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21

Establishment of Embryonic Stem Cells and Generation of Genetically Modified Rats

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1. Introduction

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

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

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

2. Mouse ES cells

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

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

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

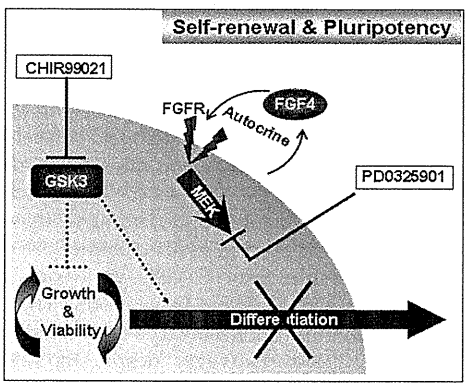

Fig. 1. Mechanism of maintenance of self-renewal and pluripotency

3. Rat

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

3.1 Background of rat ES cells

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

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

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

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

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

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

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

3.2 Maintenance of pluripotency and self-renewal by signal inhibitors

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

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

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

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

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

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

3.3 Problems with current rat ES cells

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

3.4 Importance to establish high-quality rat ES cells

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

4. Establishment of rat ES cells

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

4.1 An effect of Rho kinase inhibitor

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

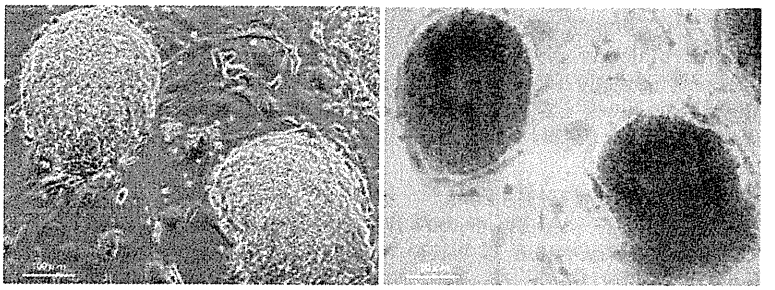

Fig. 2. Colonies cultured in a Y27632-containing medium. At passage 6, the colonies were stained blue, which means that they were positive for alkaline phosphatase activity (right). (scale bar, 100 μm)

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

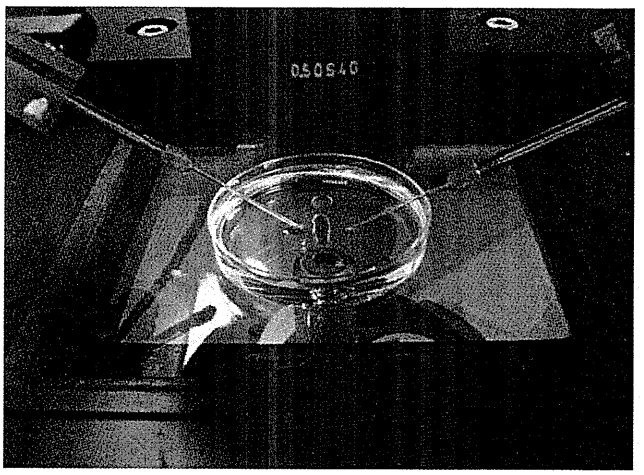

Fig. 3. Device for blastocyst injection with ES cells

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

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

4.2 Four signaling inhibitors are necessary to establish rat ES cells

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

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

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

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

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

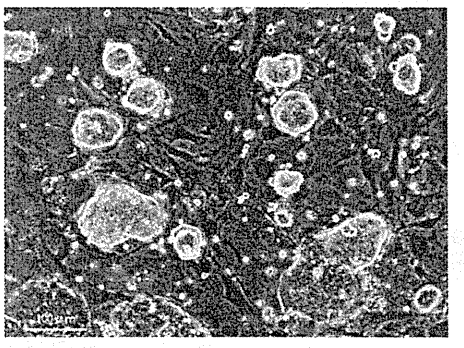

Fig. 4. Colonies cultured in a YPAC-containing medium (passage 3). (scale bar, 100 μ m)

