known to induce B-cell differentiation [19,20], but it is not rich in miR-150, which suppresses B-cell differentiation [21,22]. Our miRNA microarray detected many different types of miRNAs in human breast milk (Figure 1c); however, the functions of many of these are still unknown. Because immune-related miRNAs are well studied and their functions are clarified in the present report, we focused on their presence in human breast milk in which we believe miRNAs should have many more functions, especially in immunologic conditions such as allergy, including atopy and asthma [23,24].

The transfer of miRNA among cells means that miRNA is not only a regulatory molecule within the cell, but also, like cytokines, is a regulatory molecule for cell-cell communication. Our study clearly suggests that miRNA is a transferable genetic material from mother to infant. It is estimated that approximately 1.3×10^7 copies/liter/day of miR-181a are received by a breastfed infant. Further studies are needed to examine the potential clinical use of immune-related miRNAs in breast milk and the mechanisms by which these miRNAs act as a tool for molecular communication between mother and infant (Figure 5). As shown in Figure 1c, although there was more variation between individuals, the expression pattern of miRNA in human breast milk samples from the same mother did not differ much with time after birth. These observations suggest that human breast milk reflects a mother's constitution and her living environment such as food intake and climate. The present study provides insight into how breast milk may protect infants from various infections; proposing that the miRNAs there act as immune-regulatory agents.

Materials and methods

Ethics statement

All the women gave their signed informed consent to participate. The study was approved by Dr. Tadao Ishii,

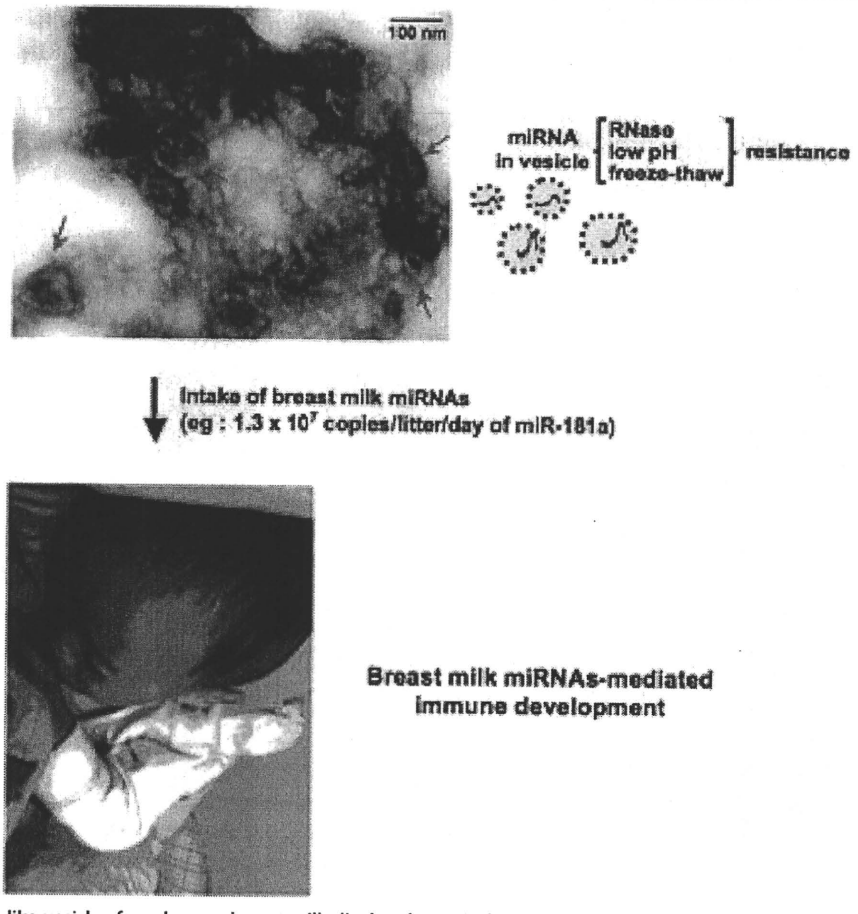

Figure 5 Exosome-like vesicles from human breast milk display the typical size (30–300 nm) and ultrastructure of electron-dense and electron-lucent microvesicles (arrows). miRNAs in the vesicles within breast milk are received by infants and facilitate many aspects of the infant's development. Scale bar, 100 nm.

Manager of Morinaga Co., Ltd and the company's ethics committee (the Ethical Committee of Functional Food Creation).

Sample collection

Human breast milk samples were collected from eight women enrolled in a breastfeeding study at Morinaga Milk Industry Co., Ltd. Human breast milk samples were collected when the infant were aged between 4 days and 11 months (see Table 1). The milk was collected and then the collected samples totaling 50-100 ml were put into in storage bags. All samples were stored at - 80°C until analyzed.

Sample preparation

We chose the samples given <6 months after birth and samples given between 6 and 12 months after birth. Cells and large debris within the breast milk were removed by centrifugation at 2,000 × *g* for 10 min twice; the supernatant was then centrifuged at 12,000 × *g* for 30 min to remove cellular debris. The clear supernatants were used for the analysis.

Total RNA extraction

Total RNA from breast milk was extracted using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA). Breast milk was thawed on ice, diluted with two volumes of *mir*Vana Lysis/Binding Solution, mixed thoroughly by vortex for 30 s and incubated for 5 min. Then 1/10 volumes of miRNA homogenate additive was added, mixed thoroughly by vortex for 30 s and incubated on ice for 10 min. An equal volume of acid/phenol/chloroform (Ambion) was then added to each aliquot. The resulting solutions were mixed by vortex for 1 min and spun for 10 min at 10,000 × *g*. The resulting aqueous volume was mixed thoroughly with 1.25 volumes of 100% molecular-grade ethanol and passed through a *mir*Vana column in sequential 700 μl aliquots. The column was washed according to the manufacturer's protocol, and RNA was eluted in nuclease-free water at 95°C. RNA extraction from the serum was performed using the same method.

Table 1 Time of sample collection

Volunteer	Sample collection (months after birth)
1	6, 8, 8
2	4, 6, 7
3	6, 7, 8, 8
4	7, 7, 8, 9
5	9, 10
6	4 samples within 1 month
7	No information
8	2, 4, 10, 12

Microarray analysis

To detect the expression of miRNAs in human breast milk, 70 ng of total RNA was labeled and hybridized using a Human microRNA Microarray Kit (Agilent Technologies) according to the manufacturer's protocol (Protocol for Use with Agilent Microrna Microarrays Version 1.5). Hybridization signals were detected by a DNA microarray scanner (Agilent Technologies), and the scanned images were analyzed using Agilent Feature Extraction software.

qRT-PCR

qRT-PCR of miRNA expression was performed using the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. To normalize the sample-to-sample variation in the RNA isolation step, synthetic *Caenorhabtidis elegans* miRNA cel-miR-39 (synthetic RNA oligonucleotides synthesized by Qiagen, Valencia, CA, USA) was added as a mixture of 25 fmol of each oligonucleotide in a total volume of 1 ml to each denatured sample (that is, after combining the breast milk and serum samples with Lysis Solution (mirVana miRNA isolation kit; Ambion). All experiments were repeated three times.

RNase and freeze-thawing treatment

To confirm that the miRNA is resistant to RNase digestion, human breast milk was treated with 10 U/ml RNase A and 400 U/ml RNase T1 (Ambion) for 60 min at 37°C. After these treatments, the RNA was extracted from the milk as described above.

To investigate the stability of miRNAs in human breast milk, the milk was subjected to three freeze-thaw cycles of at -20°C or treated for 3 h in a low pH solution (pH 1). miRNA levels were assessed by TaqMan qRT-PCR.

Isolation of CD63 positive exosome

Exosomes in human breast milk were specifically isolated by magnetic beads, using anti-CD63 antibody (BD, Erembodgem, Belgium). Human breast milk (0.3 ml) was incubated with anti-CD63 antibody (BD) or mouse IgG1 (Sigma-Aldrich, St Louis, MO, USA) coupled to magnetic microbeads (50 μl). These were mixed and incubated for 16 h at room temperature. The magnetic immune complexes were washed four times with 500 μl of PBS, then the RNA was extracted as described above.

Transmission electron microscopy

Clear supernatants from human breast milk were centrifuged at 100,000 × *g* for two hours, then washed in phosphate-buffered saline (PBS) and pelleted by ultracentrifugation (100,000 × *g*). The pellet was diluted in PBS. Resuspended exosomes were fixed in 1% glutaraldehyde in PBS (pH 7.4). The samples were stained for

10 min with 1% uranyl acetate. Excess fluid was removed with a piece of Whatman filter paper. All transmission electron micrographs were obtained using JEM1220 electron microscopy at 120 kv.

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Authors' contributions
NK designed and performed the experiments and wrote the manuscript. HI and KS prepared the samples and helped to draft the manuscript. TO designed experiments and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The Morinaga Milk Industry Co., Ltd is a commercial for-profit company. HI and KS are employed/funded by salaries from Moringa Milk Industry Co., Ltd. The Morinaga Milk Industry Co., Ltd has filed patent applications on aspects of this work. NK, KS, HI and TO are named as inventors with the Morinaga Milk Industry Co., Ltd on patent application number 2009-165991/July14, 2009.

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Review

Commitment of stem cells into functional hepatocytes ☆

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ABSTRACT

Liver transplants represent the only way to treat patients suffering from terminal liver failure, but they are associated with numerous problems, including a chronic shortage of donors, high cost, rejection, and side effects for the donor. It is anticipated that regenerative medicine will provide an alternative to liver transplants for such patients. Regenerative medicine refers to the academic field of eliciting the inherent capacity of organisms for self-regeneration to the greatest possible extent in order to develop new methods of treatment for intractable disorders. From this perspective, much is expected from the use of human embryonic stem cells (ES cells) or induced pluripotent stem cells (iPS cells), and the vigorous development of technology to induce the differentiation of such stem cells into cells possessing hepatic functions is underway. Clinical applications of these human stem cells, however, have yet to reach even the earliest stages of implementation. Facing off against these versatile ES cells are stem cells derived from somatic cells present within organisms, which are attracting attention owing to their superiority in terms of ethics and safety, with many research institutes now in the process of elucidating the details of stem cell separation and identification as well as their plasticity and pluripotency. Bone marrow cells are the best-known somatic-cell-derived stem cells, but the use of mesenchymal stem cells (MSCs) found in adipose tissue has also recently attracted attention. This paper will review the differentiation ability and mechanisms of these various stem cell types to hepatocytes and their application to liver regeneration and the future outlook.

Contents

Abbreviations: AT-MSCs, adipose-tissue-derived mesenchymal stem cells; BMP, bone morphogenetic protein; Dex, dexamethasone; EGF, epidermal growth factor; ES cells, embryonic stem cells; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; iPS cells, induced pluripotent stem cells; MET, mesenchymal-to-epithelial transition; MSCs, mesenchymal stem cells; NGF, nerve growth factor; OsM, oncostatin M; VEGF, vascular endothelial growth factor

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1. Introduction

Many studies on stem cell plasticity are challenging the concept that stem cells contain an intrinsically predefined, unidirectional differentiation program. This means that the developmental fate of a stem cell is dependent on the general potential of the cell as well as on microenvironmental cues, such as stimuli from stem cell niche (Eckfeldt et al., 2005). Despite many reports on the differentiation potential of stem cells, there is little understanding of the molecular basis of stem cell plasticity. Array-based gene expression analyses of "stemness" have been reported. Under normal conditions, the expression of "stemness genes" is tightly regulated by a dynamic array of mediators, including the spatial and temporal expression of inhibitors and the epigenetic modulation of the genome. When stem cells are exposed to microenvironmental cues of tissue or organ injury and regeneration, the balance of regulatory mediators is restored, with the plasticity of stem cells being induced towards differentiation into a specific cell lineage.

