 3-4 days. Floated colonies were also passaged.

Domed colonies were formed from dissociated single cells and could be expanded infinitely. The morphology of their domed colony (Fig. 4) was similar to that of the mouse ES cell colony but slightly different from that of the rat ES cell under the condition of a Y medium (Fig. 2).

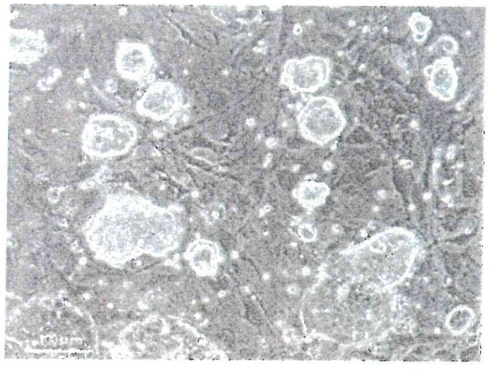

Fig. 4. Colonies cultured in a YPAC-containing medium (passage 3). (scale bar, 100 μm)

4.3 Characteristics of rat ES cells

The YPAC condition was indispensable for maintenance of the rat ES cells. Y was necessary for adherence on MEF, while PAC was necessary for maintaining their pluripotency. If Y was removed from the YPAC medium, few colonies appeared with proliferating. Thus, Y is the most important cell-signaling inhibitor in the YPAC medium.

The rat ES cell colonies tend to detach from MEF, differently from mouse ones. The domed colonies are easily detached by pipetting, followed by dissociation and passaging steps.

Recent reports have indicated that the domed colony possesses a normal karyotype, while the tightly adherent monolayer colony shows chromosomal instability (Kawamata & Ochiya, 2010; Tong et al., 2010). In fact, our rat ES cells possessed a normal karyotype during long-term culturing due to the passaging of floated colonies.

An alkaline phosphatase activity was positive in the rat ES cells. A microarray analysis showed the rat ES cells kept high levels of marker gene expression such as *Oct4, Nanog, Sox2, Rex1, Dppa3, Cdh1, and Tbx1*. Immunocytochemistry also showed that Oct4, Nanog, and Sox2 proteins were expressed in undifferentiated cells.

The classical method to induce ES cell differentiation is to allow the cells to grow in suspension and to form three-dimensional aggregates known as embryoid bodies (EBs) (Keller 1995). Dissociated ES cells were plated into Low-Cell-Binding-Dishes in the basal (without YPAC) medium. EBs were formed from the ES cells at a much lower efficiency compared with their formation from mouse ES cells. The expression of marker genes decreased during the process of EB differentiation. In the presence of PAC, cells aggregated with high efficiency and formed a clear three-dimensional structure. The EBs with PAC at day 7 still sustained high expression levels of the marker genes.

The rat ES cells formed teratomas 34 days after transplantation under the skin of an immunodeficient SCID mouse. A histological examination showed that the teratomas contained all three germ layers, namely, the intestinal epithelium (endoderm), cartilage (mesoderm), and neuronal rosette (ectoderm).

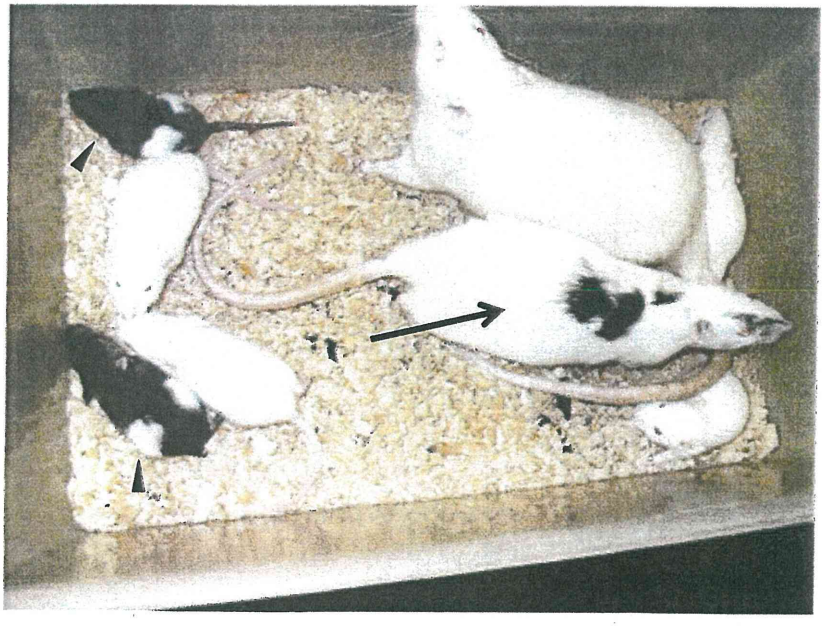

Fig. 5. Germline transmission from chimeric rats. The ES cell line derived from the LEA rat strain was injected into Wistar rat blastocysts, leading to the production of a female chimeric rat with an agouti coat color (arrow). The chimera was mated with a male Wistar rat, and ES cells were transmitted to the next generation with agouti coat color as a Wistar and LEA hybrid strain (arrowheads)

4.4 Production of germline chimeras

We first used a basic ES cell medium without YPAC. However, a coat-color chimera was hardly produced despite the fact that the ES cell line at early passages (6 to 8) was used. Only one male chimera out of 44 pups was obtained, but the chimerism was very sparse.

