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(71) Applicant: **KURARAY CO., LTD.** [JP/JP]; 1621, Sakazu, Kurashiki-shi, Okayama, 7100801 (JP).

(72) Inventor; and
(71) Applicant : **SUMI, Shoichiro** [JP/JP]; 23-1, Nishiyamawaki, Yawata-shi, Kyoto, 6148352 (JP).

(72) Inventors: **HAYASHI, Takashi**; c/o NAKAMURA-Hospital, Medical corporation midori-kai, 1-2834-5, Nagaoharimadani, Hirakata-shi, Osaka, 5730104 (JP). **YANAI, Goichi**; c/o Kyoto University, 36-1, Yoshidahonmachi, Sakyo-ku, Kyoto-shi, Kyoto, 6068501 (JP).

(74) Agent: **IEIRI, Takeshi**; HIBIKI IP Law Firm, Asahi Bldg. 10th Floor, 3-33-8, Tsuruya-cho, Kanagawa-ku, Yokohama-shi, Kanagawa, 2210835 (JP).

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(54) **Title**: METHOD OF CELL FUSION AND FUSION CELLS

(57) **Abstract**: A method of cell fusion includes preparing tissue-derived mesenchymal cells; preparing dispersed islet cells prepared from isolated islets; and performing electrofusion of the prepared tissue-derived mesenchymal cells and the dispersed islet cells to yield fusion cells. The fusion cells have a beta-cell function that includes glucose-responsive insulin release, and a function of the tissue-derived mesenchymal cells that includes a proliferation capacity.

Description

Title of Invention: METHOD OF CELL FUSION AND FUSION CELLS

Technical Field

[0001] The present invention relates to a method of cell fusion of tissue-derived mesenchymal cells and dispersed islet cells, and fusion cells.

Background Art

[0002] Diabetes mellitus (DM) is a leading cause of morbidity and mortality in industrialized countries, and the number of patients affected is estimated to be 366 million in 2011 with an increase to 552 million by 2030 (1). Among several types of DM, Type 1 DM (T1DM) is characterized by the selective destruction of pancreatic beta-cells caused by an autoimmune attack or other unknown causes. Beta-cell reconstruction is currently achieved only by either pancreas or islet transplantation in clinical setting. Although clinical trials of encapsulated islets that enable transplantation without immune suppression are on-going (2), these transplantation therapies share common problems of donor scarcity and adverse effects related to immune suppression.

[0003] Islet transplantation is an effective therapy for T1DM, but limited donor sources restrict it from becoming a major treatment option (3, 4). In islet transplantation, a diabetic patient often requires two or even three donor pancreata to accomplish insulin-independence in current mainstream protocols, which makes the problem of a donor shortage even more serious (5). Even though insulin-independence is achieved by islet transplantation, islet graft function is rarely sustained with only 7.5% of these patients remaining insulin-independent at 5 years post transplantation (3).

[0004] Loss of functional isolated islets occurs during the culture period after isolation and purification (6). It is established that apoptosis triggered by withdrawal of growth factors (7), disruption of extracellular matrix (6, 8), and endotoxin contamination (9) participates in islet loss under culture conditions. From these reports, beta-cells in isolated islets are susceptible to immune and inflammatory factors and have minimal proliferation capacity, if any.

[0005] Mesenchymal stem cells (MSCs), which were first identified by Friedenstein and his colleagues (10), are known to be highly proliferative and with anti-apoptotic potential (11). MSCs derived from bone marrow and other organs such as liver, umbilical cord blood, placenta, and adipose tissue (12-15) have high proliferation capacity and multipotency to differentiate toward various cell types such as muscle, cartilage, and bone (16). In addition, MSCs have been shown to promote angiogenesis in vivo (17, 18).

[0006] Recent studies have shown that MSCs secrete several factors that improve survival

and function of transplanted islets. MSCs co-cultured with islets secrete higher levels of anti-apoptotic signaling molecules and improve glucose-stimulated insulin secretion indexes (19). Bone marrow cells are also shown to induce endogenous beta-cell proliferation and improvement of islet function in vivo (20). Furthermore, in co-transplantation of MSCs with islets, MSCs improved the capacity of islet grafts to reverse hyperglycemia compared with islets alone (21). MSCs may also enhance islet resistance to hypoxia/reoxygenation-induced apoptosis and dysfunction by promoting anti-apoptotic gene expression (22). Because of these favorable effects on islets, MSCs provide an important approach for improvement of islet engraftment, thereby decreasing the numbers of islets needed to achieve insulin-independence (23).

[0007] Cell fusion occurs in physiological conditions such as normal development and immune reaction (24-26). Bone marrow-derived stem cells fuse to several types of cells under normal condition or after cell injury (27) and Tada M et al. have shown that the nuclei of somatic cells can be reprogrammed by cell fusion with embryonic stem cells (28). Therefore, cellular transformation may be induced by cell fusion between different types of cells through nuclear reprogramming.

[0008] Methods for producing cells having an insulin secretion capacity derived from islet cells and proliferation capacity derived from tissue-derived mesenchymal cells have been studied in order to cure diabetic patients.

For example, PLT 1 discloses a technique for fusing a pancreatic beta cell with a MSC by using a PEG (polyethyleneglycol) method.

Citation List

Patent Literature

[0009] PTL 1: International Patent Publication No. WO 2008/036349

Summary of Invention

Technical Problem

[0010] However, isolation of beta-cells from islet cells (isolated islet) requires complicated operations. In addition, it is very difficult to isolate a sufficient amount of beta-cells for use in treatment. For example, in the case of treatment for human, several hundred thousand cells are necessary.

Solution to Problem

[0011] The inventors have found a method for efficiently producing a cluster of cells including fusion cells having an insulin secretion capacity derived from beta-cells and a proliferation capacity derived from tissue-derived mesenchymal cells by using dispersed islet cells including beta-cells.

In order to solve the above-mentioned problems, a method according to a first exemplary aspect of the present invention provides a method of cell fusion. The

method includes preparing tissue-derived mesenchymal cells; preparing dispersed islet cells prepared from isolated islets; and performing electrofusion of the prepared tissue-derived mesenchymal cells and the dispersed islet cells to yield fusion cells, the fusion cells having a beta cell function that includes glucose-responsive insulin release, and a function of the tissue-derived mesenchymal cells that includes a proliferation capacity.

[0012] Further, fusion cells according to a second exemplary aspect of the present invention includes a beta cell function that includes glucose-responsive insulin release; and a function of tissue-derived mesenchymal cells that includes a proliferation capacity, wherein the fusion cells are yielded by a fusion of dispersed islet cells and the tissue-derived mesenchymal cells.

Advantageous Effects of Invention

[0013] According to an exemplary embodiment of the present invention, it is possible to improve the success rate of cell fusion by fusing the dispersed islet cells and the tissue-derived mesenchymal cells

Brief Description of Drawings

[0014] [fig.1A]Fig. 1A shows Giemsa staining in electrofusion cells (Scale bar: 20 micrometers).

[fig.1B]Fig. 1B shows Giemsa staining in non-fused cells (scale bar: 20 micrometers).

[fig.2A]Fig. 2A shows a fluorescence staining in electrofusion cells (light microscopic view).

[fig.2B]Fig. 2B shows a fluorescence staining in electrofusion cells. Islet-cells were stained by Vybrant-Dil (red).

[fig.2C]Fig. 2C shows a fluorescence staining in electrofusion cells. MSCs were stained by SYTO11 (green).

[fig.2D]Fig. 2D shows a fluorescence staining in electrofusion cells (merge).

[fig.2E]Fig. 2E shows a fluorescence staining in non-fused cells (light microscopic view).