In the natural milieu, the hepatic differentiation of stem cells involves multiple pathways (Zaret and Grompe, 2008). This in vivo process may be mimicked in vitro by using a combination of various factors and culturing conditions. It is anticipated that, over the next few years, we will see profound investigations of hepatic stem cells and, particularly, of their mechanisms, transduction pathways, and epigenetic modulations. Hepatic differentiation may certainly be enhanced by further studies and a combination of various techniques, including tissue-engineering technologies. In considering stem cell-based therapy, MSCs have emerged with great potential (Franco Lambert et al., in press). An assortment of studies has documented their contribution in hepatogenic generation in vivo and in vitro. Preliminary results of only a few clinical studies on the administration of bone marrow stem cells to liver cirrhotic patients seem to be very promising, but additional well-designed and controlled studies are needed. We herein present the current knowledge what we obtained from differentiation of functional hepatocytes from several types of stem cells.

2. Differentiation of stem cells

In Table 1, key features of several stem cells including ES cells, iPS cells, MSCs, and tissue stem cells (TSCs) are summarized. Recent years have seen substantial progress in research on stem cell plasticity and regenerative medicine using stem-cell-derived cells, and technologies are now being developed to induce the differentiation of numerous cell types from ES cells and TSCs present in adult organs. For cells such as skin and cartilage, regeneration is already underway at the tissue rather than the cellular level, and clinical applications are being launched. In the field of liver regenerative medicine, the future introduction of clinical applications for real human hepatocytes with multiple liver-specific functions is now anticipated (Fig. 1). Numerous laboratories have reported the differentiation induction of ES cells or MSCs as a stem cells source for hepatocytes (Banas et al., 2006, 2007).

3. Current status of hepatic differentiation of ES cells

The first report of hepatic differentiation of mouse ES cells was in 2001 by Hamazaki et al., who produced an embryoid body from an ES cell and subsequently added fibroblast growth factor (FGF), then hepatocyte growth factor (HGF), and finally oncostatin M (OsM), and dexamethasone (Dex) to induce the differentiation of cells exhibiting hepatocyte-like properties (Hamazaki et al., 2001). In 2003, Yamamoto et al. produced hepatic cells with a high level of liver function by transplanting ES cells into mice with regenerated livers. Viewed under an electron microscope, even in terms of ultrastructural analysis, these ES-derived hepatocytes were genuinely similar to normal hepatocytes, and our work was the first in which ES cell differentiation was induced, in which the resulting cells were cultivated in vitro, in which their hepatic functions were measured, and in which they were transplanted into the livers of immunodeficient mice (Yamamoto et al., 2003). Soto-Gutiérrez et al. (2007) successfully induced the differentiation of hepatocytes with the ability to produce glucose and

Table 1
Characteristics of stem cells.

	Established artificial stem cells		Adult stem cells	
	ES	iPS	MSC	TSC
Autologous transplantation	No	No (possibly Yes)	Yes	Yes
In vitro differentiation	Yes	Yes	Yes	Partial commitment
In vivo differentiation	Yes	Yes	Yes	Yes
Differentiation potency	Very high	Very high	High(limited)	Limited
Growth in vitro	Infinite	Infinite	Semi-infinite	Limited
Ethical issues	Yes	Low or No	No	No
Availability in medicine	No	YES	Yes	Potentially Yes
Legislative/governmental law	YES	No	No	No
Tumorigenesis	YES	YES	Low or No	No
Rejection	YES	No	No	No
Trophic activity *in vivo*	No	No	YES	?

ES: embryonic stem cell; iPS: induced pluripotent stem cell; MSC: mesencymal stem cell; TSC: tissue stem cell.

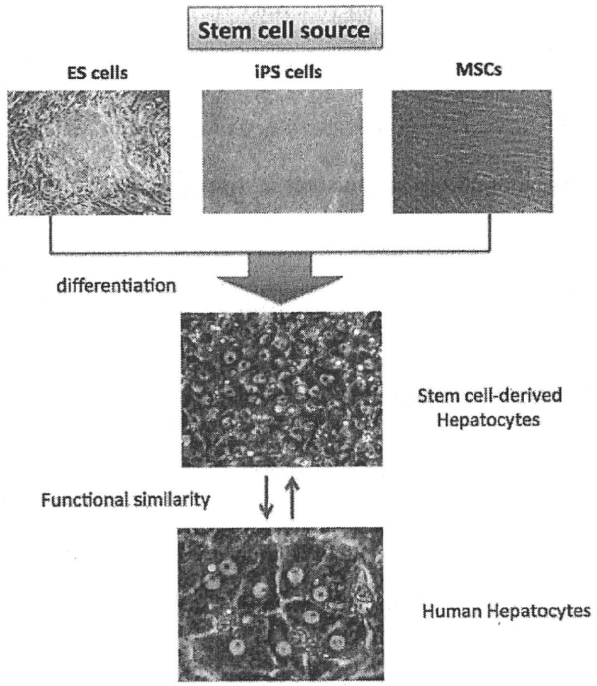

Fig. 1. Differentiation of hepatocytes from stem cells. Many types of stem cells have been differentiated in vivo and/or in vitro into hepatocyte-like cells using different induction strategies. However, there is no defined strategy to produce real human hepatocytes from stem cells that exhibited whole liver specific functions, and more study is needed.

detoxify ammonia by adding Activin A, FGF-2, a deleted variant of HGF, and Dex to a co-culture of mouse ES cells and liver nonparenchymal cell lines. A number of research groups have also developed procedures for the differentiation induction of human ES cells, and Cai et al. (2007) succeeded in obtaining human ES-cell-derived hepatocytes with numerous hepatic functions by treating ES cells first with Activin A and then with FGF-4, bone morphogenetic protein (BMP)-2, and HGF before finally promoting hepatocyte maturation with OsM and Dex. As it was possible to confirm that the cytochrome P450 gene, which is involved in drug metabolism, was activated in the hepatocytes thus obtained, it is hoped that these cells may also be of service as substitute human hepatocytes. Recently, Kume's group used P13 kinase inhibitor and other factors to increase the efficiency of ES cell differentiation to endoderm, achieving a comparatively high frequency of hepatocyte induction (Shiraki et al., 2008). As yet, there have been no reports of hepatocyte differentiation from mouse or human induced pluripotent stem cells (iPS cells), of which much is expected.

In light of the above summary, although attempts have been made to apply the technology for hepatocyte differentiation of mouse ES cells to primate ES cells, as well as to human ES cells, when the same techniques for combining multiple cytokines are used, the induction rates for hepatic differentiation are no more than a few percent. The fact that the number of cells resulting from induction differentiation is so small is one of the problems preventing the clinical application of such techniques to the creation of regenerative therapies for large organs such as the liver (Banas et al., 2007).

Currently, Basma et al. (2009) reported that human ES cells differentiate into hgepatocytes with 50%-albnumin positive and ~25% positive for asialoglycoprotein-receptor, a highly specific marker of hepatocytes. These cells could then be transplanted in mice and function by secreting albumin and alpha1-antitrypsin at a similar level to primary porcine hepatocytes. This paper also demonstrates levels off expression of key hepatocyte genes are similar to those found in primary hepatocytes. When the differentiated cells were transplanted in animals, they found homing of ES-derived hepatocyte population to the liver. Developing such a cell line from human embryonic stem cells or human iPS cells would provide a valuable tool for pharmacology studies, as well as for use in cell-based therapeutics.

4. Are MSCs useful in regenerative therapies for the liver?

In adults, there is a spectrum of stem cells with a different scale of quantity and potentiality. Among adult stem cells, MSCs, which are present in a range of tissues within the living body, are pluripotent cells similar to ES cells that are currently the subject of much attention. As MSCs are originally mesodermal cells, it is known that they differentiate into three different types of cell – bone, cartilage or adipose tissue – under varying culture conditions. In comparison with methods involving ES cells, autologous transplants using MSCs, with their inherent plasticity, have the benefit of avoiding both ethical issues and the risk of rejection.

The best-known stem cells are hematopoietic stem cells, found in bone marrow, and MSCs, an intermediate type of stem cell found in bone marrow, adipose tissue, umbilical cord blood, amniotic fluid, and the placenta. Similar procedures to those for ES cells have been reported for the hepatic differentiation of hematopoietic stem cells by adding suitable cytokines and growth factors or by using the regenerative capacity of the damaged liver, but several of these reports conclude that differentiation results from the fusion of hepatocytes from the recipient with hematopoietic stem cells from the donor (Banas et al., 2006, 2007). There have also been both reports of induction differentiation by means of in vitro cultivation only, and reports denying this, indicating that the question of whether cell fusion is required for hepatic differentiation of hematopoietic stem cells remains open to debate.

MSCs as a cell population are positive for cell membrane antigens such as SH3, CD29, CD44, CD71, CD90, CD105, CD106, CD120a, and CD124, and possess adhesive properties. They are found in numerous living tissues, and there have been reports that their cell membrane antigens and secretory proteins differ in various tissues. It has been reported that among MSCs obtained from bone marrow, adipose tissue, umbilical cord blood, and placental tissue, several hepatocyte-like cells have the ability to differentiate. Lee et al. (2004) added EGF, FGF-2, HGF, Dex, and nicotinamide to MSCs obtained from bone marrow and succeeded in obtaining cells that not only resembled hepatocytes in form but also possessed capacities for urea synthesis and drug metabolism. There have also been numerous reports of similar hepatic differentiation of MSCs from animals such as mice and rats, and the multilineage potential that enables the hepatic differentiation of MSCs may be described as existing across the species barrier (Pittenge et al., 1999). The 'immunosuppressive' property of human MSC makes them an important candidate for cellular therapy in allogeneic settings. Use of allogeneic MSC for repair of large defects may be an alternative to autologous and allogeneic tissue-grafting procedures. An allogeneic approach would enable MSC to be isolated from any donor, expanded, and cryopreserved, providing a readily available source of progenitors for cell replacement therapy. In fact, MSC from bone marrow (Popp et al., 2006; Sato et al., 2005) and umbilical cord blood (Yan et al., 2009) is considered as a safe and effective therapeutic tool in regenerative medicine in liver disease.

We believe there are numerous points to be noted with regard to the latent capacity of human subcutaneous adipose-tissue-derived MSCs (AT-MSCs) (Basma et al., 2009; Lee et al., 2004). First, they are easily sampled in comparison with MSCs derived from bone marrow. Bone marrow fluid is extracted from the pelvic bone (ilium) with a syringe, a procedure not without risk that normally involves a general anesthetic. In the case of subcutaneous adipose tissue, however, simple, highly safe procedures such as liposuction have been developed that require no more than a local anesthetic and are simple to carry out. In addition, their stem cell yield is between a hundred and a thousand times higher for the same amount of tissue. Although the MSCs themselves have somewhat different properties from those found in bone marrow, they nevertheless still possess plasticity and differentiate into cartilage, bone and adipose tissue with high frequency. Genome-wide transcriptome comparison of MSCs from bone marrow and adipose tissue was done by Izadpanah et al. (2008) and found that comparison between human bone marrow MSC versus human adipose tissue MSC was minimal. Furthermore, as discussed at the end of this article, various advantages of undifferentiated MSCs are gradually becoming apparent. They have already begun to be applied clinically in breast reconstruction following conservative surgery for breast cancer, and research is now also underway on their clinical applications in disorders such as chronic heart disorders, acute myocardial infarction and stroke.