Next, we added YPAC to the medium during the process of microinjection and blastocyst incubation. This idea was came from the results showing that PAC maintains the pluripotency of cultured cells or EBs. Indeed, PAC addition significantly improved the efficacy to produce chimeric rats. Eight of 23 coat-color chimeras were obtained from the same ES cell line, as reported above, at passage 11 or 12. The generation of coat-color chimeras was successful in all 6 cell lines. Moreover, after mating with male rats, germline transmission was accomplished in adult female chimeras derived from all the 6 cell lines independently of the rat strain (Fig. 5). This efficiency is considerably higher than that seen in previous reports (Buehr et al., 2008; Li et al., 2008), which might be due to the maintenance of a normal karyotype in the ES cells during long-term culture.

4.5 Generation of genetically modified rats

To monitor the ability of stem cells by observing fluorescence, we introduced a transgene in which a Venus protein was expressed by an *Oct4* promoter/enhancer. Venus is a mutant protein of yellow fluorescent protein (YFP) (Nagai et al., 2002). The 3.9 kb *Oct4* promoter was obtained from Wistar rat genomic DNA. This region is known to include both the proximal and the distal enhancer, which gives *Oct4* expression in the morula, inner cell mass (ICM), epiblast, primordial germ cells (PGCs), and ES cells (Chew et al., 2005). Oct4 mRNA is slightly expressed in somatic stem cells in adult mice (Lengner et al., 2007). Details of the gene introduction are described below.

Methods; For gene introduction, a nucleofector was used. After dissociating ES cells with Accutase, they were washed with PBS (-). Ten μg pOct4-Venus transgene linearized by SalI was transfected into 3x 10^6 LEA rat ES cells with the Mouse ES Cell Nucleofector Kit (Amaxa Inc.). The cells were plated on MEFs in the YPAC medium with 2% matrigel (BD Biosciences). Here, the use of 2% matrigel is important to maintain the attachment of colonies on MEF. A single colony of a Venus-positive transfectant was picked up using a hand-made capillary and expanded without drug selection.

In this expansion process of each clone, we found that dominant clones showed a Venus expression pattern with heterogeneity, indicating a complex with a strong positive, a weak positive, and a negative. In addition, a small number of clones possessed a Venus expression pattern with homogeneity. A homogeneous expression pattern was also demonstrated by immunocytochemistry for the Oct4 protein. Thus, we injected this clone into Wistar rat blastocysts, leading to the production of chimeric rats. After mating with LEA male rats, Oct4-Venus transgenic rats were delivered from the female chimera via germline transmission. Genotyping for the Oct4-Venus transgene was performed by PCR analysis to amplify the Venus DNA fragment. The transgenic rats were healthy and could produce a new generation. Venus fluorescence was detected in PGCs in fetal gonad at 17.0 days post-coitum (Fig. 6).

We further investigated the Oct4-Venus expression during the outgrowth of ICM and expansion of ES cells. The Venus-positive blastocyst was plated on MEFs in the YPAC medium. ICM cells rapidly expanded, and Venus fluorescence was observed in some of the cells. After replating the ICM by dissociation with Accutase, domed colonies possessing homogeneous expression of Oct4-Venus appeared. The domed colonies could be infinitely

continued to passage (Fig. 7). This result suggests that we had generated Oct4-Venus transgenic rats, which enabled us to monitor authentic rat ES cells with Venus fluorescence.

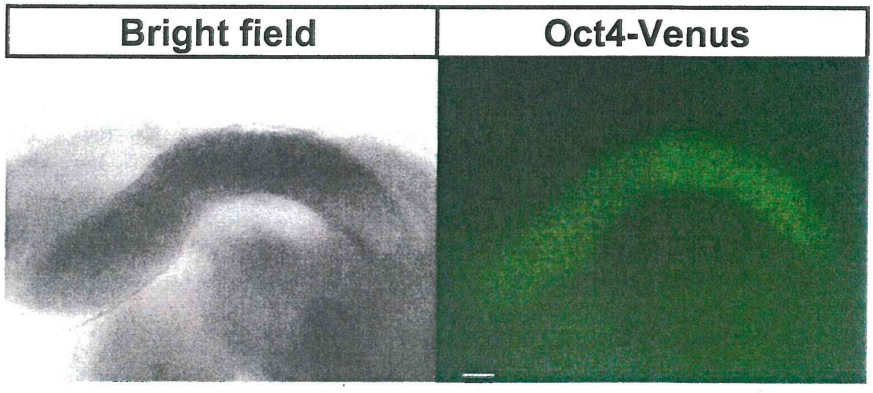

Fig. 6. Oct4-Venus expression in PGCs in fetal gonad. Fetal gonad of Oct4-Venus transgenic female embryo at 16.0 days of gestation was dissected, and Venus fluorescence was observed. (scale bar, 100 μ m)