[fig.2F]Fig. 2F shows a fluorescence staining in non-fused cells. Islet-cells were stained by Vybrant-Dil (red).

[fig.2G]Fig. 2G shows a fluorescence staining in non-fused cells. MSCs were stained by SYTO11 (green).

[fig.2H]Fig. 2H shows a fluorescence staining in non-fused cells (merge).

[fig.3A]Fig. 3A shows in vitro insulin secretion after a 1-day culture (mean plus or minus SEM: n=3, *:$p<0.05$).

[fig.3B]Fig. 3B shows in vitro insulin secretion after a 10-day culture (mean plus or minus SEM: n=3, *:$p<0.05$).

[fig.3C]Fig. 3C shows in vitro insulin secretion after a 20-day culture (mean plus or

minus SEM: n=3, *:p<0.05).

[fig.3D]Fig. 3D shows microscopic morphology of MSCs in vitro insulin secretion after a 1-day culture.

[fig.3E]Fig. 3E shows microscopic morphology of islet in vitro insulin secretion after a 1-day culture.

[fig.3F]Fig. 3F shows microscopic morphology of MSCs and islet in vitro insulin secretion after a 1-day culture.

[fig.3G]Fig. 3G shows microscopic morphology of MSCs and dispersed islet cells in vitro insulin secretion after a 1-day culture.

[fig.3H]Fig. 3H shows microscopic morphology of fusion cells in vitro insulin secretion after a 1-day culture.

[fig.3I]Fig. 3I shows microscopic morphology of MSCs in vitro insulin secretion after a 10-day culture.

[fig.3J]Fig. 3J shows microscopic morphology of islet in vitro insulin secretion after a 10-day culture.

[fig.3K]Fig. 3K shows microscopic morphology of MSCs and islet in vitro insulin secretion after a 10-day culture.

[fig.3L]Fig. 3L shows microscopic morphology of MSCs and dispersed islet cells in vitro insulin secretion after a 10-day culture.

[fig.3M]Fig. 3M shows microscopic morphology of fusion cells in vitro insulin secretion after a 10-day culture.

[fig.3N]Fig. 3N shows microscopic morphology of MSCs in vitro insulin secretion after a 20-day culture.

[fig.3O]Fig. 3O shows microscopic morphology of islet in vitro insulin secretion after a 20-day culture.

[fig.3P]Fig. 3P shows microscopic morphology of MSCs and islet in vitro insulin secretion after a 20-day culture.

[fig.3Q]Fig. 3Q shows microscopic morphology of MSCs and dispersed islet cells in vitro insulin secretion after a 20-day culture.

[fig.3R]Fig. 3R shows microscopic morphology of fusion cells in vitro insulin secretion after a 20-day culture.

[fig.4A]Fig. 4A shows results of reverse transcription PCR carried out by primers designed for rat genes. F: Fusion cells between mouse MSCs and rat islet-cells, MM: mouse MSCs, M6: MIN-6, RM: rat MSCs, and RI: rat-islets.

[fig.4B]Fig. 4B shows results of reverse transcription PCR carried out by primers designed for mouse genes. F: Fusion cells between mouse MSCs and rat islet-cells, MM: mouse MSCs, M6: MIN-6, RM: rat MSCs, and RI: rat-islets.

[fig.5A]Fig. 5A shows results of blood glucose in in vivo transplantation study.

[fig.5B]Fig. 5B shows results of body weight in in vivo transplantation study.

[fig.6]Fig. 6 shows Gene expression of CD34, CD45, CD73 and CD105 in rat MSCs, rat bone marrow, mouse MSCs and mouse bone marrow.

[fig.7A]Fig. 7A shows results of reverse transcription PCR for reprogramming after 1-day culture. PCR was carried out by primers designed for rat genes. F: Fusion cells between mouse MSCs and rat islet cells, MM: mouse MSCs, M6: MIN-6, RM: rat MSCs, RI: rat islets and MM+RI: co-culture of mouse MSCs with rat islets (nonfused).

[fig.7B]Fig. 7B shows results of reverse transcription PCR for reprogramming after 1-day culture. PCR was carried out by primers designed for mouse genes. F: Fusion cells between mouse MSCs and rat islet cells, MM: mouse MSCs, M6: MIN-6, RM: rat MSCs, RI: rat islets and MM+RI: co-culture of mouse MSCs with rat islets (nonfused).

[fig.8A]Fig. 8A shows Caspase3 gene expression for each group by 28, 30, 32, 34 and 36 PCR cycles.

[fig.8B]Fig. 8B shows annexin V staining for each group. Upper panels show light microscopic view. Middle panels show annexin V staining (green: cell membrane), and lower panels show propidium iodide staining (red: nuclear). Scale bar: 20 micrometers.

[fig.9]Fig.9 shows Rat Ki-67 gene expression of each group by RT-PCR. MM: mouse MSCs, RM: rat MSCs, RI: rat islets, Mix: co-culture of mouse MSCs with rat islets (non-fused) and F: Fusion cells between mouse MSCs and rat islet cells.

Description of Embodiments

[0015] 1. Overview

On the basis of the above-mentioned knowledge, it was hypothesized that cell fusion between MSC and beta-cells may produce a novel type of cells that combines beta-cell function with MSC characteristics including a proliferation capacity and anti-apoptotic ability.

In this study, we have established a method of electrofusion between tissue-derived mesenchymal cells and pancreatic islet cells and examined an insulin secretion capacity and gene expression of fusion cells in vitro and verified the potential application of these cells to regenerative medicine for diabetes mellitus in vivo.

[0016] The term "tissue-derived mesenchymal cells" refers to mesenchymal cell populations sampled from a tissue and having a proliferation capacity. Herein, it has been confirmed that the mesenchymal cell populations satisfy some functions in the definition of MSCs given in reference 39. However, since it has not been confirmed that the mesenchymal cell populations satisfy all the functions in this definition of MSCs, we cannot conclude that the mesenchymal cell populations are mesenchymal stem cells (MSCs). Therefore, the mesenchymal cell populations are represented as

tissue-derived mesenchymal cells. The mesenchymal cell populations are hereinafter referred to as "MSCs". However, the term "MSCs" herein described refers to tissue-derived mesenchymal cells. In other words, the terms "tissue-derived mesenchymal cells" and "mesenchymal stem cells (MSCs)" herein used are not distinguished from each other.

[0017] The term "dispersed islet cells" are cells obtained by dispersing isolated islets which are isolated from pancreas, and are composed of various cells including beta-cells.

The term "isolated islet " is a cluster of cells including various cells including beta-cells. Isolated islets are clusters of cells obtained by isolating islets from pancreas, and are distinguished from islet cells existing in a living organism. Here, we call the component cells in the isolated islets as islet cells. In other words, isolated islets (islet cells) exist in an in-vitro environment, whereas islets exist in a living organism. In this manner, isolated islets and islets are distinguished from each other depending on the environment in which they exist.

[0018] A cell fusion method according to an embodiment of the present invention includes the steps of: preparing tissue-derived mesenchymal cells and dispersed islet cells; and fusing the prepared tissue-derived mesenchymal cells and dispersed islet cells by electrofusion. Then, as a result of the fusing the two types of cells by electrofusion, a fusion cell having a beta-cell function that includes glucose-responsive insulin release and a function of the tissue-derived mesenchymal cells that includes a proliferation capacity is produced.

[0019] As described above, the tissue-derived mesenchymal cells and dispersed islet cells are fused by electrofusion.