5. Validation of hepatic differentiation potential of human AT-MSCs

When AT-MSCs are treated with a cytokine cocktail consisting of HGF, FGF1, and FGF4 (Teratani et al., 2005; Yamamoto et al., 2005), followed by further stimulation with OsM and Dex to promote their maturation into hepatocytes, they differentiate into cells that display hepatocyte characteristics. They could not be described as completely identical in form to human hepatocytes, but a pseudo-biliary duct structure appeared between the cells, and albumin and other liver-specific characteristics of hepatocytes were observed.

Under the informed consent of the patients involved, we sampled 5 g of subcutaneous fat from multiple patients who underwent surgery for abdominal cancer in that institution, isolated and cultured MSCs from this tissue, added the three types of cytokine listed above, and cultured the cells for approximately 40 days, after which they had almost all changed into cells that exhibited properties resembling those of hepatocytes. When we investigated the properties of these hepatocytes, we detected over 14 different proteins only synthesized in the liver, such as the serum component albumin and drug-metabolizing enzymes (Banas et al., 2007). Exhaustive gene expression analysis using microarrays and pathway analysis, carried out in collaboration with DNA Chip Research, Inc., revealed the activation of clusters of liver-specific genes involved in areas such as sugar metabolism, blood coagulation, and lipid metabolism (Yamamoto et al., 2008).

When approximately 10^7 of these hepatocytes were transplanted by injection into mice with artificial drug-induced liver failure, the increased ammonia concentration fell to near-normal levels within 24 hours, thus confirming that the ammonia-decomposing function of the liver, which is essential for maintaining life, was operating normally (Banas et al., 2007). We also showed that these cells possessed a range of the basic metabolic activities of cytochrome P450, which is important for drug metabolism in the liver. In particular, enzymes such as CYP2C9 and CY2B6 were equivalent to those of control human first-generation cultured hepatocytes, while enzymes such as CYP1A1, 2C9, 2D6, and 3A4 were present at levels between one-fifth and one-tenth that of control hepatocytes (unpublished observations).

6. Production of cells with hepatocyte functions in two weeks

As our previous method for the production of cells with hepatocyte functions required at least 35 days, it had numerous inconvenient aspects in terms of actual clinical application. For this reason, we improved our method to induce the differentiation of AT-MSCs into hepatocytes in a shorter period of time. First, after exploring methods for promoting differentiation from mesoderm to endoderm, we used Activin A to induce the expression of liver-specific transcription factors such as HNF4 at an early stage. Then, as a result of a gradual conversion to already-known growth factors, it became possible to transform almost 100% of undifferentiated AT-MSCs into albumin-positive cells within two weeks (Banas et al., 2009). We investigated these cells in detail, and confirmed that they expressed a range of liver functions and liver-specific genes.

7. Gene ontology analysis of hepatocytes obtained by differentiation induction of stem cells

It has been confirmed in both mice and human cells that the expression profiles of hepatocytes obtained from stem cells are similar to those of the normal liver. Clustering analysis, however, selects only those genes with a confirmed change in expression as a result of treatment to induce hepatic differentiation, and demonstrates nothing more than the expression patterns of these genes being similar to those of hepatocytes. That is, it has not been shown that genes with up-regulated expression are actually involved in liver function.

Yamamoto et al. (2008) provide a detailed analysis of the properties of the genes for which up-regulated expression has been confirmed. We have also carried out gene ontology analysis (gene categorization) of those genes with altered expression. In particular, as numerous genes related to liver function were confirmed in the differentiation induction of MSCs, we used a specialized microarray for detecting gene signaling pathways to carry out a comparative investigation of the gene signaling pathways of hepatocytes derived from human MSCs and the normal human liver. These analyses have revealed the roles of genes with altered expression as a result of hepatocyte induction treatment, and enabled the functions possessed by stem-cell-derived hepatocytes to be predicted. Here we describe and compare gene ontology of hepatocytes originated from ES cells and MSCs.

7.1. Mouse ES-derived hepatocytes

It has been confirmed that genes with up-regulated expression in ES-derived hepatocytes include many that are involved in liver function, such as alcohol dehydrogenase and transthyretin. We utilized a database to identify the categories of the genes that were significantly induced among those for which there were alterations during the hepatic differentiation process. We investigated these genes on the basis of microarray data, classified by the biological processes of gene ontology.

Within the parent population (9172 clones), we compared genetic data for 6699 clones, excluding genes with unidentified function, and among those genes (232 clones) for which expression had at least doubled, we compared data from 183 clones, also excluding genes of unidentified function, deriving a

significantly contracted gene catalog. It is noteworthy that all these gene ontologies are involved in liver function. It can therefore be inferred that genes involved in liver function are induced by hepatic differentiation treatment. For example, electron transport includes the CYP2EI and CYP2D10 genes, which are involved in drug metabolism. The results of gene ontology analysis confirmed that the group of genes found in clustering analysis with expression resembling that in ES-derived hepatocytes and normal mouse liver also includes numerous genes involved in liver function from the perspective of gene function.

7.2. Human MSC-derived hepatocytes

The results of gene ontology analysis of mouse ES cells revealed that genes involved with numerous liver functions are induced by hepatocyte induction treatment. We then carried out a similar analysis of human MSC-derived hepatocytes. When we checked for genes with up-regulated expression in stem-cell-derived hepatocytes, we confirmed that these included characteristic hepatocyte genes such as albumin, TD02, and transthyretin.

Unlike ES analysis, numerous genes in the MSC analysis exhibited altered expression. For this reason, we performed gene ontology analysis of genes with both up-regulated and down-regulated expression.

Within the population (25,721 clones), we compared genetic data for 12,441 clones, excluding genes of unidentified function, and among those genes for which expression was up-regulated (1252 clones) or down-regulated (387 clones) by $\geq$10-fold, we compared data from 739 and 215 clones respectively. Among the genes with increased expression were numerous categories of genes involved in liver functions such as blood coagulation and lipid metabolism. Categories of genes with down-regulated expression were mainly concentrated in gene categories involved in cell proliferation, including the cell cycle or cytoskeletal organization, such as the cyclin family and E2F. This suggests that when MSCs are treated to induce hepatic differentiation genes involved in liver function are induced, and as the cells mature, proliferation ceases.

When the expression of individual genes was specifically confirmed, expression of the transcription factors HNF3beta and HNF6, which are selectively expressed in hepatocytes, had also increased in stem-cell-derived hepatocytes. Among the cytochrome P450 gene family, which is involved in the important liver function of drug metabolism, genes such as CYP3A4, CYP2A6, and CYP2C8 also exhibited high expression (Table 2), and within the ABC transporter gene family, which acts as a drug excretion pump, numerous genes such as MDR1 and MRP were also found to have been induced. We also showed that numerous genes associated with the extracellular matrix, which is required for the adhesion and conservation of hepatocytes, as well as those concerned with the well-known liver functions of complement activation and blood coagulation factors, were induced. From these data, it may be concluded that according to gene ontology analysis, MSC-derived hepatocytes possess an extremely large number of liver functions.

8. Elucidation of molecular mechanisms underlying the process of hepatic differentiation of stem cells

8.1. ES cells

When we focused on the expression of transcription factors with tissue-selective expression in order to elucidate the process of hepatic differentiation of ES cells, the expression of transcription factors involved in endoderm differentiation were induced in the early stages of differentiation induction, and the expression of factors believed to be necessary for hepatic differentiation was induced from the intermediate stages of differentiation onward. It is reported that the endoderm, which forms the basis for the splanchnic tissue in the living body, must first activate these transcription factors, and is required for the activation of factors such as the endoderm/hepatocyte-specific genes transthyretin, albumin, and L-pyruvic acid phosphorylase. It has also been reported that HNF4alpha is necessary for the liver's morphological and functional differentiation, accumulation of sugar, and epithelial structure formation. The effect of OsM is also required for the in vitro maturation of hepatocytes originated by mediation with HNF4alpha. In the hepatic differentiation induction treatment used in the present study, we confirmed by using the RT-PCR method that expression of HNF4alpha was clearly up-regulated as differentiation progressed. Accordingly, these data show that endodermal cells are induced in hepatic differentiation from ES cells.

HNF3beta is believed to be an important transcription factor in the differentiation of endoderm and its subsequent induction to hepatocytes. However, no such importance has been shown in in vitro experiments on the hepatic differentiation of ES stem cells. In our present research, we showed that using HNF3beta-siRNA to suppress the expression of HNF3beta inhibited differentiation to albumin-expressing cells. Accordingly, we are the first to reveal the function of HNF3beta in hepatic differentiation in vitro. These results are also consistent with the results of experiments using mouse models, which showed that HNF3beta is required in the early stages of hepatic differentiation. This system of hepatic differentiation induction may thus become an important tool to reveal the network of hepatocyte transcription factors in vivo.

The development mechanisms for ectoderm and mesoderm have been comparatively well investigated, but much remains unknown about the generation of endoderm cells and their subsequent construction of tissues. This may be due to the fact that there have been no optimum models for investigating these processes, making in vitro research difficult. The data from our present research have shown that the hepatic differentiation induction system follows a route resembling the developmental

Table 2
Profile of CYPs expression in hepatic cells differentiated from various human stem cells.

Stem cells	Expressed CYPs (mRNA level)
Human ES cells	1A1, 1A2, 2B6 2C8, 2C9, 2C19, 3A4, 3A7, 7A1
Human BM-MSC	1A1, 1A2, 2B1, 2B6, 2C6, 2C8, 2C9, 2C12, 2C19, 2E1, 3A4
Human UCB-MSC	1A1, 1A2, 1B1, 2B6, 3A4
Human P-MSC	3A4
Human AT-MSC	1A1, 2A6, 2C8, 2C9, 2E1, 3A4, 3A5, 4F3, 7A1, 8B1

ES: Embryonic stem, BM: Bone marrow, P: Placenta, MSC: Mesenchymal stem cell, UCB: Umbilical cord blood, AT: Adipose tissue.

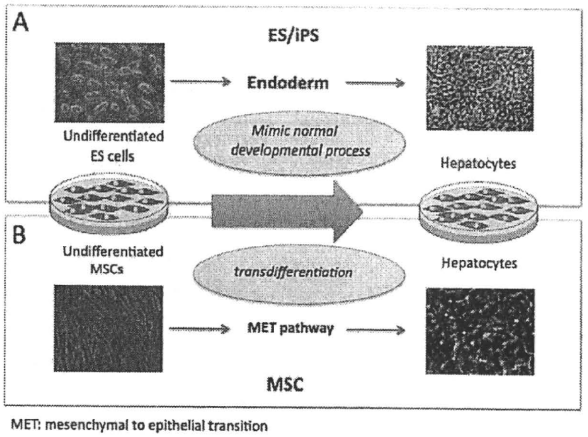

Fig. 2. Differentiation process of hepatocytes form stem cells. Numerous cytokines and growth factors have been shown to have a potent effect on hepatic growth and differentiation in vitro. They include the hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF), acidic FGF/FGF1, insulin, insulin-like growth factor (IGF), and oncostatin M (OsM). It is noteworthy that for each of the cytokines/growth factors, the timing, dosage, and combinations must be carefully chosen according to the stem cell type and desired effect. Among the non-proteinaceous chemical compounds that are known to promote hepatic differentiation and/or maintenance in vitro are dexamethasone (DEX), retinoic acid (RA), sodium butyrate, nicotinamide, norepinephrine, and dimetylsulfoxide (DMSO). Sequential treatment with growth factors that mimic in in vivo development of the liver has been demonstrated. (A) Hepatic induction from ES cells/iPS cells may highly mimic molecular event of normal liver developmental process. Endoderm-associated transcription factors HNF1/TCF2, HNF3/FoxA1, HNF3/FoxA2, and HNF4 were upregulated in the process of hepatic differentiation from ES cells. (B) MET occurs during the hepatic differentiation of MSCs. Morphologically, this may be understood as a mesenchymal-to-epithelial transition (MET), and in fact the expression of EMT regulator molecules such as Twist and Snail is suppressed by hypermethylation of their promoter region.

phases of mouse ES cells for differentiation into hepatocytes (Fig. 2).