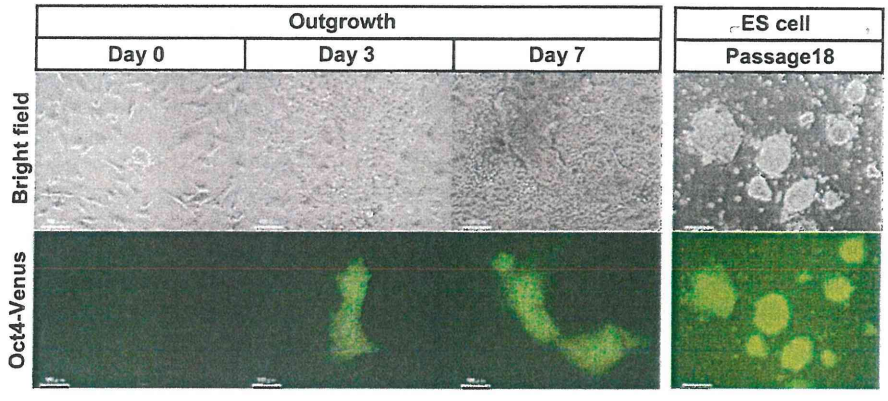

Fig. 7. Oct4-Venus expression during outgrowth of ICM and expansion of ES cells. Blastocyst derived from Oct4-Venus transgenic rats was outgrown on MEFs in the YPAC medium for 7 days. Venus fluorescence was detected in the ICM cells, while it was not detected in differentiated cells. Homogenous Venus fluorescence was observed in ES cells at passage 18. (scale bar, 100 μ m)

5. Conclusion

Our results demonstrated that the use of a combination of serum and cell-signaling inhibitors during outgrowth, cell culture, and blastocyst injection leads to the generation of germline chimeras with extremely high efficiency. Furthermore, we generated genetically modified rats from ES cells. The complete generation of Tg rats might be based on the use of

a culture medium containing 20% serum and YPAC, which might strongly protect from cell damage during gene introduction with electric stimuli and maintain pluripotency with a stable karyotype during the cloning and expansion process. Previous works suggested that failure in the establishment of authentic rat ES cells over the two past decades was due to the presence of serum (Buehr et al, 2008; Li et al., 2008). Indeed, serum may contain various kinds of nutrient factors as well as differentiation ones for rat ES cells. Our reason for the present success in the establishment of such significant pluripotent cell lines might be not only the signaling inhibitors shielding ES cells from differentiation but also the utilization of the nutrients in the serum.

Although two groups have reported the establishment of authentic rat ES cells, only one out of several cell lines accomplished germline transmission in each group (Buehr et al., 2008; Li et al., 2008). Thus, trials to produce more potent cell lines and to find the optimal combination of rat strains for donor ES cells, host blastocysts, and recipient foster females remain to be addressed (Buehr et al., 2008; Li et al., 2008). In this study, our YPAC-culture and -injection method overcame the difficulty of completing germline transmission in all the six ES cell lines independently of the rat strain. The YPAC condition will enable the selection of preferable rat strains for the generation of genetically modified rats from ES cells, bringing great advantages to research for strain-specific disease models. We believe that the availability of our rat ES cells and the YPAC-injection technique will also open up a valuable platform for routinely generating knockout/knockin rats, holding out the promise for the generation of new disease models.

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The Progression of Liver Fibrosis Is Related with Overexpression of the miR-199 and 200 Families

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Abstract

Background: Chronic hepatitis C (CH) can develop into liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Liver fibrosis and HCC development are strongly correlated, but there is no effective treatment against fibrosis because the critical mechanism of progression of liver fibrosis is not fully understood. microRNAs (miRNAs) are now essential to the molecular mechanisms of several biological processes. In order to clarify how the aberrant expression of miRNAs participates in development of the liver fibrosis, we analyzed the liver fibrosis in mouse liver fibrosis model and human clinical samples.

Methodology: In a CCL_4-induced mouse liver fibrosis model, we compared the miRNA expression profile from CCL_4 and olive oil administrated liver specimens on 4, 6, and 8 weeks. We also measured expression profiles of human miRNAs in the liver biopsy specimens from 105 CH type C patients without a history of anti-viral therapy.

Principle Findings: Eleven mouse miRNAs were significantly elevated in progressed liver fibrosis relative to control. By using a large amount of human material in CH analysis, we determined the miRNA expression pattern according to the grade of liver fibrosis. We detected several human miRNAs whose expression levels were correlated with the degree of progression of liver fibrosis. In both the mouse and human studies, the expression levels of miR-199a, 199a*, 200a, and 200b were positively and significantly correlated to the progressed liver fibrosis. The expression level of fibrosis related genes in hepatic stellate cells (HSC), were significantly increased by overexpression of these miRNAs.