In general, when dispersed islet cells are left for a long period of time (for example, for a day), after the preparation thereof, the cells aggregate. For this reason, it is preferable to process the dispersed islet cells within a short period of time of the preparation thereof. For example, the electrofusion is preferably carried out within 60 minutes after the preparation of dispersed islet cells. More specifically, it is preferable to treat the dispersed islet cells within 60 minutes, and more preferably, within 20 minutes, after the preparation thereof.

Further, the tissue-derived mesenchymal cells are preferably cells derived from one of bone marrows, adipose tissues, dental pulps, dermal cells, placentas, endometrial membranes, umbilical cord blood, pancreases, and livers.

[0020] The electrofusion is carried out by performing electrification for cell fusion on a suspension of the tissue-derived mesenchymal cells and dispersed islet cells. Accordingly, cell fusion randomly occurs with an arbitrary combination of cells. Such cell fusion enables production of a desired fusion cell.

[0021] A fusion cell produced by the cell fusion method according to an embodiment of the

present invention includes at least a beta-cell function that includes glucose-responsive insulin release, and a function of the tissue-derived mesenchymal cells that includes a proliferation capacity.

According to the present invention, cell fusion is carried out using the dispersed islet cells having the beta-cell function, thereby facilitating the preparation of the cells, as compared with the case of using beta-cells.

[0022] Materials and Methods:

Animals

Rats and mice were purchased from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). These animals were housed in climate-controlled rooms with free access to pellet food and water. The approval to conduct this experiment was obtained from the Animal Care Committee of the Institute for Frontier Medical Sciences, Kyoto University, and the animals were treated according to the experimental protocols under its regulations.

[0023] Reverse Transcription Polymerase Chain Reaction (RTPCR)

In the present study, total RNA was extracted using PureLink RNA Mini kit (Invitrogen). RT-PCR was carried out using a SuperScriptIII First-Strand cDNA synthesis (Invitrogen) and a thermal cycler, iCycler (Bio-Rad). The primers used for PCR are shown in the tables designated for each experiment. Unless otherwise noted, PCR was performed for 35 cycles with each cycle comprising 20 sec at 94 degrees Celsius, 30 sec at 65 degrees Celsius, and 1 min at 72 degrees Celsius. A final cycle comprised 5 min at 72 degrees Celsius. After PCR, electrophoresis was performed by E-Gel iBase Power System and E-Gel 4% Agarose (Invitrogen).

[0024] Preparation of MSCs

The bone marrow was isolated from the tibias and femurs of Lewis rats (male, 5 weeks old) or C57/BL6 mice (male, 4 weeks old). After washing 3 times with Hank's balanced salt solution and centrifugation (1,000 rpm for 5 min at 4 degrees Celsius), the cells were plated to a 225cm2 flask in a 1:1 mixture of Dulbecco's modified Eagle's Medium and F12 (DMEM/F12; Gibco, NY, USA) with 12.5% fetal bovine serum (FBS) and 1% antibiotics solution (mixture of 100 units/mL penicillin G sodium, 100 micrograms/mL streptomycin sulfate and 25 micrograms/mL amphotericinB; Gibco) at 37 degrees Celsius in a humidified atmosphere of 5% CO2 and 95% air. Non-adherent cells were removed after a 3-day culture. Adherent cells were detached with 0.5% Trypsin-EDTA (Gibco) when the cells become confluent after a 4- to 5-day culture. MSCs of passages 5-10 were used in the following experiments.

To characteraize MSCs, the cells were examined by RT-PCR about four genes, i.e., CD34 and CD45 as negative markers and CD73 and CD105 as positive markers. Bone marrow was served as the control for MSCs. Primers are shown in Table 1. Table 1:

Primer sequence for RT-PCR.

[0025] [Table 1]

Animal	Gene		
Rat	CD34	Forward	AGCCATGTGCTCACACATCA
		Reverse	CAAACACTCGGGCCTAACCT
	CD45	Forward	TTGCTCCCCATCCGATAAGAC
		Reverse	AGCGTGGATGAAAAACCATCG
	CD73	Forward	TGCATCGATATGGCCAGTCC
		Reverse	AATCCATCCCCACCGTTGAC
	CD105	Forward	ACTGAGTTGCACATCTGGGG
		Reverse	TTCCGAAGTGGTGGTAAGCC
Mouse	CD34	Forward	CAGGAGAAAGGCTGGGTGAA
		Reverse	GTTGTCTTGCTGAATGGCCG
	CD45	Forward	ACTGAATCCACACCCCCAAG
		Reverse	AGCTTGGCTGCTGAATGTCT
	CD73	Forward	AGTTCTCTCTGTTGGCGGTG
		Reverse	GGATGCCACCTCCGTTTACA
	CD105	Forward	TGTACCCACAAGTCTCGCAG
		Reverse	ATGCTTTGGGGGTCATCCAG

[0026] Islet isolation

Islets were isolated from male Lewis rats (11 weeks old, 280-300g) as described previously (29). Briefly, rat pancreata were digested by collagenase (typeXI, Sigma, St. Louis, USA) and then the islets were separated by a dextran gradient. The islets were further purified by handpicking and then were cultured in a CMRL-1066 medium (Gibco) with 10% FBS and antibiotics at 37 degrees Celsius in a humidified atmosphere of 5% CO2 and 95% air. After a 24-hour culture, the islets were treated with 0.5% Trypsin-EDTA for 10 min at 37 degrees Celsius in order to prepare dispersed islet cells. Then, dispersed cells were collected by centrifugation (1200rpm, 5 min at 4 degrees Celsius) and temporarily kept in the same medium at 37 degrees Celsius until used for an experiment within 20 min.

[0027] Cell Fusion

MSCs and dispersed islet cells were washed once in fusion medium containing 5% glucose, 0.1 mM CaCH3(COO)2, 0.5 mM MgCH3(COO)2 and 0.3% bovine serum albumin (BSA). The pH of the fusion medium was adjusted to 7.2-7.4 with L -histidine (all chemicals were from Sigma). After centrifugation, the cells were re-suspended in the fusion medium without BSA. MSCs (1x106) and dispersed islet cells (1x106) were suspended in 100 microliters of fusion medium and placed in a specially designed fusion chamber made of two concentric oval electrodes (6cm x 16cm, Cat. No. CUY480G2, NEPA GENE Co., Ltd. Chiba, JAPAN). For electrofusion, a pulse generator (ECM 2001, BTX Instrument, Genetronics, CA, USA) was used. Electrofusion involved two independent but consecutive steps. The first treatment is to bring cells in close contact by dielectrophoresis, which can be accomplished by exposing cells to an alternating electric field (AC) of relatively low voltage. Then, cell fusion was triggered by applying a single squarewave pulse (DC) to induce reversible cell membrane break-down in the zone of membrane contact. For this study, electrofusion was perfomed by AC of 35 V for 20 sec followed by DC of 350 V for 25 microseconds based on our past study (30).

[0028] Validation of cell fusion

To determine fusion efficiency, cell mixtures before cell fusion and fusion cells were stained by Giemsa staining after a 24-hour culture. In order to confirm cell fusion between MSCs and islet-cells, MSCs and dispersed islet cells were pre-labeled with the SYTO11 (Invitrogen, CA, USA) and Vybrant Dil (Santa Cruz, CA, USA), respectively, following manufacturer's instructions. Cells before and after cell fusion were examined under a fluorescence microscope (Olympus IX70, Tokyo, Japan) after an overnight culture.