It has been reported that, using this system, expression of the AFP gene, which is expressed by undifferentiated hepatocytes, is induced in the early stages of differentiation. AFP expression has been confirmed at the 4th somite stage of mouse embryonic development. In addition, its expression decreases immediately after birth, and the amount of mRNA expressed in the adult liver is known to be less than 0.01% of that during the fetal period. Re-expression of AFP during periods of hepatocyte proliferation, such as liver regeneration or tumor formation, has been reported. From these data, it can be inferred that AFP is a marker gene for liver progenitor cells known as oval cells. For this reason, the use of this hepatic differentiation system may enable hepatocytes to be confirmed during differentiation induction. When we used RT-PCR to investigate the expression of CK19, Thy-1, GGT, and D1k, which have been reported as being expressed in hepatic stem cells (Yamamoto et al., 2005), we confirmed their expression (unpublished observation). These data remain provisional, but their expression in the intermediate phase of hepatic differentiation supports the possibility that this system could be used to obtain biliary epithelial cells or hepatocytes with the ability to differentiate in two directions. In summary, hepatic induction from ES cells may highly mimic molecular event of normal liver developmental process (Fig. 2).

8.2. MSCs

As a different microarray substrate was used, a simple comparison could not be made, but when the categories of genes detected as having significant changes in expression in hepatocytes derived from the differentiation induction of ES cells and MSCs were compared, we found that an extremely large number of liver functions were induced in MSC-derived hepatocytes. We believed that by investigating the workings of the gene networks in MSC-derived hepatocytes, we could identify the gene signal pathways involved in liver function and cellular maintenance. We thus increased the sensitivity of detection by limiting the number of probes on the chip to 5000, and carried out analysis by microarray with a specialized substrate for investigating signal pathways (Concise Pathway, Conpath). Gathering data from the same sample using a microarray with a different substrate can also be described as enabling the revalidation of the results from the microarray used for the MSC analysis.

The genes mounted on the ConPath microarray were selected from a signaling pathway diagram obtained from the GenMAPP database, and the expression data obtained from the microarray were returned to the GenMAPP diagram in order to enable a visual representation of the alterations in gene expression for each pathway. Expression data from primary cultured hepatocytes were used as reference, and ConPath used to obtain expression data from undifferentiated MSCs, MSC-derived hepatocytes, and normal liver. A comparison with the reference of pathways involved in liver function showed that the number of activated genes was similar in MSC-derived hepatocytes and normal liver, and it may be inferred that signaling pathways are functional in both. In the blood coagulation pathway in particular, out of a total of 20 genes, up-regulation of expression was observed in 14 genes in MSC-derived hepatocytes and 15 in normal liver. Furthermore, the signaling pathways involved in complement activation are clearly unlike those of undifferentiated MSCs, while the gene expression patterns of MSC-derived hepatocytes and normal liver are extremely similar. In the same way, we also confirmed that the expressions of fatty acid oxidation and steroid synthesis pathways are markedly up-regulated. We have therefore shown that numerous signaling pathways involved in liver function can be prominently detected in MSC-derived hepatocytes.

9. Analysis of molecular mechanisms of MSC-derived hepatocytes

We confirmed that MSCs differentiate into hepatocyte-like cells, but many questions still remain regarding the transdifferentiation and its molecular mechanisms. To reveal the molecular processes of the shift from MSCs to hepatocytes, we focused particularly on mesenchymal-to-epithelial transition (MET). MET represents the process whereby interstitial cells (MSCs) undergo a morphological change into hepatocytes, which are epithelial cells. Microarray analysis has shown that Twist and Snail, which are factors that induce epithelial-to-mesenchymal transition (EMT), were down-regulated in the hepatic differentiation process. In addition, expression of E-cadherin and alpha-catenin, genes expressed in epithelial cells, was up-regulated in MSC-derived hepatocytes.

In contrast, expression of N-cadherin and vimentin, genes expressed in interstitial cells, was down-regulated following differentiation. We observed that cell morphology changed markedly during hepatic differentiation, from fibroblast-like to epithelium-like morphology. These data support the hypothesis that MET occurs during the hepatic differentiation of MSCs (Fig. 2). Further experiments are required to reveal the molecular mechanisms of transdifferentiation from MSCs to hepatocytes, but it may be inferred from the microarray data given here that MET is an important factor for determining stem cell transdifferentiation and plasticity.

As shown by the microarray data, MSCs expressed N-cadherin and vimentin, factors that are expressed in the interstitium. In addition, expression of E-cadherin, which is expressed in epithelial cells, was up-regulated 81-fold by hepatic differentiation. Further provisional data from exhaustive methylation analysis of gene promoter regions based on microarrays indicate that the promoter regions of 39 genes in MSC-derived hepatocytes were methylated, while those of eight genes were demethylated. It is extremely interesting that Twist is among the methylated genes, suggesting that the Twist promoter region is methylated in cells in which hepatic differentiation has progressed and that its expression is suppressed through the epigenetic modification of the genome (unpublished observations).

These data support the hypothesis of transdifferentiation by MET. This analysis does not reveal a close relationship between the methylated genes in the genome and the hepatic differentiation process, but it is conceivable that investigating the mechanisms of transdifferentiation from a range of viewpoints may reveal the mechanisms of hepatic differentiation of MSCs.

10. Therapeutic effectiveness of differentiated hepatocytes

10.1. ES cells

There have been numerous reports of transplants of cells possessing liver function induced from ES cells into liver-damaged mice (Banas et al., 2007). The results of Thorgeirsson's group (Heo et al., 2006) are particularly noteworthy, as they report that liver precursor cells induced from ES cells also have the ability to overcome liver damage. As described above, however, when ES cells are used as the cell source, a high differentiation induction rate cannot be obtained irrespective of the method used, and the reality is that it is currently impossible to guarantee a sufficient number of cells for transplantation to treat disorders of the liver, the largest organ in the human body. Expectations are focused on the possible level and differentiation induction rate of potential future technologies for the production of hepatocytes from iPS cells.

10.2. MSCs

We transplanted human hepatocyte-like cells differentiated from adipose-tissue-derived MSCs (AT-MSCs) into animal models with liver damage induced by administration of carbon tetrachloride (Banas et al., 2009). Tissue staining and other analyses showed that the transplanted *hepatocytes* adhered to the liver after 24 hours, and were incorporated into cord-like structures with other hepatocytes. We estimated that approximately one-tenth of the cells adhered. We showed that liver figures, such as total albumin, ammonia, AST, and ALT, were restored to near-normal levels in damaged livers that received these transplants. Further histological investigation revealed that hepatocyte apoptosis itself was significantly suppressed in the group that received transplants of AT-MSC-derived hepatocytes.

11. Homing effect of undifferentiated MSC

The pool of MSCs within the human body will be a highly effective tool to realize the potential of regenerative medicine using autologous cells. To understand the molecular basis of the stability of the differentiated state and of niche compartments, along with elucidation of stem cell mobilization and homing, must be achieved. To understand homing ability of undifferentiated MSCs in animal body, systemic injection of MSCs into mice were carried out. As shown in Fig. 3, left panel, MSCs were

In vivo distribution of AT-MSC in healthy mice

Organs	% accumulated
Spleen	17
Thymus	14
Lung	12
Spinal cord	9
Brain	5
Liver	2
Heart	1
Others (unknown)	40

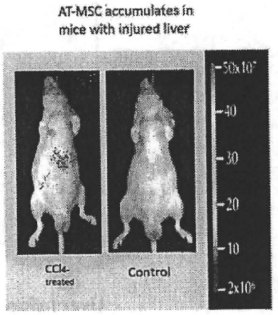

Fig. 3. Homing effect of MSCs. The autologous MSCs are a particularly promising source of cells for many clinical applications in the evolving field of regenerative medicine. Furthermore, MSCs represent an advantageous cell type for allogenic transplantation since MSCs are immune-privileged, with low MHC I and no MHC II expression, therefore reducing the risks of transplant rejection. MSCs have been found to be immunosuppressive, through a mechanism thought to involve paracrine inhibition of T- and B-cell proliferation and as such have been used in trials investigating their effects on autoimmune diseases and graft versus host disease (GVHD) (Uccelli et al., 2007). Co-infusion of donor-derived MSCs together with HSCs has been shown to reduce the incidence and severity of GVHD in sibling allografts (Le Blanc, 2006). It was reported that a patient suffering from severe therapy-resistant GVHD was treated with human AT-MSC and revealed a complete recovery (Fang et al., 2008). The hypo-immunogenic properties of MSCs are considered to be sufficient to allow transplantation even between individuals who are not HLA-compatible. Therefore, MSCs represent a population of cells with the potential to contribute to future treatments for a wide range of acute or degenerative diseases including liver. To further elucidate such a homing ability, MSCs were intraperitonealy injecyted into healthy immunodeficient mice (left panel). Two days after the injection, mice were analyzed for the presence of MSCs. Data represents % of cells distriubuted in each organs. In the next, luciferase-positive MSCs were inoculated into mice with carbon tetrachroride (CCl_4) administered mice (right panel). In vivo bioluminescence analysis revealed that MSCs were accumulated into injured liver, indicating that the injured liver produces key regulatory factors that are necessary for the homing of stem cells to the site of injury. Photons from animal whole bodies were counted by using the IVIS imaging system (Xenogen). Bar indicates $\times 10^6$ photon per s.

distributed in a variety of organs/tissues in healthy immunodeficient mice (unpublished observations). In contrast, as can be seen in vivo imaging analysis of bioluminescence, human MSCs, as well as murine ES cells (Teratani et al., 2005), were accumulated in the injured liver (Fig. 3, right panel). These results indicate that the injured liver produces key regulatory factors that are necessary for the homing of stem cells to the site of liver injury. Though it is reported that CXCR4 and its ligand SDF-1 are crucial for the homing effect of adipose tissue stromal cells (Cho et al., 2006; Sengenès et al., 2007; Yamamoto et al., 2008; Banas et al., 2009), the precise molecular mechanisms how do MSCs find their way to injured site are not well understood.