4.3 Characteristics of rat ES cells

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

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

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

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

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

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

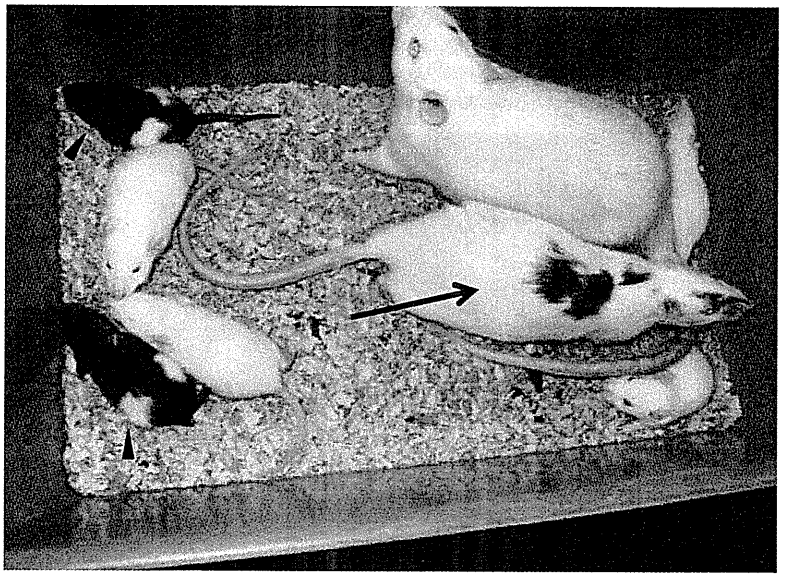

Fig. 5. Germline transmission from chimeric rats. The ES cell line derived from the LEA rat strain was injected into Wistar rat blastocysts, leading to the production of a female chimeric rat with an agouti coat color (arrow). The chimera was mated with a male Wistar rat, and ES cells were transmitted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

4.4 Production of germline chimeras

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

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

4.5 Generation of genetically modified rats

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

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

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

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

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

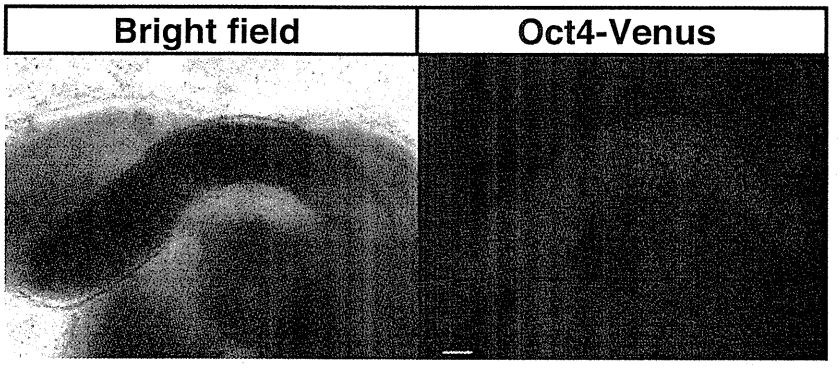

Fig. 6. Oct4-Venus expression in PGCs in fetal gonad. Fetal gonad of Oct4-Venus transgenic female embryo at 16.0 days of gestation was dissected, and Venus fluorescence was observed. (scale bar, 100 μ m)

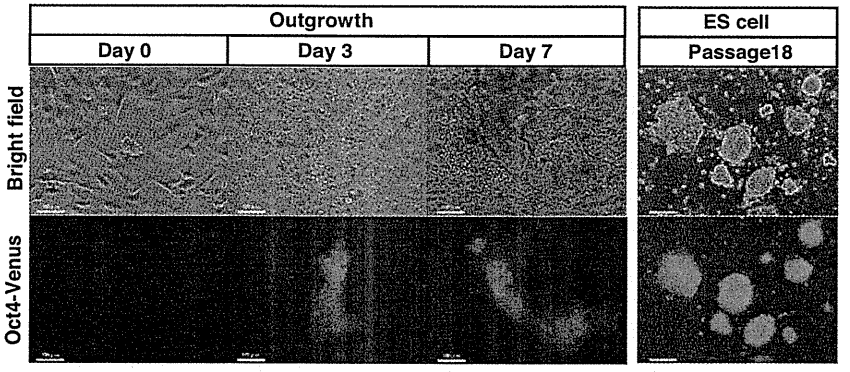

Fig. 7. Oct4-Venus expression during outgrowth of ICM and expansion of ES cells. Blastocyst derived from Oct4-Venus transgenic rats was outgrown on MEFs in the YPAC medium for 7 days. Venus fluorescence was detected in the ICM cells, while it was not detected in differentiated cells. Homogenous Venus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

5. Conclusion

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

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

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

6. Acknowledgements

This work was supported by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control.