[0029] Insulin secretion test

In vitro glucose challenge test was performed in the prepared cells as follows after 1-, 10- and 20-day culture: (1) MSCs only (2x104 cells per well), (2) Islets only (20 Islets), (3) Non-fused MSCs (2x104 cells) with islets (20 islets), (4) Non-fused MSCs (2x104 cells) with dispersed islet cells prepared from 20 islets, (5) Fusion cells of MSCs (2x104 cells) and dispersed islet cells prepared from 20 islets. For the glucose challenge test, all groups were pre-incubated in RPMI-1640 with 0.1% BSA containing 3.3 mM glucose at 37 degrees Celsius for 1 hour. After pre-incubation, the medium was replaced with the same medium for 1 hour. Then, the medium was replaced with RPMI-1640 with 0.1% BSA containing 16.7 mM glucose for 1 hour. Finally, the medium was replaced with RPMI-1640 with 0.1% BSA containing 3.3 mM glucose for 1 hour. Insulin concentration of the media was measured using a rat insulin ELISA kit (Shibayagi, Gunma, Japan).

[0030] RT-PCR (Nuclear Reprogramming)

In order to investigate whether nuclear reprogramming occurs in MSCs and/or islet cells, mouse MSCs and rat islet cells were fused and, after an overnight culture, expressions of typical MSC genes (Oct3/4, CD106, and Sca1) and islet genes (Insulin-1, Pdx-1 and Ngn3) were examined by a reverse transcription polymerase chain reaction (RT-PCR) using the primers designed for both rat and mouse genes.

Total RNA was extracted from mouse or rat MSCs, rat islets, MIN-6 cells (31) and the fusion cells using PureLink RNA Mini kit (Invitrogen). RT-PCR was carried out using a SuperScriptIII First-Strand cDNA synthesis (Invitrogen) and a thermal cycler, iCycler (Bio-Rad). The primers used for PCR are shown in Table 2 and 3. Table 2 is designed for rat genes. Table 3 is designed for mouse genes. All PCR were performed for 35 cycles with each cycle comprising 20 sec at 94 degrees Celsius, 30 sec at 65 degrees Celsius, and 1 min at 72 degrees Celsius. A final cycle comprised 5 min at 72 degrees Celsius. After PCR, electrophoresis was performed by E-Gel iBase Power System and E-Gel 4% Agarose (Invitrogen).

Total RNA was extracted from MSCs of mouse and rat, rat islets, MIN-6 cells (31) and the fusion cells. Co-culture of mouse MSCs with rat islets (MM+RI) was served as the control for fusion cells. The primers are shown in Tables 2 and 3.

[0031] [Table 2]

Inslin-1	Forward	GAGGACCCGCAAGTGCCACA
	Reverse	GGCGGGGAGTGGTGGACTCA
pdx1	Forward	TTGCAGGCTCGCTGGGAACG
	Reverse	AGCAGCTGGGCCCGAGTGTA
Ngn3	Forward	TGGGCCCCCGTTGCTGATTG
	Reverse	ACGCGGGACTAGAGTACGCCC
CD106	Forward	GGTGGCTGCACAGGTTGGGG
	Reverse	ACCCACAGGGCTCAGCGTCA
sca1	Forward	TCTTTGCAACGCAGCAGGGC
	Reverse	CACGTGCCTCCAGGGCCAAG
oct3/4	Forward	GGGGAGCCCACCTTCCCCAT
	Reverse	ACGGGGAGATCCCCAGCACC
β-actin	Forward	GCGAGTACAACCTTCTTGCAGCTC
	Reverse	TGGCATGAGGGAGCGCGTAA

[0032]

[Table 3]

Inslin-1	Forward	TGGAGCTGGGAGGAAGCCCC
	Reverse	ATTGCAAAGGGGTGGGGCGG
pdx1	Forward	CAAAGCGATCTGGGGTGGCGT
	Reverse	CGCTGAACTCTGGCACCGGG
Ngn3	Forward	TGCCCGCTACATGCAGGGTT
	Reverse	AGGAACCGTCCCTGCAACTCAC
CD106	Forward	CGTGGGGACTTGGCTGGCTG
	Reverse	AGCCGGGCTGGTGTGAGTGA
sca1	Forward	GCCCCTGCTGGGTAGGTAGGT
	Reverse	TGTGCTGGCTGTGTGCCTCC
oct3/4	Forward	CTGCCCCCAGGTCCCCACTT
	Reverse	AGCATCCCCAGGGAGGGCTG
β-actin	Forward	AGGCGGACTGTTACTGAGCTGC
	Reverse	CTCAGGGCATGGACGCGACC

[0033] Transplantation Experiment

In order to investigate whether fusion cells can really control hyperglycemia, the effect of fusion cell transplantation was observed in comparison to optimal (2,000 islet per rat) and suboptimal (1,000 islet per rat) islet mass transplantation. Lewis rats (male, 11 weeks old, 280-300 g) were made diabetic with streptozotocin (55 mg/kg body weight, i.p.) for recipients. One week after the injection, blood glucose (BG) was measured by tail vein sampling using Fuji Dry Chem system (Dri-chem 3000 colorimetric analyzer, Fujifilm, Tokyo, Japan) and rats with non-fasting BG higher than 500 mg/dL were used for recipients. Rats were divided into seven groups as follows: Group1; Normal control group (n=6), Group 2; sham operated DM group (n=6), Group 3; sub-optimal islet group (1000 islets: n=9), Group 4; optimal islet group (2000 islets: n=5), Group 5; MSCs group (1x106 cells: n=9), Group 6; Non-fused cell group (dispersed islet cells from 1000 islets and MSCs 1x106 cells: n=9), Group 7; Fusion cell group (fusion cells processed from dispersed cells of 1000 islets and MSCs 1x106 cells: n=9). Cells were transplanted into the left renal subcapsular space through the skin incision on the lumbar dorsum under general anesthesia of isoflurane inhalation. After transplantation, BG and body weight were measured on postoperative days (POD) 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, and 91.

[0034] Islet Cell Apoptosis

In order to investigate whether electrofusion of rat MSCs induce changes on rat islet cell apoptosis, rat caspase3 gene expression was examined by RT-PCR in five groups

described in insulin secretion test after 1-day culture. The primers used for PCR are shown in Table 4. PCR were performed for 28, 30, 32, 34 and 36 cycles with each cycle comprising 10 sec at 98 degrees Celsius, 30 sec at 65 degrees Celsius, and 1 min at 72 degrees Celsius. A final cycle comprised 5 min at 72 degrees Celsius. After PCR, electrophoresis was performed by E-Gel iBase Power System and E-Gel 4% Agarose (Invitrogen). Moreover, in order to detect apoptotic cells, all groups were stained with annexin V and propidium iodide (PI) using Annexin V-FITC (Beckman Coulter, Tokyo, Japan, IM2375) following manufacturer's instructions. Briefly, cell samples were suspend in accompanying buffer on ice. FITC-labeled annexin V solution and PI solution were added and the samples were kept on ice for 10 minutes in the dark. Then, the samples were observed under a fluorescence microscope (KEYENCE BZ-8000, Tokyo, Japan).

[0035] [Table 4]

Animal	Gene	
Rat	caspase3	Forward CTTTGCGCCATGCTGAAACT
		Reverse ATGACGACCTGGAACATCGG

[0036] Islet Cell Proliferation

In order to investigate whether islet cell proliferation occurs in fusion cells, fusion cells were made from mouse MSCs and rat islet cells. Expressions of rat Ki-67 gene was examined by RT-PCR using the primers shown in Table 5 after 1- and 5-day culture. Mouse MCSs, rat MSCs, rat islets and co-culture of mouse MSCs with rat islets were served as controls.

[0037] [Table 5]

Animal	Gene	
Rat	Ki-67	Forward CTTTGCGCCATGCTGAAACT
		Reverse ATGACGACCTGGAACATCGG

[0038] Statistical Analysis

Results are presented as mean plus or minus SE as indicated in the figure legends. Two-way ANOVA for repeated-measures in SPSS 14.0 for windows was used, and $p < 0.05$ was chosen as the level of significance.