12. Therapeutic potential of undifferentiated MSCs

In the concurrent therapeutic experiments, we used both AT-MSC-derived hepatocytes and undifferentiated AT-MSCs from the same donor. These undifferentiated AT-MSCs did not exhibit any type of liver-specific function in vitro, but when they were transplanted in the same way as described above, they had a remarkable ability to restore liver damage (Banas et al., 2008). The levels of serological recovery and suppression of histopathological damage were not inferior to those achieved by differentiated cells possessing liver functions. Protein array and other analyses suggested that this therapeutic capacity for liver disorders possessed by undifferentiated AT-MSCs may be a trophic effect resulting from factors such as the various types of cytokines and chemokines produced by these cells (Banas et al., 2008). In particular, not only is it a plausible supposition that factors such

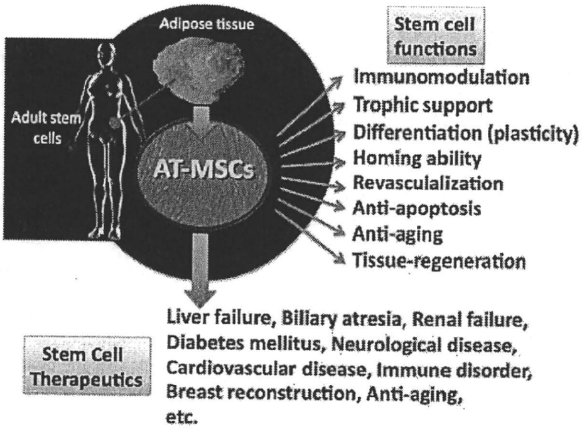

Fig. 4. The advantages of MSCs as a source of regenerative cells. A variety of humoral factors (cytokines and chemokines) are expressed in MSC culture supernatant. Their actions are varied, but they include a range of factors believed to be of use in repairing tissue/organ damage. In addition to the trophic and immnoregulatory effect of such cytokines, tissue repair mechanisms engendered by intercellular interactions are also an important element. AT-MSCs may account for their broad therapeutic efficacy in animal models of several diseases such as biliary atresia, renal failure, diabetes mellitus, neurological disease, cardiovascular disease, immune disorder, breast reconstruction, anti-aging, etc. and in the clinical settings for their treatment.

as VEGF, HGF, NGF, and FGF are active in hepatocyte proliferation but these cells are also rich in the production of anti-inflammatory cytokines such as IL-1Rα and IL-10, thus indicating a relationship between mechanisms for suppressing concentrations of inflamed cells and recovery from liver damage. In addition, according to several recent noteworthy reports, generalized administration of the culture supernatant from MSCs alone has been successful in effectively prolonging the lives of model animals with life-threatening liver damage (Parekkadan et al., 2007; van Poll et al., 2008). It may be that the day is coming in which humoral factors emitted by MSCs play a leading role in liver failure therapy.

In summary, these findings suggest that AT-MSCs may account for their broad therapeutic efficacy in animal models of several diseases such as biliary atresia, renal failure, diabetes mellitus, neurological disease, cardiovascular disease, immune disorder, breast reconstruction, anti-aging, etc. and in the clinical settings for their treatment (Fig. 4).

13. Future prospects

When we investigated the gene categories that exhibited significance among 232 clones of genes with altered expression in ES-derived hepatocytes, all were involved with liver function. Integrated analysis with a database of gene expression profiles confirmed the process of hepatic differentiation. Consequently, a detailed investigation of microarray data may provide the key to revealing the networks and signaling pathways of factors and genes that regulate the hepatic differentiation of ES cells.

The same may be said about the hepatic differentiation of human MSCs. When we investigated the gene categories that exhibited significance among genetic data from 1639 clones, those involved with liver functions such as steroid metabolism and lipid metabolism were identified. In addition, signaling pathway analysis using a different microarray revealed that numerous signals were activated in the differentiation process for MSC-derived hepatocytes, in the same way as normal human liver. Accordingly, this analysis not only revealed the factors required for the hepatic differentiation of MSCs, but may also be of use in identifying part of the plasticity required for the hepatic differentiation of stem cells.

It is known that expression of many enzymes involved in liver metabolism is induced after birth, and that the liver then attains its fully mature condition by acquiring further metabolic functions. It is also known that the expression of various CYP genes is also induced after birth, and that these exhibit metabolic functions with respect to drugs. Microarray analysis has shown that numerous cytochrome P450 genes are induced at high frequency in stem-cell-derived hepatocytes during the hepatic differentiation of mouse ES cells and human MSCs. In addition, when we analyzed MSC-derived hepatocytes, we confirmed the activity of CYP1A2, CYP2B6, 2C19, 2D6a, CYP2C9, and CYP3AC in those differentiated cells. In Table 2, profile of CYPs genes expression in hepatic cells differentiated from various stem cells is summarized.

The enzyme CYP3A4 plays an extremely important role, being responsible for 45–60% of cytochrome P450 drug metabolism reactions in human liver. Our microarray data confirmed that CYP3A4 mRNA expression was up-regulated 170-fold in MSC-derived hepatocytes when compared with undifferentiated MSCs. We further confirmed that expression of MDR-1, an ABC transporter gene that is extremely important in drug excretion, was substantially up-regulated. CYP3A4 and MDR-1 are genes that fulfill extremely important roles in reactions with foreign substances, and the fact that their expression is up-regulated in stem-cell-derived hepatocytes raises the expectation that these cells may find applications in experiments to validate the biotransformation of new drugs. A particularly important point is that if these cells can be used as substitute cells in screening and other drug experiments currently used, they may play an important role in overcoming ethical and cost issues. Primary cultured hepatocytes are appropriate cells for drug experiments, but there are limits to cell proliferation and maintenance under normal culture conditions, and problems such as a shortage of donors mean that, under current circumstances, the amount of usable cells for experimental purposes is limited.

Accordingly, as ES- or MSC-derived hepatocytes in our research express multiple genes that contribute to drug transport, such as CYP3A4 and MDR-1, in the same way as primary cultured hepatocytes, it may be possible to use them as drug safety testing. The discovery that AT-MSCs can be differentiated into patients' own hepatocytes, with the possibility of autologous transplantation, is extremely interesting. Numerous problems remain to be answered, however, regarding the differentiated cells thus induced and called "hepatocytes," including the extent to which they truly possess the capacities of mature hepatocytes, whether they pose the risk of tumor formation, and whether there is any danger that they will change back into the original undifferentiated cells, and these questions must be answered with caution.

In the current news, the U.S. Food and Drug Administration (FDA) has granted clearance of the US company's Investigational New Drug (IND) application for the clinical trial of a human embryonic stem cell (hESC) in patients with acute spinal cord injury. This will be the world's first study of ES cell-based therapy in human. In the therapy of liver diseases, with a view to the future achievement of regenerative therapies for liver disorders, the fact that not only hepatocytes differentiated by induction but also human MSCs in their undifferentiated state have the capacity to restore liver disorders is important for future applied research (Fig. 4). The research achievements described in this paper may not only be expected to open the way for the establishment of revolutionary new therapeutic methods for treating life-threatening liver failure caused by disorders such as viral hepatitis

in adults and biliary atresia in children; the human hepatocytes obtained in our research will also be of use in toxicity and safety testing for new drugs. Furthermore, should the use of highly reliable human hepatocytes in place of existing animal and other testing systems become possible, this would be of great social benefit. We have not yet arrived at the stage when we can claim a full understanding of the unknown possibilities possessed by stem cells, however, and while keeping pace with the progress of research on pluripotent human iPS cells, realizing the potential of stem cells and understanding their molecular mechanisms from a range of angles will require both effort and patience.

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Systemic Delivery of Synthetic MicroRNA-16 Inhibits the Growth of Metastatic Prostate Tumors via Downregulation of Multiple Cell-cycle Genes

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Recent reports have linked the expression of specific microRNAs (miRNAs) with tumorigenesis and metastasis. Here, we show that microRNA (miR)-16, which is expressed at lower levels in prostate cancer cells, affects the proliferation of human prostate cancer cell lines both *in vitro* and *in vivo*. Transient transfection with synthetic miR-16 significantly reduced cell proliferation of 22Rv1, Du145, PPC-1, and PC-3M-luc cells. A prostate cancer xenograft model revealed that atelocollagen could efficiently deliver synthetic miR-16 to tumor cells on bone tissues in mice when injected into tail veins. In the therapeutic bone metastasis model, injection of miR-16 with atelocollagen via tail vein significantly inhibited the growth of prostate tumors in bone. Cell model studies indicate that miR-16 likely suppresses prostate tumor growth by regulating the expression of genes such as *CDK1* and *CDK2* associated with cell-cycle control and cellular proliferation. There is a trend toward lower miR-16 expression in human prostate tumors versus normal prostate tissues. Thus, this study indicates the therapeutic potential of miRNA in an animal model of cancer metastasis with systemic miRNA injection and suggest that systemic delivery of miR-16 could be used to treat patients with advanced prostate cancer.

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INTRODUCTION

Advanced prostate cancer is frequently difficult to treat and causes substantial symptoms, including severe pain from metastasis to bone or other sites. Numerous experimental therapeutics are being pursued in clinical trials and offer some hope of improved treatments, but most have so far demonstrated only modest results.

Mounting evidence suggests that the altered expression of specific microRNA's (miRNA's) accuracy contributes to the development of a variety of cancers. Cancer types including prostate cancers can be classified based on their distinct miRNA expression profiles.[1–5]

MiRNAs have been implicated in prostate cancer. Volinia *et al.* identified >40 miRNAs with expression levels that were significantly different in prostate tumors versus normal prostate tissue.[5] Furthermore, the need for additional therapies in metastasis due to hormone-refractory prostate cancer is considerable. Mattie *et al.* found that miRNA expression in human prostate cancer cell lines could distinguish androgen hormone-insensitive PC3 from hormone-sensitive LNCaP cells.[6] LNCaP cells showed upregulation of microRNA (miR)-200c, miR-195, and several let-7 family members, whereas miR-10a, miR-27b, miR-221, miR-222, and mir-210 were lower than in PC3. The serum prostate-specific antigen is the most useful tumor marker for diagnosis and monitoring of prostate cancer. However, its low specificity in distinguishing prostate carcinoma from benign prostatic hyperplasia limits its use as an early detection biomarker. Investigators used custom designed arrays to compare the expression profiles of 319 miRNAs in prostate tumors, cancer cell lines, xenografts, and benign prostatic hyperplasia.[7] MiRNAs could be used to cluster the androgen receptor status of cell lines and xenografts. Among a small set of benign prostatic hyperplasia, hormone refractory, and untreated prostate carcinomas they found 51 differentially expressed miRNAs, 37 of which were downregulated. MiRNAs in this set accurately clustered the benign prostatic hyperplasia, untreated and hormone-refractory prostate carcinomas providing evidence that miRNA expression profiles are altered by changes in disease status. More recently, Bonci *et al.* showed that miR-15a and miR-16-1 cluster inhibit the tumor cell proliferation and invasion via targets CCND1 (cyclinD1), WNT3A, and BCL2 in prostate cancer cell line and clinical samples.[8] These miR-15a and miR-16-1 were coded on chromosome 9 13q14. In this region, loss of heterogeneity was detected in chronic lymphocytic leukemia[9] and prostate cancer patients.[10] These results suggest that miR-15a and/or miR-16 could be a novel target for prostate cancer therapy.

To supplement the expression studies that have been published for prostate cancer, we used a library of synthetic miRNAs

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to identify the small RNAs that alter the proliferation of prostate cancer cells. Among the miRNAs that were identified in a functional screen featuring 22Rv1 prostate cancer cells was miR-16, an miRNA that has been implicated in chronic lymphocytic leukemia[1,11,12] and prostate cancer.[9,10] Our studies of miR-16 revealed that it has the capacity to affect the proliferation of a variety of human-derived prostate cancer cells. For the evaluation of miRNA therapy for bone metastasis of prostate cancer, the mouse model of bone-metastatic prostate cancer using bioluminescence-based *in vivo* imaging analysis was selected. We have already established small-interfering RNA (siRNA) molecules that can be delivered to tumor cells in a bone metastatic site using an atelocollagen delivery method.[13] The properties of synthetic miRNA molecules are similar to synthetic siRNA; therefore, it is speculated that synthetic miRNA can also be used for systemic treatment mediated by atelocollagen. In this article, the systemic delivery of synthetic miR-16 using atelocollagen inhibited bone-metastatic human prostate tumor growth in a mouse bone site. We further analyzed the altered expression of cancer-related genes in miR-16-transfected prostate cancer cells and verified that genes associated with cell-cycle progression were mostly affected by miR-16. These results suggest a therapeutic potency of miR-16 in bone-metastatic prostate cancer.