Conclusion: Four miRNAs are tightly related to the grade of liver fibrosis in both human and mouse was shown. This information may uncover the critical mechanism of progression of liver fibrosis. miRNA expression profiling has potential for diagnostic and therapeutic applications.

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Introduction

Chronic viral hepatitis is a major risk factor for hepatocellular carcinoma (HCC) [1]. Worldwide 120–170 million persons are currently chronically Hepatitis C Virus (HCV) infected [2]. Due to repetitive and continuous inflammation, these patients are at increased risk of developing cirrhosis, subsequent liver decompensation and/or hepatocellular carcinoma. However, the current standard of care; pegylated interferon and rivabirin combination therapy is unsatisfied in the patients with high titre of HCVRNA and genotype 1b. Activated human liver stellate cells (HSC) with chronic viral infection, can play a pivotal role in the progression of liver fibrosis [3]. Activated HSC produce a number of profibrotic cytokines and growth factors that perpetuate the fibrotic process through paracrine and autocrine effects.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading target mRNA or suppressing their translation [4]. There are currently 940 identifiable human miRNAs (The miRBase Sequence Database - Release ver. 15.0). miRNAs can recognize hundreds of target genes with incomplete complementary; over one third of human genes appear to be conserved miRNA targets [5][6]. miRNA is associated several pathophysiologic events as well as fundamental cellular processes such as cell proliferation and differentiation. Aberrant expression of miRNA can be associated with the liver diseases [7][8][9][10]. Recently reported miRNAs can regulate the activation of HSCs and thereby regulate liver fibrosis. miR-29b, a negative regulator for the type I collagen and SP1, is a key regulator of liver fibrosis [11]. miR-27a and 27b allowed culture-activated rat HSCs to switch to a more quiescent HSC phenotype,

with restored cytoplasmic lipid droplets and decreased cell proliferation [12].

In this study, we aimed to reveal the association between miRNA expression patterns and the progression of liver fibrosis by using a chronic liver inflammation model in mouse. We also sought to identify the miRNA expression profile in chronic hepatitis (CH) C patients according to the degree of liver fibrosis, and to clarify how miRNAs contribute to the progression of liver fibrosis. We observed a characteristic miRNA expression profile common to both human liver biopsy specimens and mouse CCL_4 specimens, comprising the key miRNAs which are associated with the liver fibrosis. This information is expected to uncover the mechanism of liver fibrosis and to provide a clearer biomarker for diagnosis of liver fibrosis as well as to aid in the development of more effective and safer therapeutic strategies for liver fibrosis.

Results

The expression level of several mouse miRNAs was increased by introducing mouse liver fibrosis

In order to identify changes in the miRNA expression profile between advanced liver fibrosis and non-fibrotic liver, we intraperitoneally administered CCL_4 in olive oil or olive oil alone twice a week for 4 weeks and then once a week for the next 4 weeks. Mice were sacrificed at 4, 6, or 8 weeks and then the degree of mouse liver fibrosis was determined by microscopy (Figure S1). miRNA expression analysis was performed from the liver tissue collected at the same time. Histological examination revealed that the degree of liver fibrosis progressed in mice that received CCL_4 relative to mice receiving olive oil alone (Figure 1A). Microarray analysis revealed that in CCL_4 mice, the expression level of 11 miRNAs was consistently higher than that in control mice (Figure 1B).

miRNA expression profile in each human liver fibrosis grade

We then established human miRNAs expression profile by using 105 fresh-frozen human chronic hepatitis (CH) C liver tissues without a history of anti-viral therapy, classified according to the grade of the liver fibrosis (F0, F1, F2, and F3 referred to METAVIR fibrosis stages)(Figure 2, Table S2). Fibrosis grade F0 was considered to be the negative control because these samples were derived from patients with no finding of liver fibrosis. In zebrafish, most highly tissue-specific miRNAs are expressed during embryonic development; approximately 30% of all miRNAs are expressed at a given time point in a given tissue [13]. In mammals, the 20–30% miRNA call rate has recently been validated [14]. Such analysis revealed that the diversity of miRNA expression level among specimens was small. Therefore, we focused on miRNAs with a fold change in mean expression level greater than 1.5 ($p<0.05$) in the two arbitrary groups of liver fibrosis.