[0039] Results:

Validation of cell fusion

Rat MSCs and dispersed rat islet-cells were successfully fused by our method. By

Giemsa staining, multinuclear cells were observed after cell fusion whereas such cells were not observed without cell fusion (Figs. 1A and 1B). By electrofusion of SYTO11-labeled MSCs and Vybrant Dil-labeled islet cells, cells double positive for SYTO11 and Vybrant Dil were observed after cell fusion, whereas such cells were not observed without cell fusion (Figs. 2A to 2H).

[0040] Insulin secretion test

In a comparison among the cultured cells of MSCs alone, islets alone, MSCs and islets without cell fusion, MSCs and dispersed islet cells without cell fusion and fusion cells of MSCs and dispersed islet cells, all groups except for MSCs alone showed glucose-responsive insulin release after a 1-day culture (Fig. 3A). After a 10-day culture, islets alone lost the glucose responsiveness but dispersed islet cells with MSCs and fusion cells maintained it (Fig. 3B). Although undispersed islets with MSCs seemed to maintain responsiveness, the insulin concentration was lower and the response was not statistically significant (Fig. 3B). After a 20-day culture, all groups except fusion cells showed loss of glucose-responsiveness (Fig. 3C). In dispersed islet cells with MSCs, although a significant increase was observed between the first 3.3 mM and 16.7 mM, decrease between 16.7 mM and the last 3.3 mM was not observed. Therefore, these cells were considered to have lost good glucose-responsiveness.

[0041] Figs. 3D to 3R shows microscopic morphology of the tested cells. Figs. 3D to 3H show the cells after a 1-day culture, Figs. 3I to 3M show those after a 10-day culture and Figs. 3N to 3R show cells after 20-day culture. In islets alone, most of the islets were destroyed after a 20-day culture (Fig. 3O). In the co-cultures of MSCs and islets, a few islets maintained their morphology and they were attached to colonies of MSCs (Fig. 3P). In a co-culture of MSCs and dispersed islet-cells, re-clustering of islet cells was observed although it is not known if such clusters contained MSCs (Fig. 3Q). In fusion cells, most cells were attached to the bottom of the culture dish (Figs. 3H, 3M and 3R).

[0042] RT-PCR (Nuclear Reprogramming)

MSC- and beta-cell-related gene expression of fusion cells between mouse MSCs and rat islets was examined. Primers designed for rat genes (Table 1) did not react with the cDNA derived from mouse MSCs and MIN-6, a mouse beta-cell line. Rat islet markers such as Insulin-1, Pdx-1 (Pancreatic duodenal homeobox 1), and Ngn3 (Neurogenin 3) were not expressed in rat MSCs and conversely, rat MSC markers such as Sca-1, CD106 and Oct3/4 were not expressed in rat islets. Fusion cells prepared from rat islets and mouse MSCs showed new expression of the rat MSC markers (Figs. 4A and 7A). Similarly, primers designed for mouse genes (Table 2) did not react with the cDNA derived from rat islets and MSCs. Mouse islet markers were not expressed in mouse MSCs. Mouse MSC markers except for Oct3/4, were not expressed in MIN-

6. Fusion cells showed new expression of the mouse islet markers (Figs. 4B and 7B). On the other hand, such changes in gene expression were not observed in a co-culture of mouse MSCs and rat islets (Figs. 7A and 7B). These results indicate that nuclei of both MSCs and islet cells were mutually reprogrammed by one another in fusion cells.

[0043] In vivo transplantation

Effect of renal subcapsular transplantation of fusion cells was examined in seven groups as follows: Group1; Normal control (n=6), Group 2; sham operated strep-tozotocin-induced DM rats (n=6), Group 3; sub-optimal islet mass (1000 rat islets: n=9), Group 4; optimal islet mass (2000 islets: n=5), Group 5; MSCs alone (1x106 cells: n=9), Group 6; Non-fused cell mix of dispersed islet cells from 1000 islets and MSCs 1x106 cells (n=9), Group 7; Fusion cell processed from dispersed cells from 1000 islets and MSCs 1x106 cells (n=9). Figs. 5A and 5B show changes in blood glucose (BG) and body weight, respectively. In group 4 (2000 islets), BG was decreased promptly and hyperglycemia was corrected to the normal range ever since POD 40. In the same group, body weight also showed a significant increase with a slope similar to the control group. There were no significant parametric changes either in BG or body weight in group 3 (1000 islets). Transplantation of MSCs alone in group 5 did not show any effect on BG or body weight in comparison to DM control of group 2. In group 6 (non-fused cell), BG did not decrease less than 400 mg/mL and body weight was slightly increased. Group 7 (fusion cell) showed a continuous decrease in BG during the observation period and a greater increase in body weight compared to group 6 was observed. A significant difference was detected between group 7 and group 6 both in BG and body weight as well as between group 7 and group 3 ($p<0.05$, Fig. 5A and 5B).

[0044] Characterization of MSCs

MSCs have been established using various methods by many researchers. In the present study, our rat and mouse bone marrowderived cells showed morphology similar to typical MSCs and expressed several MSC-specific markers such as CD73 and CD105 (Fig. 6) in addition to sca-1 and CD106 (Figs. 7A and 7B). On the other hand, CD34 and CD45 that were expressed in original bone marrow cells were not detected (Fig. 6). These results indicate that our bone marrow-derived cells were consistent with putative MSCs.

[0045] Islet Cell Apoptosis

Gene expression of caspase3 was detected in all groups except MSCs. PCR product was readily detected after 28 cycles in islet group and MSCs and dispersed islet cells group. On the other hand, in MSCs and islet group and fusion group, it was detected after 30 cycles. All groups reached plateau by 36 cycles (Fig. 8A). Therefore, caspase3 expression was reduced in fusion cells in comparison to that in co-culture of MSCs and

dispersed islet cells. Annexin and PI positive cells were scarcely detected in MSCs. However, in other groups (Islet, MSCs and islet, MSCs and dispersed islet cell, fusion cell), they were observed after a 1-day culture. A co-culture of MSCs with islets appeared to reduce annexin and PI positive cells in comparison to islets alone. Annexin and PI positive cells appeared to be reduced in fusion cells in comparison to co-culture of MSCs and dispersed islet cells (Fig. 8B).

[0046] Islet Cell Proliferation

On day 1, only rat MSCs expressed rat Ki-67. However, on day 5, fusion cells newly expressed rat Ki-67. Ki-67 expression was not detected in a co-culture of mouse MSCs and rat islet (Fig. 9). This result suggests that rat islet nuclei obtained a proliferative capability after cell fusion with mouse MSCs.

[0047] Discussion:

In this study, fusion cells between islet cells and MSCs were successfully prepared by electrofusion as shown in Figs. 1A, 1B and 2A to 2H. The fusion cells maintain sustained a beta-cell function in vitro and ameliorate hyperglycemia in a progressively-increasing manner, suggesting potential clinical use of these fusion cells for diabetes therapy. In the literature, Barrera-Escorcia E, et al. have reported electrofusion of donor islet cells with poorly characterized dermal cells of an allogeneic recipient in rats. They transplanted the fusion cells to examine the modulation of immunogenicity without in vitro characterization (32). Although a slight improvement was seen in several parameters including blood glucose levels, they failed to obtain the reversion of the diabetic profile with transplantation of their fusion cells.

[0048] Artificial cell fusion was first enabled through the discovery of the Sendai virus by Okada Y, et al. (33). Then, polyethylene glycol method (34) and electrofusion (35) were put to practical use. Among these methods, electrofusion appears to be the most efficient in order to prepare the large number of cells necessary for transplantation therapy. In addition, electrofusion is suitable for clinical use because it does not need special chemicals or biological materials that may affect the safety of the processed cells.