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特集／外来で診る肥満症

肥満の合併症

がん

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はじめに

肥満は洋の東西を問わず増加の兆しを見せ，心血管イベントのみならず多くのがんのリスクを増加させることがメタアナリシスなどから明らかにされている[1)2)]。ごく近年まで人類の歴史は飢餓との闘いであり，その環境に順応すべくヒトは飢餓に対応するエネルギー蓄積やエネルギー消費の制限などの巧妙なメカニズムを獲得したが，飽食の現在それに対応する術として余剰エネルギーを排泄するメカニズムを持ち合わせていない。結果的に肥満となり，これに引き起こされる各種生活習慣病やがんが深刻な問題となってきている。本稿では肥満とがんの関係，特に内臓脂肪と内臓脂肪などから分泌されるアディポサイトカインとがんとの関係およびメカニズムを検討した。

Ⅰ．肥満とがんに関する疫学的エビデンス

世界がん研究基金（WCRF）と米国がん研究協会（AICR）は，食物や食品成分と発がん性やがん予防効果について，科学論文を系統的にレビューすることによりこの因果関係の確からしさを評価している[3)]。肥満が確実にリスクを上げるがんとして，食道腺がん，膵がん，大腸がん，乳がん（閉経後），子宮内膜がん，腎がんが，また内臓脂肪型肥満では大腸がんが挙げられている（表1）。このメカニズムとして，インスリン，インスリン様成長因子-Ⅰ（insulin-like growth factor-Ⅰ；IGF-Ⅰ），アディポネクチン，レプチン，慢性炎症，閉経後女性におけるエストロゲンの減少などの関与が示唆されている。

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Ⅱ．肥満関連因子とがん

肥満に伴う発がんの促進を媒介する因子としては，内臓脂肪外ではインスリン，IGF-Ⅰが，内臓脂肪からはアディポネクチン，遊離脂肪酸（FFA），レプチン，TNF-α，IL-6などが重要である。これらのホルモンは全身への代謝作用と，局所への直接作用を有すため，これらの因子の発がんへの作用解析を難しくしている。

1．インスリンおよびインスリン抵抗性

肥満はインスリン抵抗性・高インスリン血症の主因となる。インスリンは肝臓からの遊離IGF-Ⅰ産生亢進および活性化を介して発がんを促進すると考えられるが，ヒトにおける解析ではインスリン分泌を反映するCペプチドが大腸がんと相関することが報告されている[4)]。一方，インスリン投与は培養細胞の増殖やラットにおける上皮の増殖を促進するが[5)]，使用されたインスリン濃度が非常に高く，インスリンの直接作用は非生理的なものであったため，実際のところインスリンは後述するIGF活性化を介して，またはインスリン抵抗性により発がん促進に働くものと考えられる。

2．IGF-Ⅰ（insulin-like growth factor-Ⅰ）

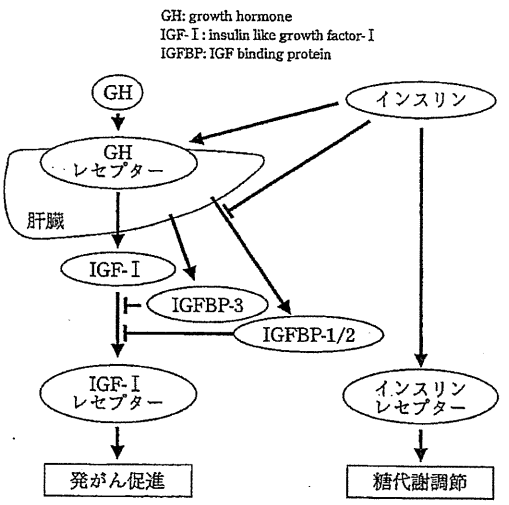
IGFは成長ホルモンの刺激により肝臓で産生される体細胞の増殖刺激因子で，インスリンと対照的に長期的な体細胞の増殖を促進して成長などに関与している。IGF-Ⅰの産生は成長ホルモン（GH）刺激によるが，GHの肝臓でのレセプターの発現はインスリンによって促進される。

インスリンはその生理的濃度では肝臓での

表 1　肥満とがんのリスク

World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington, D. C.: AICR, 2007. より一部改変

	増加因子	がんの部位
Convincing（確実なリスク）	体脂肪	食道腺がん
		膵臓がん
		大腸がん
		乳がん（閉経後）
		子宮内膜がん
		腎がん
	内臓脂肪	大腸がん
Probable（ほぼ確実なリスク）	体脂肪	胆嚢がん
	内臓脂肪	膵臓がん
		乳がん（閉経後）
		子宮内膜がん
	成人での体重増加	乳がん（閉経後）