RESULTS

Effect of miR-16 on proliferation of human prostate cancer cell lines

22Rv1 prostate cancer cells were transiently transfected in triplicate with individual synthetic mimics for ~200 miRNAs. Three days after transfection, the cells were monitored for proliferation and apoptotic activity. Among the most active miRNAs identified in the functional screen was miR-16, which reduced the proliferation of the prostate cancer cells by 25% and increased apoptosis by 40% (data not shown). Follow-up studies for measuring the proliferation; using the alamar blue assay with another prostate cancer cell line, PC-3M-luc, revealed that miR-16 reduces proliferation by 60% (**Figure 1a**) relative to the cells transfected with a negative control (NC) miRNA. Further studies of the antiproliferative effect of miR-16 on prostate cancer cells revealed that synthetic miRNA can significantly affect the expansion of cultured 22Rv1, PPC-1, and Du145 cells (**Figure 1a**). The only prostate cancer cell line that proved to be unaffected by the transfection of miR-16 was LNCaP (**Figure 1a**). The amount of miR-16 in the PC-3M-luc cells transfected with synthetic miR-16 was >500-fold higher than that in the control cells (**Figure 1b**). This result suggests that the induced increase of intracellular miR-16 concentrations is capable of suppressing the proliferation of the prostate cancer cells.

miR-16 expression levels in prostate cancer cell lines

Although four of the five prostate cancer cell lines exhibit significant reductions in proliferation following transfection with synthetic miR-16, it is interesting that there is a variation in the level of the effect. To address whether this might be due to variation in the levels of endogenous miR-16 in the various cell lines, we used quantitative reverse transcription (qRT)-PCR to measure the relative abundance of mature miRNA. As shown in **Figure 1c**, most of the cell lines expressed miR-16 at reduced levels. The extent

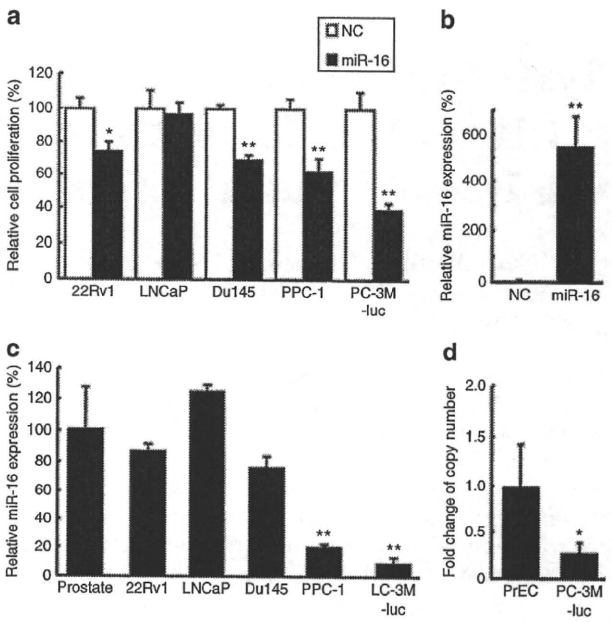

Figure 1 The expression and function of miR-16 in human prostate cancer cell lines. (**a**) Effect of miR-16 on proliferation of human prostate cancer cell lines. Percent (%) proliferation values were normalized to values from cells treated with negative control (NC) microRNA (miRNA). Data represent the mean ($n = 4$) ± SD $^*P < 0.05$, $^{**}P < 0.01$ versus NC miRNA. (**b**) The amount of miR-16 in PC-3M-luc cells transfected with synthetic miR-16. The cellular level of miR-16 was detected by quantitative PCR. The data represent the mean ($n = 3$) ± SD $^{**}P < 0.01$ versus NC miRNA. (**c**) Expression level of miR-16 in human prostate cancer cell lines. The relative expression of miR-16 for each of the cell lines was calculated by comparing the level in normal prostate tissue samples. The data represent the mean ($n = 3$) ± SD $^{**}P < 0.01$ versus normal human prostate tissue. (**d**) The copy number change of the miR-16 loci on chromosome 13q14 in PC-3M-luc cells. The copy number of miR-16 genes were quantified by real-time PCR with genomic DNA. Cultured normal human prostate epithelial cells (PrEC) was used as the control for this experiment for comparison to the PC-3M-luc cells. The data represent the mean ($n = 3$) ± SD $^*P < 0.05$ versus PrEC.

of downregulation correlated with the phenotypic response in these cell lines: *e.g.*, PPC-1 and PC-3M-luc cells, which showed the strongest response to miR-16, had the lowest levels of endogenous miR-16 (**Figure 1a**). The DNA copy numbers on chromosome 13q14, a genomic region that is frequently deleted in chronic lymphocytic leukemia and prostate cancer[14] in the PC-3M-luc cells were reduced to half that of normal prostate cells (**Figure 1d**). However, because the DNA sequence data did not show any mutations on chromosomes coding miR-16 of other copy in the PC-3M-luc cells (data not shown), the remarkable reduction of miR-16 expression might be invoked by a combination of DNA copy number alteration and other factors to affect the expression. LNCaP cells, which showed no response to the miR-16 mimic, were the only cells that tend to have higher miR-16 expression levels than the normal prostate (**Figure 1c**). Additionally, the transfection of miRNAs, which are not down-regulated in PC-3M-luc cells, such as miR-10a and miR-188, did not inhibit the growth of PC-3M-luc cells (data not shown). The expression and function data suggest that reduced expression of miR-16 is critical for sustained proliferation in some prostate

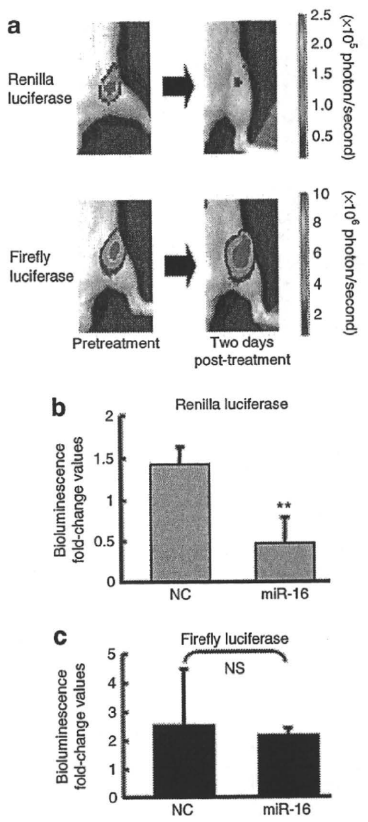

Figure 2 Evaluation of delivery for synthetic microRNA (miRNA) molecules to tumors in bone. A dual-luciferase expressing PC-3M cells that have 3′-UTR of Bcl2 under the renilla luciferase gene, PC-3M-luc/Rluc-Bcl2 3′UTR cells, were generated. These cells were used for dual assay system, for monitoring of tumor growth by firefly luciferase, for monitoring of delivery efficacy of synthetic miR-16 by renilla luciferase. (**a**) Representative images of bone metastasis in the femur of mice. To examine the efficacy of synthetic miR-16 in tumor cells, PC-3M-luc/Rluc-Bcl2 3′UTR cells were injected into the heart of nude mice. Nine weeks after tumor injection, bioluminescence from renilla luciferase was detected. Intravenous injection of miR-16 complexed with atelocollagen suppressed the expression of renilla luciferase (top). In contrast, bioluminescence from firefly luciferase was not affected (bottom). (**b**) Normalized fold change (2 days post/pre-miR-16 administration) of bioluminescence emitted from whole body of mice. This figure is graphically shown of the results of **Figure 2a** by fold change of photon counts. Data represent the mean (n = 3) ± SD *P < 0.01 versus NC miRNA. NS, not significant. NC, negative control.

cancer cell lines and that reintroduction of miR-16 can interfere with that phenotype.

Evaluation of miRNA delivery to bone-metastatic tumors in mice

In order to assess the capacity of the synthetic miR-16 to affect prostate tumor growth in mice, we chose to use a mouse model featuring PC-3M-luc cells that have the capacity to form prostate tumors in the bones of mice.[13,15,16] To evaluate that atelocollagen can efficiently deliver synthetic miRNA molecules to metastatic prostate tumors in bone, we generated a PC-3M-luc metastatic prostate cancer cell line stably expressing the renilla luciferase gene fused to the 3′UTR of Bcl2, a validated miR-16 target (**Supplementary Figure S1a**).[17] Thus, this newly engineered cell line PC-3M-Fluc/Rluc-Bcl2 3′UTR expresses both firefly and renilla luciferase, the later of which is under control of miR-16 (**Supplementary Figure S1b**). As expected, transfection of cultured PC-3M-Fluc/Rluc-Bcl2 3′UTR cells with 30 nmol/l of miR-16 deceased the luminescence derived from renilla luciferase (**Supplementary Figure S1c**). To monitor atelocollagen-mediated delivery of miR-16 in the animal, PC-3M-luc/Rluc-Bcl2 3′UTR cells were intracardiac injected into mice and allowed the tumor cells to deposit in the bone. Nine weeks after implantation, the mice were tail-vein injected with 50 μg of miR-16 mimic that was complexed with atelocollagen. Mice injected with the miR-16/atelocollagen complex produced <50% renilla luciferase from tumors in the bone than they produced before treatment (**Figure 2a,b**). The signal from the firefly luciferase that represents tumor growth was unaffected by the synthetic miR-16, indicating that the inhibition observed for renilla luciferase was due to the binding of injected synthetic miR-16 to the 3′UTR of Bcl2. Synthetic miR-16 was detected in tumor tissue at >20 pg/mg tissue when injected systemically and it was observed to persist in tumors for 3 days after injection (data not shown). Thus, our dual-luciferase prostate cancer xenograft model clearly showed that atelocollagen can efficiently deliver active miRNAs into metastatic tumors in mice.

Inhibition of tumor growth in bone tissues in mice with systemic miR-16 treatment

To assess the therapeutic potential of the miR-16/atelcollagen complexes, prostate tumors were initiated in the bones of mice by intracardiac injection of PC-3M-luc cells. A 50 μg of miR-16 mimic complexed with atelocollagen was administered intravenously into mice at 4, 7, and 10 days after prostate tumor initiation (**Supplementary Figure S2**). The development of tumor in the bone was monitored *in vivo* by bioluminescent imaging. At the end of the experiment on day 28, mice treated with the NC miRNA/atelocollagen complex showed the presence of tumor in the thorax, jaws, and/or legs of mice frequently (**Figure 3a**). In contrast, the mice injected with miR-16/atelocollagen complex exhibited no increase in luminescence during the same observation period. There are significant differences between NC and miR-16 treatment groups on day 28 (P < 0.05) (**Figure 3b**). Histopathological analysis also revealed that growth of PC-3M-luc cells in the bone tissues of mice was significantly inhibited by the miR-16 treatment (**Figure 3c**). These data suggest that atelocollagen-mediated systemic delivery of miR-16 could be a novel strategy for inhibition of prostate tumor growth in the bone tissues.

miR-16 expression in human prostate tissues

We used qRT-PCR to quantify miR-16 levels in the tumors and normal adjacent tissues of seven prostate cancer patients as well as four additional prostate tumors. The relative expression level of miR-16 in each of the samples was calculated by comparing to the average normalized miR-16 levels in prostate samples from three normal donors. The average relative expression of miR-16 in the seven prostate normal adjacent samples was 95% with a standard deviation of 16% and the eleven prostate tumors was 73% with a standard deviation of 28% (**Figure 4**). There is a trend toward lower miR-16 expression in prostate tumors versus normal