[0049] In the literature, Soleimani M et al. (36) reported a protocol for isolation and culture of MSCs from mouse bone marrow using a primary culture with frequent medium changes, suggesting that MSCs can be obtained without positive or negative selection. Many other researchers use similar protocols without cellular selection to obtain MSCs. In the present study, tissue-derived mesenchymal cells (bone marrow derived cells) show morphology similar to that of typical MSCs and expressed several MSC-specific markers, i.e., CD73 (also known as ecto-59-nucleotidase, differentiation marker of lymphocytes), CD105 (also known as Endoglin, a component of TGF-beta receptor), sca-1 (also known as Ly-6A, expressed in hematopoietic stem cells, skeletal

muscle cells, epithelial stem cells, lymphocytes and macrophages) and CD106 (also known as VCAM-1, expression is strongly downregulated in MSCs after differentiation into adipo-, osteo-, and chondrocytes) (37-38). Expression of these markers is known to be involved in the minimal criteria for defining multipotent MSCs (39). On the other hand, the negative markers, CD34 and CD45, that are well known as hematopoietic stem cells markers, were positive in original bone marrow cells but were not detected in MSCs. Lack of expression of these markers is also involved in the minimal criteria (39). From these results, we consider that our tissue-derived mesenchymal cells (our bone marrow-derived cells) used in the present study were consistent with putative MSCs.

[0050] In the present in vitro study, a co-culture of islet cells with MSCs enhanced the sustainability of a beta-cell function to a certain extent and cell fusion of islet cells and MSCs could further enhance it. The protective effect of MSCs on islets was previously reported (22) and our co-culture study confirmed it. Additionally, the present study further showed that cell fusion of islet cells with MSCs dramatically enhances the sustainability of the beta-cell function in vitro.

[0051] The present RT-PCR examination clearly showed that nuclei of both islet cells and MSCs were mutually reprogrammed by electrofusion. Therefore, these fusion cells can be considered to be special cells with a beta-cell function and robustness of MSCs. In fact, we observed sustained insulin secretion during the experimental period in vitro and this seems to be caused by the MSCs' antiapoptotic nature. On the other hand, although the protective effect of MSCs on co-cultured islets is often attributed to signaling molecules and cytokines released from MSCs (22), the co-culture of MSCs with islet cells did not cause reprogramming of islet cell nuclei (Figs. 7A and 7B).

[0052] Palermo A. et al. (40) have reported nuclear reprogramming in fusion cells of human keratinocytes and mouse muscle cells and found that extensive changes were observed within 4 days. They also reported that, depending on the ratio of the cell number of two cell types, either phenotype could be dominant. In the present study, we examined only one ratio of 1,000 islets and 1x106 MSCs, that can be estimated approximately 1:1 cell ratio at only one time point, after an over-night culture. Apparently, the influence of a cell ratio and time point awaits further investigation.

[0053] As to the anti-apoptotic nature of MSCs, caspase3 gene expression was decreased in the co-culture of MSCs with islets in comparison to islets alone (Fig. 8A), suggesting an anti-apoptotic effect of MSCs on islets. MSCs, however, did not show the effect when co-cultured with dispersed islet cells in which a apoptotic tendency is thought to be increased (Fig. 8A). Finally, caspase3 gene expression was decreased in fusion cells between MSCs and dispersed islet cells (Fig. 8A). These results indicate that electrofusion of MSCs can inhibit apoptotsis of islet cell nuclei even after they are

dispersed.

[0054] In the present in vivo transplantation study, a sub-optimal number of islet cells fused with MSCs gradually normalized the blood glucose levels in weeks. This suggests that the beta-cell function of transplanted fusion cells was gradually enhanced. Some other studies have reported that co-transplantation of islets with MSCs could facilitate engraftment of the islets (21). However, in the present study, we have failed to show this in the group 6 of non-fused islet cells and MSCs co-transplantation, probably because islet mass of 1,000 islets was too small to show the effect even transplanted with MSCs. On the other hand, fusion cells between islet cells and MSCs showed significant effect of transplantation in comparison to the group 3 (sub-optimal islet number) and even to the group 6 (co-transplantation with MSCs). These results indicate that cell fusion with MSCs provides a more potent facilitative effect on islet transplantation than co-transplantation of MSCs.

[0055] The mechanism of this observation remains to be elucidated. But, from the present in vitro study, one possibility is the sustainability of the beta-cell function that overcomes glucose toxicity caused by hyperglycemia. Another possibility is proliferation of the fusion cells that exert a beta-cell function. Although it is not quantitatively analyzed in the present study, proliferation of fusion cells is suggested in the in vitro experiments as shown in Figs. 3H, 3M and 3R. Further studies of proliferation capacity and details of insulin secretion in fusion cells are also ongoing.

[0056] Furthermore, new expression of rat Ki-67 in fusion cells suggests that islet cell nuclei obtain proliferation capability at least 5 days after cell fusion (Fig. 9). Therefore, fusion cells between islet cells and MSCs seem durable and proliferative to exert increasing beta-cell function after transplantation. Possible relationship between carcinogenesis and interaction or cell fusion of bone marrow-derived cells is suggested (41). Although we did not find any tumor formation at the transplantation site of fusion cells at POD 91, tumorigenicity of fusion cells should be carefully addressed in future studies.

[0057] In conclusion, this study showed that electrofusion of islet-cells with MSCs is an efficient method to obtain potent and robust insulin-secreting cells that can potentially be applied to the transplantation thereby for diabetes mellitus.

[0058] As described above, we establish a method of electrofusion between the dispersed islet cells and the tissue-derived mesenchymal cells in rats. The fusion cells maintained glucose-responsive insulin release for 20 days in vitro. Renal subcapsular transplantation of fusion cells prepared from a suboptimal islet mass (1,000 islets) that did not correct hyperglycemia even if co-transplanted with the tissue-derived mesenchymal cells, caused slow but consistent lowering of blood glucose with significant weight gain within the observation period in streptozotocin-induced diabetic rats. In

the fusion cells between rat islet cells and mouse the tissue-derived mesenchymal cells, RT-PCR showed new expression of both rat MSC-related genes and mouse beta-cell-related genes, indicating bidirectional reprogramming of both beta-cell and tissue-derived mesenchymal cells nuclei. These results show that electrofusion between the tissue-derived mesenchymal cells and the islet cells yield special cells with beta-cell function and robustness of tissue-derived mesenchymal cells and seems feasible for novel therapeutic strategy for diabetes mellitus.

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[0060] This application is based upon and claims the benefit of priority from U.S. pro-

visional application for patent No. 61/734696, filed on December 7, 2012, the disclosure of which is incorporated herein in its entirety by reference.