Expression of several miRNAs was dramatically different among grades of fibrosis. In the mice study 11 miRNAs were related to the progression of liver fibrosis (mmu-let-7e, miR-125-5p, 199a-5p, 199b, 199b*, 200a, 200b, 31, 34a, 497, and 802). In the human study 10 miRNAs were extracted, and the change in their expression level varied significantly between F0 and F3 (F0<F3: hsa-miR-146b, 199a, 199a*, 200a, 200b, 34a, and 34b, F0>F3: hsa-miR-212, 23b, and 422b). The expression level of 6 miRNAs was significantly different between F0 and F2 (F0<F2: hsa-miR-146b, 200a, 34a, and 34b, F0>F2: hsa-miR-122 and 23b). 5 extracted miRNAs had an expression level that was significantly different between F1 and F2 (F1<F2: hsa-miR-146b, F1>F2: hsa-miR-122, 197, 574, and 768-5p). The expression level of 9 miRNAs changed significantly between F1 and F3 (F1<F3: hsa-miR-146b, 150, 199a, 199a*, 200a, and 200b, F1>F3: hsa-miR-378, 422b, and 768-5p). The miRNAs related to liver fibrosis were extracted using two criteria: similar expression pattern in both the human and the mice specimens and shared sequence between human and mouse. We compared the sequences of mouse miRNAs as described on the Agilent Mouse MiRNA array Version 1.0 (miRbase Version 10.1) and human miRNAs as described on the Agilent Human MiRNA array Version 1.5 (miRbase Version 9.1). The sequences of mmu-miR-199a-5p, mmu-miR-199b, mmu-miR-199b, mmu-miR-200a, and mmu-miR-200b in mouse miRNA corresponded to the sequences of hsa-miR-199a, hsa-miR-199a*, hsa-miR-199a, hsa-miR-200a, and hsa-miR-200b in human miRNA, respectively (Table S3).

Validation of the microarray result by real-time qPCR

The 4 human miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) with the largest difference in fold change between the F1 and F3 groups were chosen to validate the microarray results using stem-loop based real-time qPCR. The result of real-time qPCR supported the result of that microarray analysis. The expression level of these 4 miRNAs was significantly different between F0 and F3 and spearman correlation analysis also showed that the expressions of these miRNAs were strongly and positively correlated with fibrosis grade ($n=105$, $r=0.498$(miR-199a), 0.607(miR-199a*), 0.639(miR-200a), 0.618(miR-200b), p-values<0.0001) (Figure 3).

Over expression of miR-199a, 199a*, 200a, and 200b was associated with the progression of liver fibrosis

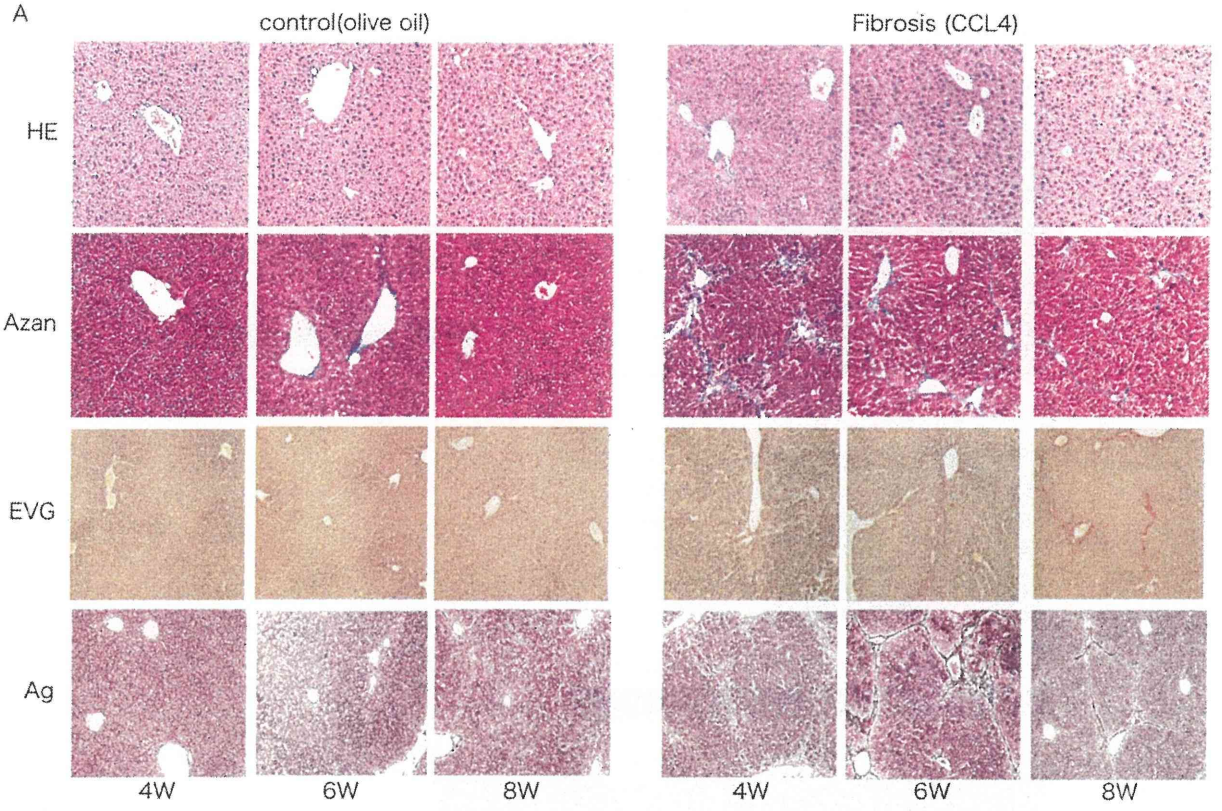
In order to reveal the function of miR-199a, miR-199a*, miR-200a, and miR-200b, we investigated the involvement of these miRNAs in the modulation of fibrosis-related gene in LX-2 cells. The endogenous expression level of these 4 miRNAs in LX2 and normal liver was low according to the microarray study (Figure S2). Transforming growth factor (TGF)β is one of the critical factors for the activation of HSC during chronic inflammation [15] and TGFβ strongly induced expression of three fibrosis-related genes include a matrix degrading complex comprised of α1 procollagen, matrix remodeling complex, comprised of metalloproteinases-13 (MMP-13), tissue inhibitors of metalloproteinases-1 (TIMP-1) in LX-2 cells (Figure 4A). Furthermore, overexpression of miR-199a, miR-199a*, miR-200a and miR-200b in LX-2 cells resulted significant induction of above fibrosis-related genes compared with control miRNA (Figure 4B). Finally we validated the involvement of TGFβ in the modulation of these miRNAs. In LX-2 cells treated with TGFβ, the expression levels of miR-199a and miR-199a* were significantly higher than in untreated cells; the expression levels of miR-200a and miR-200b were significantly lower than in untreated cells. Thus, our in vitro analysis suggested a possible involvement of miR-199a, 199a*, 200a, and 200b in the progression of liver fibrosis.