インスリンは肝において成長ホルモンレセプターの発現促進，強力なIGF-I結合タンパクであるIGFBP-1の産生を抑制し結果的にIGF-Iの生理活性を亢進させる。
日本消化器内科学会：肥満と消化器疾患，p126. より改変

図 1　インスリン，IGF-Iと腫瘍増殖

GHレセプター発現の亢進，IGFBP1，2の発現抑制を通して最終的にはIGF-I活性を亢進し発がんを促進する。従って高インスリン血症やインスリン抵抗性下では，IGF-Iの生理活性が亢進し発がん促進に働くものと考えられる（図1）。

3. アディポネクチン

脂肪細胞から分泌されるアディポネクチンにはインスリン抵抗性改善作用があるが，肥満，特に内臓脂肪型肥満に伴いその分泌は有意に減少する。アディポネクチンはこれらのレセプターを介して標的臓器でAMPキナーゼ（AMPK）やMAPキナーゼ，PPARγなどを活性化することが知られている。乳がん・前立腺がん・大腸がんなどでアディポネクチンは発がん抑制的に働くことが報告されている[6]。

4. レプチン

レプチンは脂肪組織から分泌されるアディポサイトカインの一つで，満腹中枢へ作用し摂食を抑制し，末梢ではエネルギー消費を亢進する[7]。レプチンは，エネルギー摂取が多くなり余剰エネルギーとして脂肪蓄積量が増加すると脂肪組織からの分泌が亢進し，視床下部に作用し食欲を低下させ，負のフィードバックで体内の脂肪蓄積量を一定に保つ作用がある。欧米のように肥満者が多くない我が国におけるエビデンスも少なく，今後は肥満者を対象とした検討，およびモデル動物による発がん促進の分子機序の解明が求められる[8]。

表 2　がん予防10の提言

World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer : a Global Perspective. Washington, D. C.: AICR, 2007. より一部改変

1	体脂肪	標準範囲内に体重をコントロールする
2	身体活動	日常生活を活発に
3	体重を増加させる飲食物	高カロリーの食物摂取を制限する 甘い飲み物を避ける
4	野菜摂取	野菜の豊かな食卓を
5	肉類摂取	赤身肉の摂取を制限する 加工肉の摂取を避ける
6	アルコール	アルコール摂取を制限する
7	保存，加工，調理	塩分摂取を制限する カビ臭い穀類や豆類を避ける
8	サプリメント	食事からのみの栄養摂取を目指す
9	母　乳	母乳による育児を
10	がんの既往	がん予防を心がける

Ⅲ. 肥満による発がん促進の分子メカニズム解析

これまでの検討で肥満からの発がんのメカニズムが少しずつ解明されている。PI3K/Akt シグナル経路は細胞生存，増殖，細胞周期を刺激し発がんに働くが，この経路は肥満によるインスリン，IGF-Ⅰ，レプチンなどにより活性化され，治療標的になりうることが明らかにされている[9)10)]。細胞の生存や増殖に関与し発がん過程においても重要な役割をする転写因子であるNF-κB は肥満で増加するため検討が進んでいる[11)]。細胞がエネルギーを消費すると AMP/ATP 比が増加し，AMPK が活性化されその下流の mammalian target-of-rapamycin (mTOR) が抑制され，タンパク合成が抑制される。エネルギー消費をコントロールし生存に働くメカニズムであるが，肥満において余剰エネルギーはこの経路の逆に作用するため，腫瘍形成への関与が示唆されている[12)]。この AMPK は発がんの重要なチェックポイントである p53 と相互作用し，さらに，肥満患者における p53 の減少は肥満関連発がんを促進するなど，今後さらに肥満とがんに関与するシグナル経路，転写因子，分子などは増える可能性がある[13)14)]。

Ⅳ. 肥満と腸内細菌そしてがん

肥満はエネルギー摂取と消費の収支，または貯蓄の結果である。数兆個ともいわれる正常腸内細菌叢は栄養吸収やエネルギー調節に関与しているが，肥満者はやせとは異なる細菌叢を形成している。肥満者ではやせに比べ *Bacteroides* が少なく，*Firmicutes* が多いことが知られているが，食事療法後の肥満者では腸内細菌叢中の *Bacteroides* の割合が 3 %から15%に増加した[15)]。腸内細菌叢が肥満の影響を受ける可能性が示唆されているが，そのメカニズムとして不消化多糖類の発酵，リポタンパクリパーゼの増加や PPARγ および AMPK 活性誘導，CD14 などの炎症性サイトカインの増加が考えられている[16)]。培養技術の進歩により菌種が明らかとなる可能性がある。