Claims

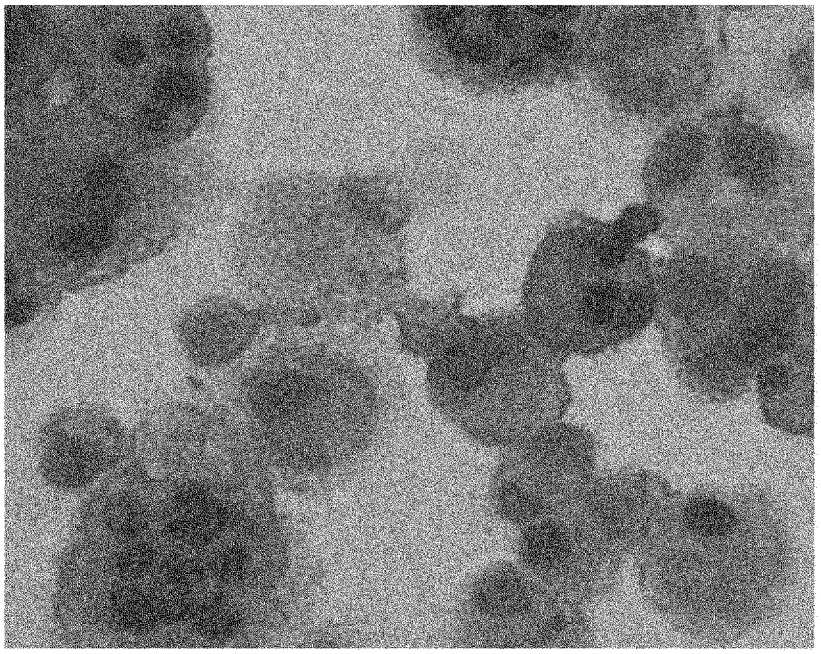
[Claim 1] A method of cell fusion comprising:
preparing tissue-derived mesenchymal cells;
preparing dispersed islet cells prepared from isolated islets; and
performing electrofusion of the prepared tissue-derived mesenchymal cells and the dispersed islet cells to yield fusion cells, the fusion cells having a beta-cell function that includes glucose-responsive insulin release, and a function of the tissue-derived mesenchymal cells that includes a proliferation capacity.

[Claim 2] The method according to Claim 1, wherein the performing electrofusion treats the dispersed islet cells within 60 minutes after the preparing of the dispersed islet cells.

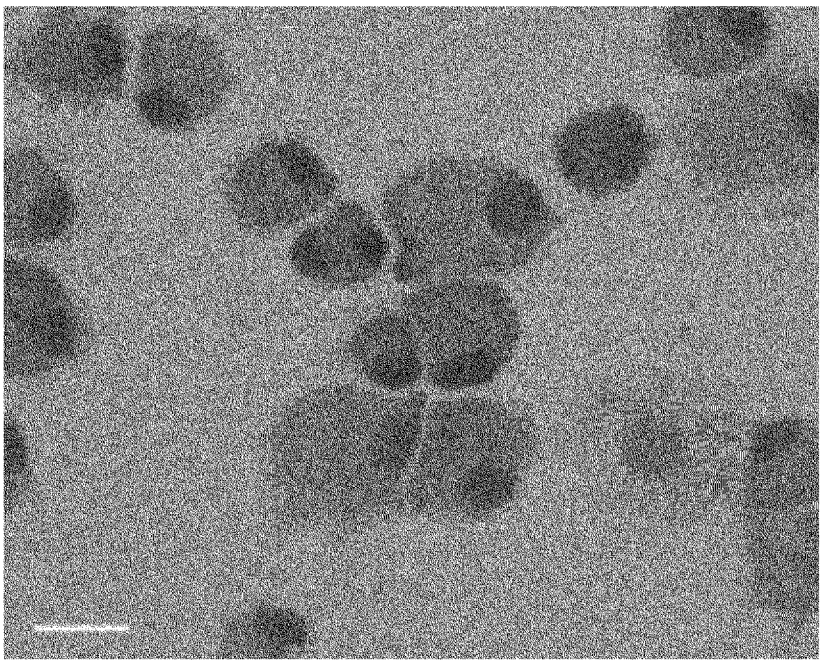
[Claim 3] A method according to Claim 1 or 2, wherein the tissue-derived mesenchymal cells are derived from one of bone marrows, adipose tissues, dental pulps, dermal cells, placentas, endometrial membranes, umbilical cord blood, pancreases, and livers.

[Claim 4] Fusion cells comprising:
a beta-cell function that includes glucose-responsive insulin release; and
a function of tissue-derived mesenchymal cells that includes a proliferation capacity,
wherein the fusion cells are yielded by a fusion of dispersed islet cells and the tissue-derived mesenchymal cells.

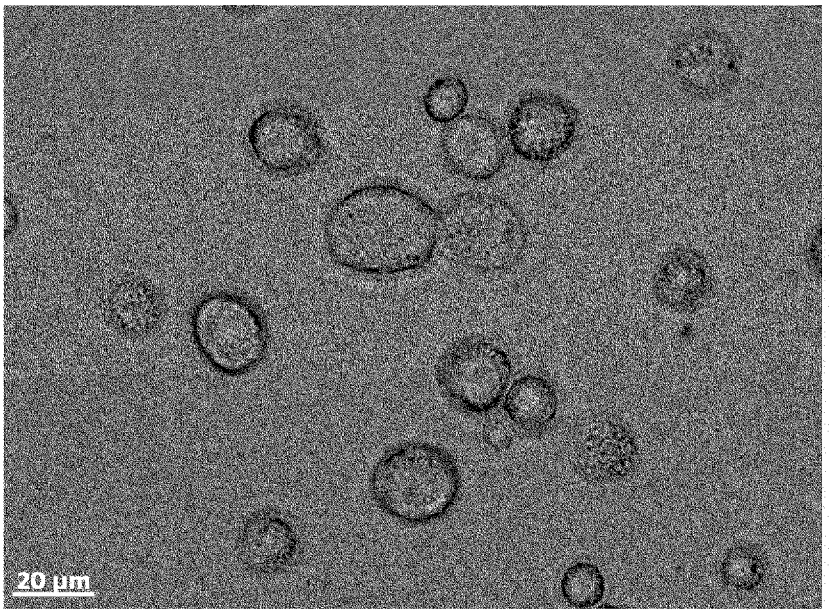
[Fig. 1A]



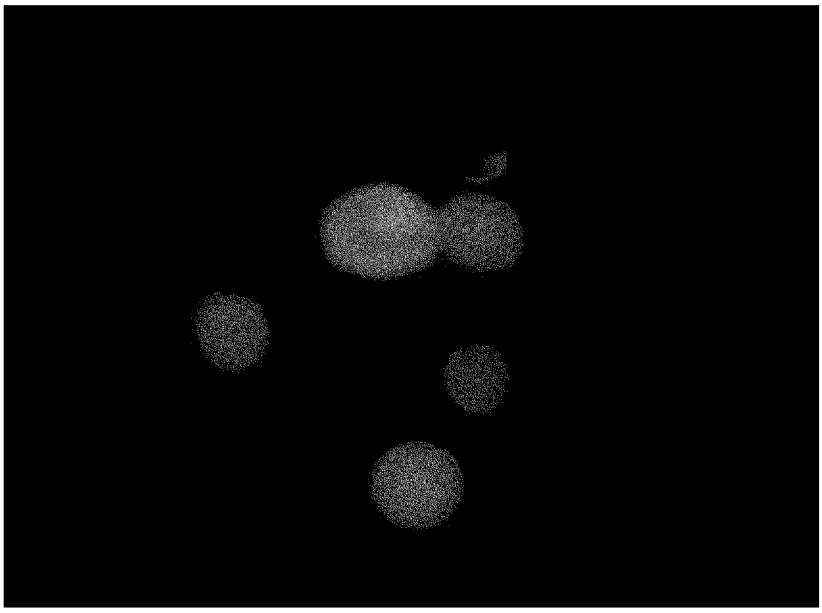
[Fig. 1B]