Discussion

Our comprehensive analysis showed that the aberrant expression of miRNAs was associated with the progression of liver fibrosis. We identified that 4 highly expressed miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) that were significantly associated with the progression of liver fibrosis both human and mouse. Coordination of aberrant expression of these miRNAs may contribute to the progression of liver fibrosis.

Prior studies have discussed the expression pattern of miRNA found in liver fibrosis samples between previous and present study. In this report and prior mouse studies and the expression pattern of

A



B

miRNA	Fold change CCL4 mouse/control		
	4week	6week	8week
mmu-let-7e	2.92	2.09	2.64
mmu-miR-125a-5p	4.29	2.35	2.67
mmu-miR-199a-5p	2.71	2.12	2.38
mmu-miR-199b	3.25	2.55	2.49
mmu-miR-199b*	5.13	4.47	3.94
mmu-miR-200a	2.23	1.63	2.02
mmu-miR-200b	2.38	2.06	2.20
mmu-miR-31	3.4	2.35	3.19
mmu-miR-34a	3.89	3.22	3.5
mmu-miR-497	2.25	2.00	2.55
mmu-miR-802	2.02	2.87	2.74

Figure 1. The change of liver fibrosis in mouse model. A. Representative H&E-stained, Azan-stained, Ag-stained, and EVG-stained histological sections of liver from mice receiving olive oil alone or CCL_4 in olive oil. Magnification is ×10. B. The expression level of mmu-miRNA in mouse liver with olive oil or CCL_4 at 4W, 6W, and 8W respectively, by microarray analysis.
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3 miRNAs (miR-199a-5p, 199b*, 125-5p) was found to be similar while the expression pattern of 11 miRNAs (miR-223, 221, 24, 877, 29b, 29a, 29c, 30c, 365, 148a, and 193) was partially consistent with fibrosis grade [16]. In low graded liver fibrosis, the low expression pattern of 3 miRNAs (miR-140, 27a, and 27b) and the high expression pattern of 6 miRNAs in rat miRNAs (miR-29c*, 143, 872, 193, 122, and 146) in rat miRNA was also similar to our mouse study (GEO Series accession number GSE19865) [11] [12] [17].

The results in this study and previously completed human studies reveal that the expression level of miR-195, 222, 200c, 21, and let-7d was higher in high graded fibrotic liver tissue than in low graded fibrotic liver tissue. Additionally, the expression level of miR-301, 194, and 122 was lower in the high graded fibrotic liver tissue than in low graded fibrotic liver tissue [18] [19] [20](GEO Series accession number GSE16922). This difference in miRNA expression pattern may be contributed to (1) the difference of microarray platform, (2) difference of analytic procedure, and (3) the difference of the species (rat, mouse, and human).

The miR-199 and miR-200 families have are circumstantially related to liver fibrosis. TGFβ-induced factor (TGIF) and SMAD