Ⅴ. 身体活動と大腸がん

身体活動が確実にリスクを減少させるがんとして大腸がんが挙げられる[3)]。

食生活と大腸がんの関連にはややばらつきが認められるが，欧米では身体活動に関する多くのコホート研究において結腸がんに対する予防効果が統一した見解となっている[17)]。我が国においても，特に男性では身体活動が大腸がん

リスクを低下させるケースコントロールスタディーが報告されている[18]。機序として，肥満の改善効果や，インスリン抵抗性改善作用，2次胆汁酸関連発がん物質やPGE2の抑制，腸管蠕動亢進，細胞性免疫の増強，活性酸素生成抑制などが考えられている。一方，結腸がんと身体活動に関してはBMIと独立した抑制因子として報告されており，直腸がんに関しては多くの報告ではBMIや身体活動との相関は認められていない。

おわりに

2007年のWCRF/AICRからの報告にがん予防の提言がされているが，体重や摂取カロリーのコントロールなど生活習慣病の予防とよく似ている点が非常に興味深い（表2）。前述した他にも遊離脂肪酸，レジスチン，グレリンなどと各種がんの関連はほとんど解析されておらず，これからの課題となる。今後より詳細な肥満からの発がんメカニズムの解析が進むことが期待される。

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特集　AMPキナーゼと糖・脂質・エネルギー代謝 update

AMPキナーゼと癌*

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Key Words：AMPK, chemoprevention, metformin

代謝と癌をつなぐ key playerであるAMPK

AMPKはエネルギーセンサーとして働き，低酸素状態や低血糖などによりATPの産生が抑制される，あるいは運動などによりATPが消費される代謝ストレス状態によって増加したAMPによってAMPKは活性化される．活性化したAMPKはATPを産生する異化経路を促進し，同時にATPを消費する細胞増殖や合成経路を抑制する．AMPKの上流にはアディポネクチンやレプチンがあり，また近年，Peutz-Jeghers症候群の原因遺伝子であるLKB1遺伝子(*Lkb1*)も上流にあることが判明し[1]，このLKB1-AMPKカスケードがメタボリックシンドロームと発癌をつなぐkey playerである可能性が示唆された．すなわち，細胞増殖(発癌)とエネルギー代謝(メタボリックシンドローム)をともに制御している上流のマスター分子があり，そのマスター分子をコードする遺伝子が「癌抑制遺伝子」であることが判明した．図1に示すようにAMPKは活性化するとmTOR経路の抑制などを通して細胞増殖の抑制を促す．

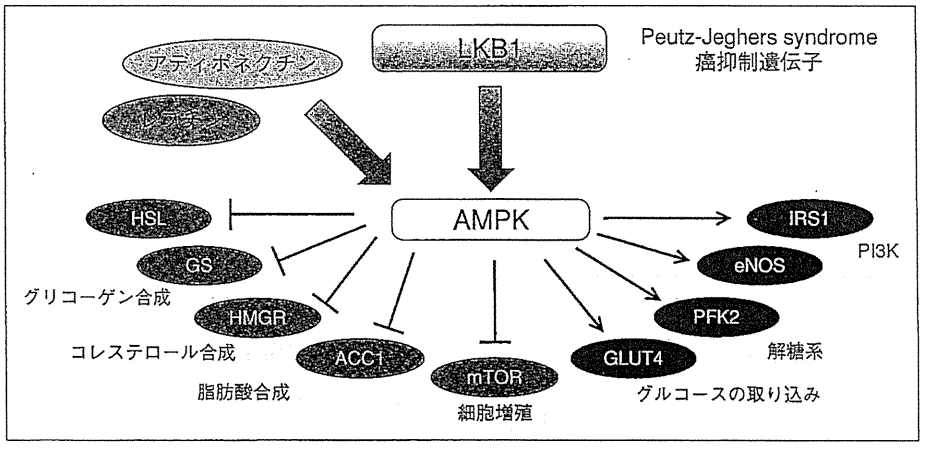

図1　AMPKの伝達経路
実際はAMPKはこれまでに図2のような各種作用を有することが知られている．正常細胞では環境に応じてapoptosis, autophagy, cell growthの調整をしている．

* AMP kinase and cancer.

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