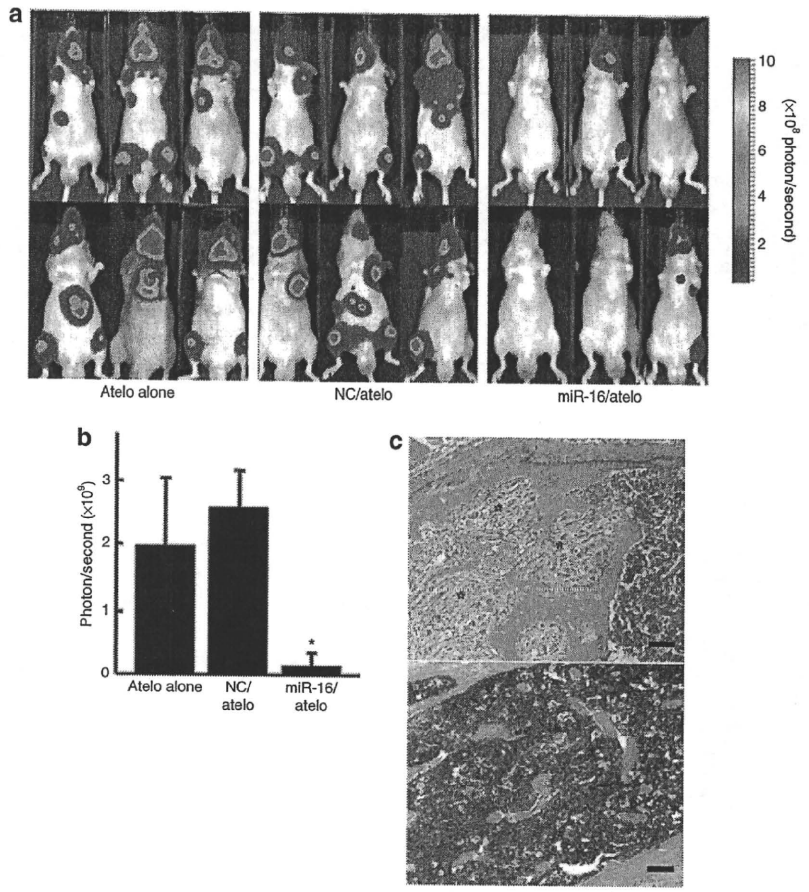

Figure 3 Inhibition of metastatic tumor growth in bone tissues by the atelocollagen-mediated miRNA treatment. Mice were injected with 2×10^6 PC-3M-luc-C6 cells into the left heart ventricle on day zero. The miR-16 and NC miRNA (50 µg) with 0.05% atelocollagen (Atelo) or Atelo alone in a 200 µl volume were injected into the tail vein on days 4, 7, and 10 after tumor injection. At the end of the experiment on day 28, the metastasis was evaluated by IVIS imaging and confirmed by subsequent necropsy. (**a**) All mice used in this experiment on day 28 were shown. There was an increase in luminescence in mice treated with atelocollagen alone and NC miRNA whereas the miR-16/atelocollagen-treated groups had no or low increase in luminescence during the same observation period. (**b**) Quantitation of bioluminescence emitted from whole body of mice on day 28. Data represent the mean ($n = 6$) ± SD *P<0.05 versus other groups. (**c**) Histopathological analysis confirmed micrometastasis in the tibia of nontreated mice (upper). Metastatic lesions are indicated by asterisk mark. In the miR-16-treated mice, any micrometastasis was not observed (lower). Bar = 100 µm.

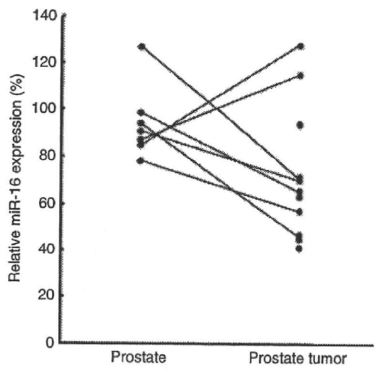

Figure 4 Clinical association of miR-16 expression with prostate cancer. The qRT-PCR analysis to quantify miR-16 levels in the tumors and normal adjacent tissues of seven prostate cancer patients and four additional prostate tumors was performed. The P value calculated by Student's t-test for the two sample sets was 0.08.

prostate tissues, but this trend did not reach statistical significance (Figure 4).

mRNA array analysis following transfection of synthetic miR-16

To get insight into the antioncogenic mechanism of miR-16, we transfected PC-3M-luc cells with the miR-16 mimic and analyzed the expressions of mRNA using mRNA array analysis. Exogenously, miR-16 might directly affect the mRNA levels of the target genes and indirectly affect the expression of genes that are downstream of these direct targets.[18] To identify the pathways that could be affected both directly and indirectly by miR-16, total RNA was isolated from the cells 72 hours after miR-16 transfection. The mRNA array data for the miR-16-transfected samples were compared to the NC miRNA-transfected samples (**Supplementary Table S1**). Fold-differential and P value calculations were used to select 285 mRNAs whose expression levels were significantly altered in the miR-16-transfected samples. A selection of genes suppressed

Table 1 Genes suppressed by miR-16

Gene title	Fold-change (miR-16 versus NC)[a]	*P* value[a]	Cellular process[b]
Aurora kinase B	0.61	2.83×10^{-5}	Chromosomal stability
BUB1	0.58	4.34×10^{-8}	Chromosomal stability
BUBR1	0.61	5.73×10^{-6}	Chromosomal stability
Cyclin D3	0.71	1.30×10^{-6}	Cell cycle
CDK1	0.58	4.99×10^{-7}	Cell cycle
CDK2	0.65	2.35×10^{-5}	Cell cycle
Double parked, DUP	0.76	4.07×10^{-5}	Chromosomal stability
Cks1	0.63	4.71×10^{-6}	Cell cycle
Forkhead box M1	0.61	8.19×10^{-6}	Transcription
Polo-like kinase 1	0.72	1.42×10^{-3}	Chromosomal stability
TACC1	0.76	1.56×10^{-4}	Cell cycle
TACC3	0.64	1.51×10^{-5}	Cell cycle
Thymidylate synthetase	0.56	2.50×10^{-8}	Nucleotide synthesis

Abbreviations: miRNA, microRNA; NC, negative control.
[a]Fold change and *P* value were determined by calculating the ratio of global normalized signals from miR-16 transfected cells to NC miRNA–transfected cells in expression array analysis. [b]Genes are clustered by cellular process, according to their gene ontology classification.

Table 2 Classes of genes affected by miR-16

Functional class	Count[a]	%[b]	*P* value[c]
M phase of mitotic cell cycle	20	12.7	9.60×10^{-18}
DNA metabolism	21	13.3	4.00×10^{-7}
Cytoskeleton organization and biogenesis	16	10.1	8.00×10^{-7}
Cytoskeleton-dependent intracellular transport	9	5.7	6.90×10^{-6}
Regulation of progression through cell cycle	15	9.5	2.50×10^{-5}
Mitotic sister chromatid segregation	4	2.5	6.10×10^{-4}
Establishment of cellular localization	13	8.2	4.10×10^{-3}
Mitotic spindle organization and biogenesis	3	1.9	5.50×10^{-3}
Regulation of progression through mitotic cell cycle	3	1.9	8.10×10^{-3}

[a]The number of genes affected in functional pathway. [b]The percentage calculated from the number of genes affected in functional pathway divided by the number of genes included on the arrays. [c]The significance of the appearance of the functional class in affected genes was calculated as *P* value using DAVID 2.0 software.

by miR-16 is listed in **Table 1**. Pathway analysis combining the Kyoto Encyclopedia of Genes and Genomes[19–21] and Database for Annotation, Visualization, and Integrated Discovery[22] was used to analyze the list of genes with altered expression to determine if there was a significant enrichment of genes associated with any known cellular pathways (**Table 2**). Overall, the statistical enrichment of pathways was moderately low, suggesting that no single pathway or network was specifically and vigorously responsive to the treatment. However, for those pathways that were considered enriched, a few strong underlying themes emerged. The gene lists were enriched for functions related to cell division and control of the cell cycle (**Table 2**). The functions associated with cell-cycle control were most enriched in miR-16-affected genes and these 12 genes that cover G1, S, G2, and M phase of cell cycle are mapped into the Kyoto Encyclopedia of Genes and Genomes Pathway Cell Cycle Map (**Supplementary Figure S3**). Thus, these data suggest that strong inhibition of prostate tumor growth in bone tissues of our animal model was due to downregulation of a key component of cell-cycle genes.

DISCUSSION

The likely involvement of miR-16 in the development of prostate cancer is apparent on multiple levels. The loss of the genomic locus at 13q14 that encompasses the miR-16-1 gene has been reported to be highly associated with human prostate cancer progression.[14] Dong *et al.* suggested loss of heterogeneity at 13q14 is associated with clinically significant high-grade and high-stage prostate cancers[10] with ties to both metastasis and tumor initiation.[23] Consistent with its genomic location, our qRT-PCR results showed that the miR-16 is significantly reduced in most prostate tumors and cultured prostate cancer cells relative to normal prostate tissues.

Based on our studies with cultured prostate cancer cells, the reduced expression of miR-16 is likely necessary to maintain high rates of proliferation. The relationship between miR-16 and apoptosis likely stems from the miRNA's apparent role in regulating BCL2 expression.[17] Our previous data also showed that the transfection of miR-16 into 22Rv1 prostate cancer cells induced apoptosis (F. Takeshita *et al.*, unpublished results). Although increased apoptosis is likely to be at least partially responsible for the reduced proliferation rates that we observed in miR-16-transfected PC-3M-luc cells, it appears that the small RNA also affects cell-cycle progression by regulating the expression of multiple cell-cycle genes. The transfection of prostate cancer cells with synthetic miR-16 reduced the expression of genes like *Cyclin D3*, *CDK1*, *CDK2*, *Cks1*, *TAAC1*, and *TAAC3* that play roles in regulating cell-cycle progression. The apparent capacity of miR-16 to simultaneously regulate cell cycle and apoptosis points to the likely importance of the small RNA in maintaining normal cell function and underscores the influence that the altered expression of the miRNA likely has on tumorigenesis.

The importance of miRNAs like miR-16 as tumor suppressors is becoming increasingly clear. Myriad array and qRT-PCR studies have revealed that the expression levels of specific miRNAs are reduced in the tumors of patients with a variety of cancers.[4,5] When transfected into cancer cells, many of these miRNAs affect proliferation, viability, cell cycle, or apoptosis[24,25] and affect the expression of multiple known oncogenes.[17,18,26–28] Although the growth inhibition of LNCaP cells was not induced by transfection of miR-16 in our study, Bonci *et al.* showed that such inhibition of LNCaP cells was induced by transduction of the *miR-15a-miR-16-1* cluster by lentiviral vector.[8] This discrepancy indicated that the growth inhibition of LNCaP might be induced mainly by induction of miR-15a, further careful studies are needed, considering any clinical application of miR-16.

The clinical application of these naturally occurring tumor suppressors represents a major opportunity for the future treatment

of cancer patients. As with other oligonucleotide-based therapies, realizing the potential of therapeutic miRNAs will require an effective delivery technology. In a previous study, we showed that intravenous injections of EZH2 and p110α siRNA complexed with atelocollagen inhibited the tumor growth in bone tissues of the mouse model.[13] These results showed that an atelocollagen-mediated systemic delivery of siRNA could reach tumor cells at metastatic sites and inhibit tumor growth *in vivo*. As demonstrated here, atelocollagen facilitates the accumulation of enough synthetic miRNA in the cancers cells of an existing prostate tumor to affect the expression of a target gene. Furthermore, the combination of synthetic miR-16 and atelocollagen strongly inhibited the development of human prostate tumors in the bones of mice. Interestingly, the effect of miR-16 appeared to be restricted to the prostate cancer cells, as the miR-16 treated mice showed no notable side effects. Follow-up studies featuring the treatment of larger tumors and more extensive toxicity studies will be required to demonstrate the therapeutic potential of atelocollagen-miR-16; however, these early results are extremely encouraging.