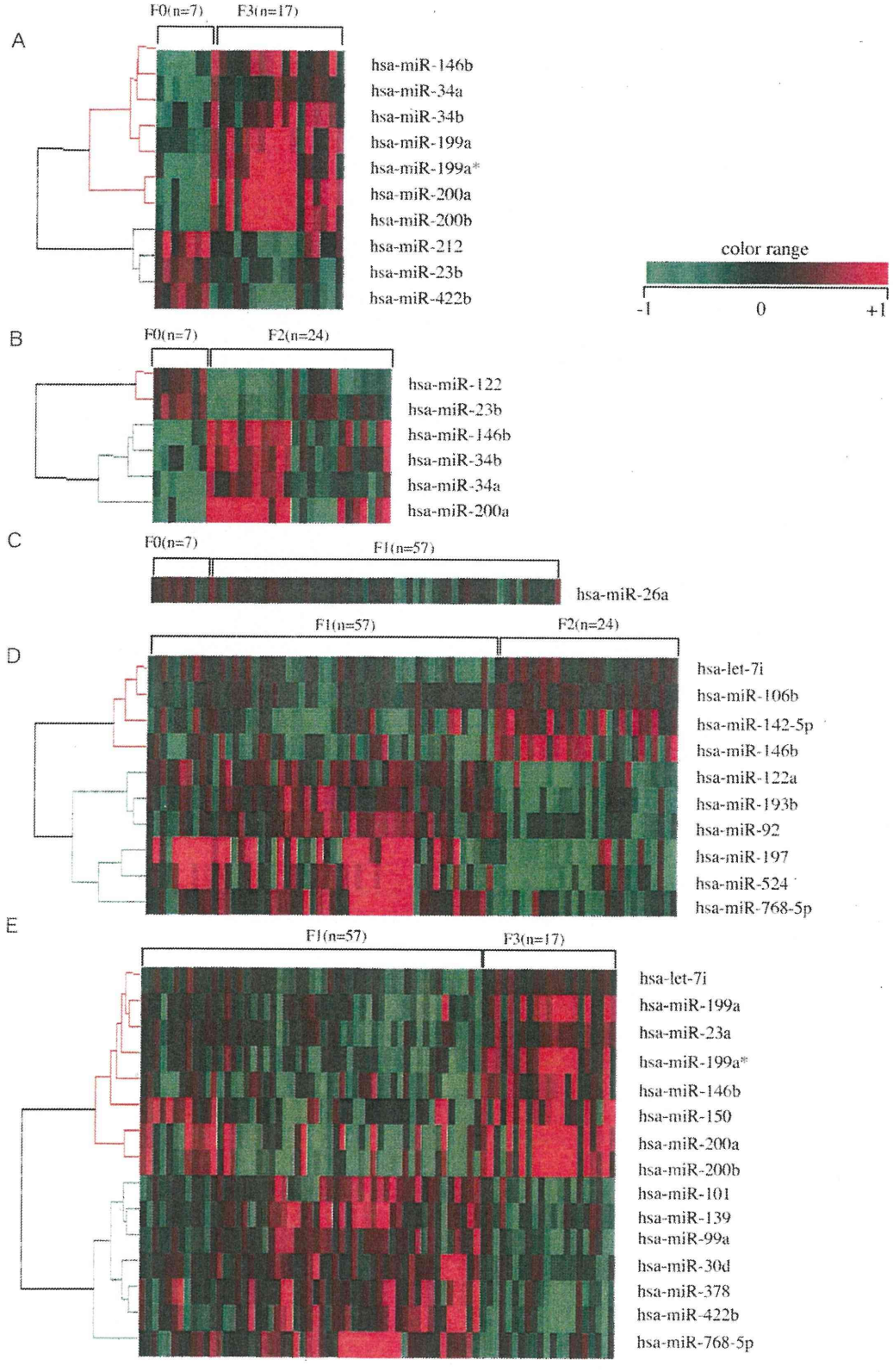
A
F0(n=7)
F3(n=17)
hsa-miR-146b
hsa-miR-34a
hsa-miR-34b
hsa-miR-199a
hsa-miR-199a*
hsa-miR-200a
hsa-miR-200b
hsa-miR-212
hsa-miR-23b
hsa-miR-422b
color range
-1
0
+1
B
F0(n=7)
F2(n=24)
hsa-miR-122
hsa-miR-23b
hsa-miR-146b
hsa-miR-34b
hsa-miR-34a
hsa-miR-200a
C
F0(n=7)
F1(n=57)
hsa-miR-26a
D
F1(n=57)
F2(n=24)
hsa-let-7i
hsa-miR-106b
hsa-miR-142-5p
hsa-miR-146b
hsa-miR-122a
hsa-miR-193b
hsa-miR-92
hsa-miR-197
hsa-miR-524
hsa-miR-768-5p
E
F1(n=57)
F3(n=17)
hsa-let-7i
hsa-miR-199a
hsa-miR-23a
hsa-miR-199a*
hsa-miR-146b
hsa-miR-150
hsa-miR-200a
hsa-miR-200b
hsa-miR-101
hsa-miR-139
hsa-miR-99a
hsa-miR-30d
hsa-miR-378
hsa-miR-422b
hsa-miR-768-5p

Figure 2. Liver fibrosis in human liver biopsy specimen. A. B. C. D. and E. miRNAs whose expression differs significantly between F0 and F3, F0 and F1, F0 and F2, F1 and F2, and F1 and F3, respectively. Relative expression level of each miRNA in human liver biopsy specimen by microarray. Data from microarray were also statistically analyzed using Welch's test and the Bonferroni correction for multiple hypotheses testing. Fold change, p-value are listed in Table S2.
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specific E3 ubiquitin protein ligase 2 (SMURF2), both of which play roles in the TGFβ signaling pathway, are candidate targets of miR-199a* and miR-200b, respectively, as determined by the Targetscan algorithm. The expression of miR-199a* was silenced in several proliferating cell lines excluding fibroblasts [21]. Down regulation of miR-199a, miR-199a* and 200a in chronic liver injury tissue was associated with the hepatocarcinogenesis [9]. miR-199a* is also one of the negative regulators of the HCV replication [22]. According to three target search algorithms (Pictar, miRanda, and Targetscan), the miRNAs that may be associated with the liver fibrosis can regulate several fibrosis-related genes (Table S4). Aberrant expression of these miRNAs may be closely related to the progress of the chronic liver disease.

Epithelial-mesenchymal transition (EMT) describes a reversible series of events during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics [23]. Although EMT is not a common event in adults, this process has been implicated in such instances as wound healing and fibrosis. Recent reports showed that the miR-200 family regulated EMT by targeting EMT accelerator ZEB1 and SIP1 [24]. From our observations, overexpression of miR-200a and miR-200b can be connected to the progression of liver fibrosis.

The diagnosis and quantification of fibrosis have traditionally relied on liver biopsy, and this is still true at present. However, there are a number of drawbacks to biopsy, including the invasive nature of the procedure and inter-observer variability. A number of staging systems have been developed to reduce both the inter-observer variability and intra-observer variability, including the METAVIR, the Knodell fibrosis score, and the Scheuer score. However, the reproducibility of hepatic fibrosis and inflammatory activity is not as consistent [25]. In fact, in our study, the degree of fibrosis of the two arbitrary fibrosis groups was classified using the miRNA expression profile with 80% or greater accuracy (data not shown). Thus, miRNA expression can be used for diagnosis of liver fibrosis.

In this study we investigated whether common miRNAs in human and mouse could influence the progression of the liver fibrosis. The signature of miRNAs expression can also serves as a tool for understanding and investigating the mechanism of the onset and progression of liver fibrosis. The miRNA expression profile has the potential to be a novel biomarker of liver fibrosis.

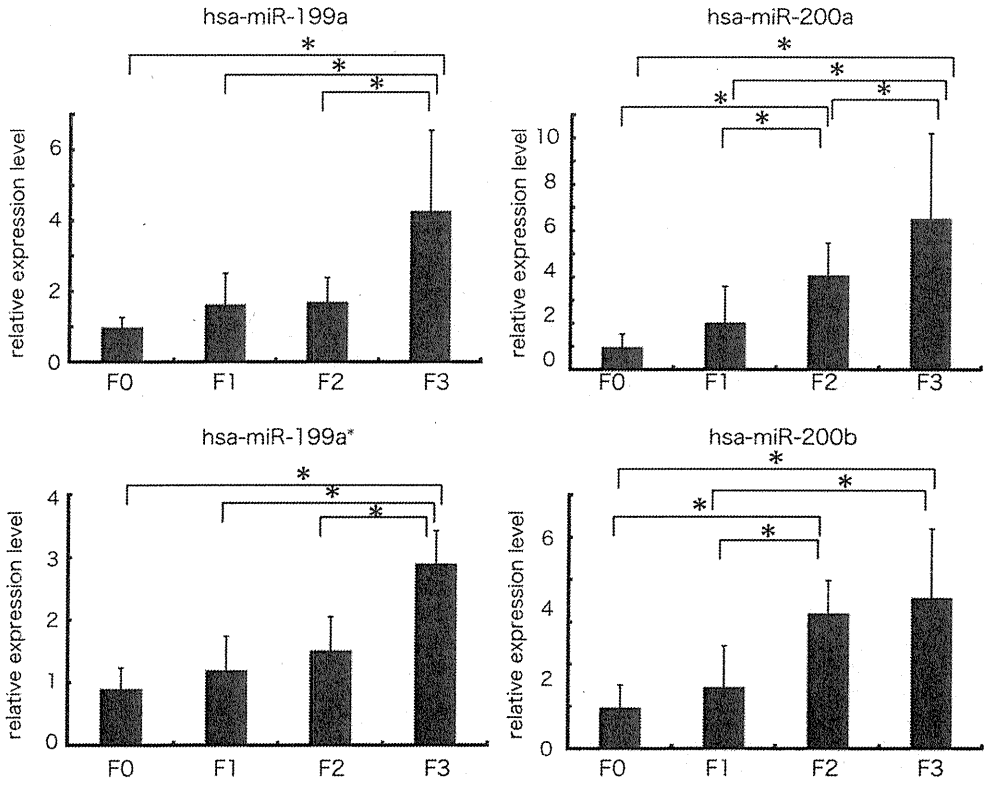

Figure 3. The expression level of miR-199 and 200 families in human liver biopsy specimen by real-time qPCR. Real-time qPCR validation of the 4 miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b). Each column represents the relative amount of miRNAs normalized to the expression level of U18. The data shown are the means+SD of three independent experiments. Asterisks indicates to a significant difference of $p<0.05$ (two-tailed Student-t test), respectively.
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