MATERIALS AND METHODS

Cell culture. The human prostate cell line 22Rv1, LNCaP, DU145, and PPC-1 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum. The PC-3M-luc cells continuously expressing firefly luciferase (Xenogen, Alameda, CA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.2 mg/ml zeocin (Invitrogen, Carlsbad, CA). For construction of 3′-UTR–renilla luciferase plasmid and reporter assays, the segment of 3′-UTR of *Bcl2* gene was amplified by PCR using genomic DNA from normal human prostate epithelial cells (CT-2555; Lonza Walkersville, Walkersville, MD) as reported.[17] The PCR product was inserted into the pGL4.75[*hRluc*/CMV] vector (Promega, Madison, WI), using *Xba*I site immediately downstream from the stop codon of renilla luciferase (pGL4.75[*hRluc*/CMV]-Bcl2 3′UTR). For reporter assays, PC-3M-luc-C6 cells were transfected with 2 μg of pGL4.75[*hRluc*/CMV]-Bcl2 3′UTR using LipofectAMINE 2000 (Invitrogen). Stable transfectants were selected in hygromycine (0.2 mg/ml; Invitrogen) and bioluminescence was used to screen transfected clones for renilla and firefly luciferase gene expression using dual-luciferase assay system (Promega), intensity of renilla luciferase was normalized by firefly luciferase. Clones expressing the both luciferase gene were named PC-3M-luc/*Rluc*-Bcl2 3′UTR. The cells were maintained *in vitro* at 37 °C in a humidified atmosphere of 5% CO_2.

Transfection with synthetic miR-16 and assay of cellular proliferation. Synthetic hsa-miR-16 (Pre-miR-hsa-miR-16; Ambion, Austin, TX) or NC miRNA (Pre-miR microRNA Precursor Molecule-Negative Control #2, cat. no. AM17111; Ambion) was delivered via lipid-based reverse transfection with 30 nmol/l final concentration of miRNA as described previously.[29] As a control for inhibition of cellular proliferation, siRNA against the motor protein kinesin 11, also known as Eg5, was used. Eg5 is essential for cellular survival of most eukaryotic cells and a lack thereof leads to reduced cell proliferation and cell death.[30] siEg5 was used in lipid-based transfection following the same experimental parameters that apply to miRNA. We observed 50–70% growth inhibition in all cell lines used in this study. Percent (%) proliferation values from the alamar blue assay (Invitrogen) were normalized to values from cells treated with NC miRNA.

Quantitative RT-PCR of miR-16. Human cultured cell line RNA was isolated using the ISOGEN (Wako Chemical, Tokyo, Japan). MiRNA-specific complementary DNA was generated using the TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA) and the miRNA-specific RT primer from the TaqMan Micro RNA Assay (Applied Biosystems). The expression of the U6 small nuclear RNA was used as an internal normalization control. miRNA levels were also measured by using the miRNA-specific probe included with TaqMan Micro RNA Assay on a Real-Time PCR System 7300 and SDS software (Applied Biosystems).

Quantitative PCR of miR-16 loci on chromosome 13q14. Genomic DNAs were extracted from PC-3M-luc and prostate epithelial cells using DNAeasy (Qiagen, Valencia, CA). Quantitative PCR for the miR-16 loci on chromosome 13q14 was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and primer sequences were 5′-CCA CCA CAC TTA ATA CTG CA-3′ and 5′-ATA CCT CTT ATG ATA CCA AT-3′. The house keeping gene, *RNase P* was also quantified as a control reference gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and TaqMan RNase P Detection Reagents Kit (Applied Biosystems). The reactions were incubated at 50 °C for 2 minutes, then heated to 95 °C for 2 minutes followed by 45 cycles of 15 seconds at 95 °C, and 30 seconds at 60 °C.

Evaluation of miRNA delivery to bone-metastatic tumors in mice. Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Seven- to ten-week-old male Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane on day zero and subsequent days. On day zero of the experiments, to generate a bone-metastatic human prostate cancer model, the anesthetized animals were injected with 2 × 10^6 PC-3M-luc/*Rluc*-Bcl2 3′UTR cells suspended in 100 μl sterile Dulbecco's phosphate-buffered saline into the left heart ventricle.[13,15,16] For *in vivo* imaging, the mice were injected with ViviRen (5 mg/kg; Promega) by intravenous tail vein injection and imaged immediately to count the photons from animal whole bodies using the IVIS imaging system (Xenogen). After the bioluminescence from renilla luciferase disappeared, photons from firefly luciferase were counted as described previously.[13]

Preparation of complex with miR-16 and atelocollagen. For preparing the complexes of miRNA and atelocollagen (Koken, Tokyo, Japan), an equal volume of atelocollagen (0.1 % in phosphate-buffered saline at pH 7.4) and miRNA solution were combined and mixed by rotating for 1 hour at 4 °C. The final concentration of atelocollagen was 0.05%. Nine weeks after tumor injection, individual mice (from cohorts containing three animals) were injected with 200 μl of atelocollagen containing 50 μg of miR-16 complexed with atelocollagen, or NC miRNA/atelocollagen by intravenous tail-vein injection.

Analysis of miR-16/atelocollagen treatment for bone-metastatic prostate cancer. Mice were inoculated with PC-3M-luc cells into the left cardiac ventricle on day zero as described previously.[13] The miR-16 and NC miRNA (50 μg) with 0.05% atelocollagen in a 200 μl volume were injected into the mouse tail vein on days 4, 7, and 10 postinoculation. Each experimental condition included six animals per group. At the end of the experiment on day 28, to confirm the presence of neoplastic cells, selected tissues were excised from the mice at necropsy. Tissues were fixed in 4% formaldehyde-phosphate-buffered saline(−), embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin.

Clinical samples. Human prostate tissue samples derived from resected prostates from treatment-naive men with an average age of 65 (range of 52–76) diagnosed with nonmetastatic T2 or T3 prostate adenocarcinoma who gave informed consent. Gleason scores for all patients were 8 or 9. The tissues from patients were formalin-fixed, paraffin-embedded, sectioned, hematoxylin and eosin stained, and subjected to microscopic analysis. Three adjacent sections comprising 60–90% (74% average) cancerous tissue were selected as cancer samples from each patient. Three adjacent sections lacking evidence of cancer cells were selected as normal adjacent

samples. RNA from the tissues were prepared using the RecoverAll Total RNA Isolation Kit (Ambion). The isolated RNA was subjected to qRT-PCR for miR-16 as described above.

MiR-16 functional pathway analysis. For preparation of RNA samples, PC-3M-luc cells were reverse transfected in quadruplicate by complexing miR-16 and NC miRNA and NeoFX transfection reagent (Ambion). The final concentration of miRNA was 30 nmol/l. Cells were harvested at 72 hours post-transfection. One microgram of total RNA per sample was used to prepare biotin-labeled cRNA using a MessageAmp II-based protocol (Ambion) and one round of amplification. Labeled cRNA was hybridized, washed, and scanned using Illumina's recommended protocols. Illumina BeadScan software was used to produce .idat, .xml, and .tif files for each array on a slide. Raw data were extracted using Illumina BeadStudio software, v 3.0 (Illumina, San Diego, CA). Following quality assessment, data from the replicate beads on each array were summarized into average intensity values and variances. The background subtracted data were used to compare the relative expression of mRNAs in cells transfected with miR-16, NC miRNA, and transfection agent only. analysis of variance was used to judge the significance of the variation observed between the various treatment groups. In total, 285 mRNAs exceeded the thresholds used to identify differentially expressed genes (log ratio greater than 0.5 or less than −0.5 for the average signal between miR-16 and NC miRNA or transfection agent only treatments and P values <0.001 for the 72 hour time-point).

Statistical analysis. The results are given as mean ± SD Statistical analysis was conducted using the analysis of variance with the Bonferroni correction for multiple comparisons. A P value of ≤0.05 was considered to indicate a significant difference.

SUPPLEMENTARY MATERIAL

Figure S1. The scheme of dual luciferase assay for monitoring of systemic miR-16 delivery.

Figure S2. Overview of experimental protocol for inhibition of metastatic tumor growth in bone tissues by the atelocollagen-mediated miRNA treatment.

Figure S3. KEGG cell cycle diagram.

Table S1. Data of the mRNA array for comparison of miR-16 and NC miR transfected PC-3M-luc cells.

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ORIGINAL ARTICLE

Mutant mouse *p53* transgene elevates the chemical induction of tumors that respond to gene silencing with siRNA

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To study the role of mutant *p53* in the induction and cure of tumors, we generated transgenic mice carrying mutant *p53* (m*p53*) containing a 9 bp deletion in exon 6 in addition to wild-type *p53*, expressing both *p53* and m*p53*. The m*p53* cDNA was cloned from a radiation-induced mouse tumor and ligated to the chicken β-actin promoter/CMV-IE enhancer in the expression vector. The presence of m*p53* suppressed *p21* expression in primary fibroblasts after ionizing irradiation, indicating the dominant-negative activity of m*p53* in the mice. These mice developed fibrosarcomas after the subcutaneous injection of 3-methylcholanthrene with an incidence 1.7-fold higher than that of wild-type mice (42% excess). The tumors were then treated via a potent atelocollagen delivery system with small interfering RNA (siRNA), that targeted the promoter/enhancer of the expression vector, resulting in the suppression of tumor growth in 30% of 44 autochthonous tumors, including four cures, and their transplants, the total fraction corresponding to the tumor excess. This suppressive effect involved the induction of apoptosis. These results indicate that m*p53* activity causes tumors that can be suppressed by subsequent silencing of m*p53* in the presence of wild-type *p53* alleles.
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Keywords: mutant *p53*; transgenic mice; tumor; siRNA gene silencing; apoptosis

Introduction

The *p53* gene is a tumor suppressor, controlling the cell cycle and apoptosis,[1,2] protecting the irradiated mouse fetus from teratogenesis[3] and regulating mouse reproduction.[4] Mutated *p53* is frequently found in human cancers.[5] and predisposes Li–Fraumeni syndrome patients to cancer.[6]

Heterozygous mice with various *p53* mutations have been used to study the effects of these mutations on tumor induction. Mice carrying *p53* mutated at codon135 are highly sensitive to spontaneous[7,8] and chemical tumor induction.[9] Mice carrying *p53* mutations analogous to the human Li–Fraumeni syndrome hot spot mutation exhibit a high incidence of spontaneous tumors with a spectrum different from that of *p53*-null mice,[10–12] a high metastatic potential for produced tumors[13] and a high incidence of chemically induced tumors.[14] Especially of note is an approach toward simulating the human context with heterozygous mice containing human *p53* and Li–Fraumeni mutant *p53* in the genome.[12]

At the initial step of carcinogenesis caused by p53 mutation, there must exist a heterozygous state of wild-type *p53* with mutant *p53*, resulting in the expression of both types of p53 proteins. Mutant p53 proteins act in a dominant-negative manner by interfering with the activity of wild-type p53 proteins, thus exerting gain-of-function activity.[12,15,16] This activity induces tumor formation by interfering with the *ATM*-controlled molecular network protecting cells from tumorigenesis. A recent study reported that mutant p53 interferes with the binding of the repair protein complex, Mre11-Rad50-NBS1, to radiation-induced DNA double strand breaks,[12] suggesting an additional role of mutant *p53* in DNA repair and tumorigenesis.

On the other hand, the absence of *p53* expression in mice results in a high incidence of tumors.[17,18] By restoring wild-type *p53* function via Cre recombinase-mediated reactivation, such tumors regress after apoptosis[19] or cellular senescence.[20] However, such restoration may not function in the presence of mutant p53 protein because of its dominant-negative action over the

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