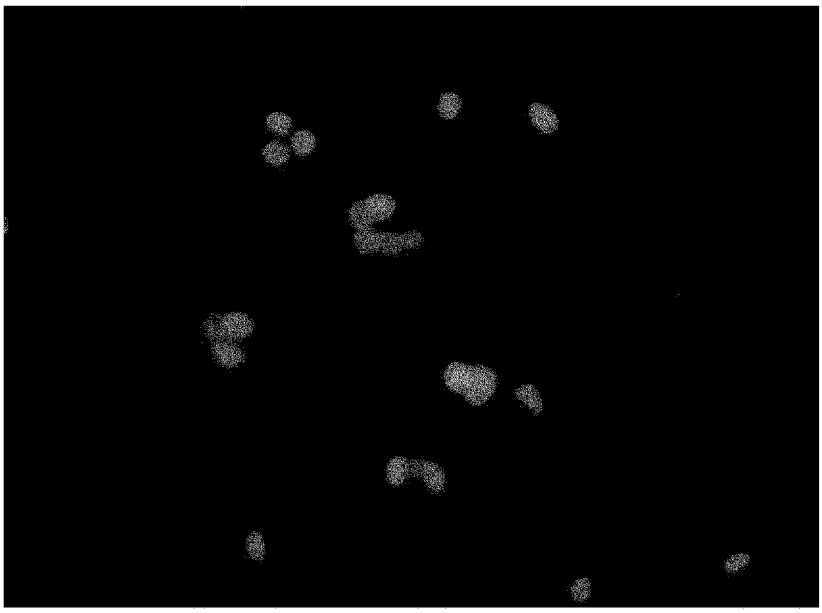
[Fig. 2A]



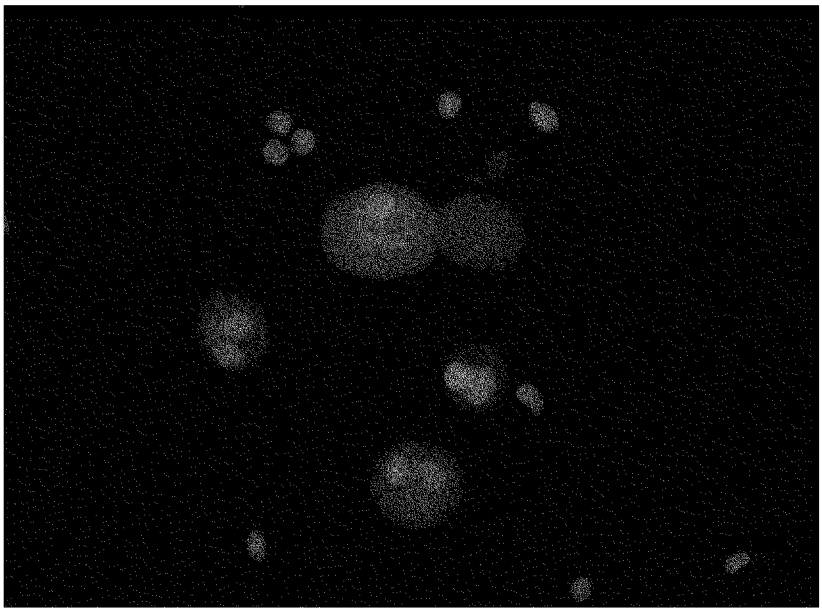
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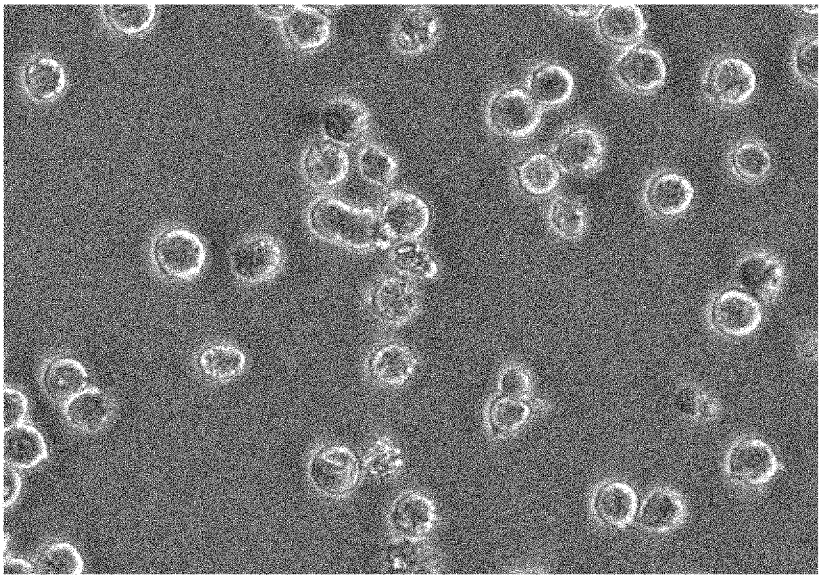
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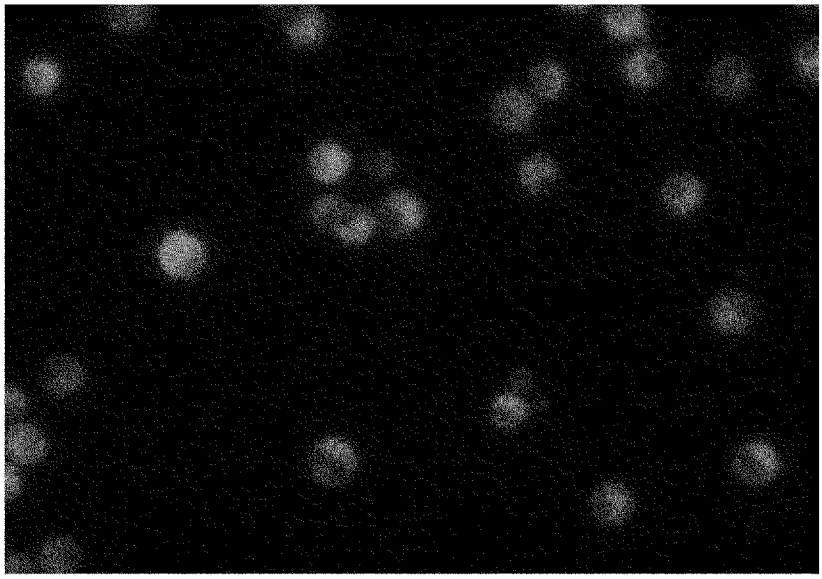
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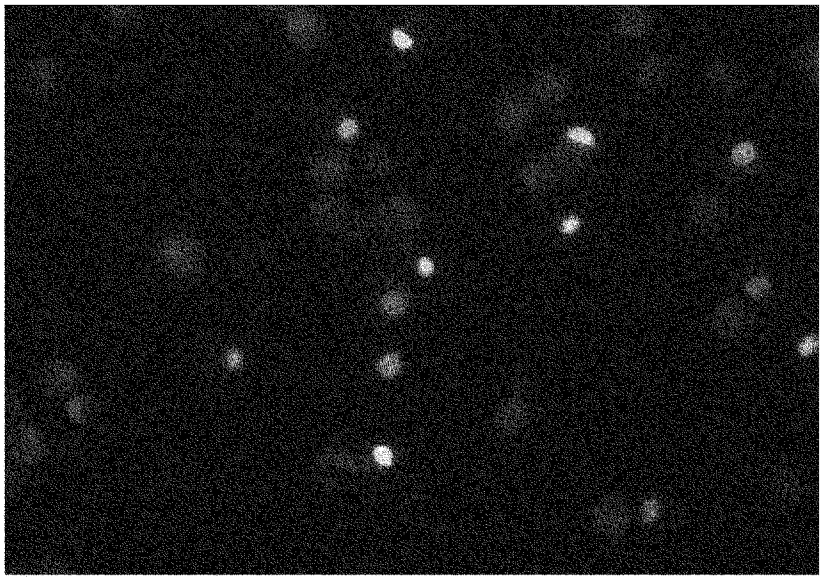
[Fig. 2E]



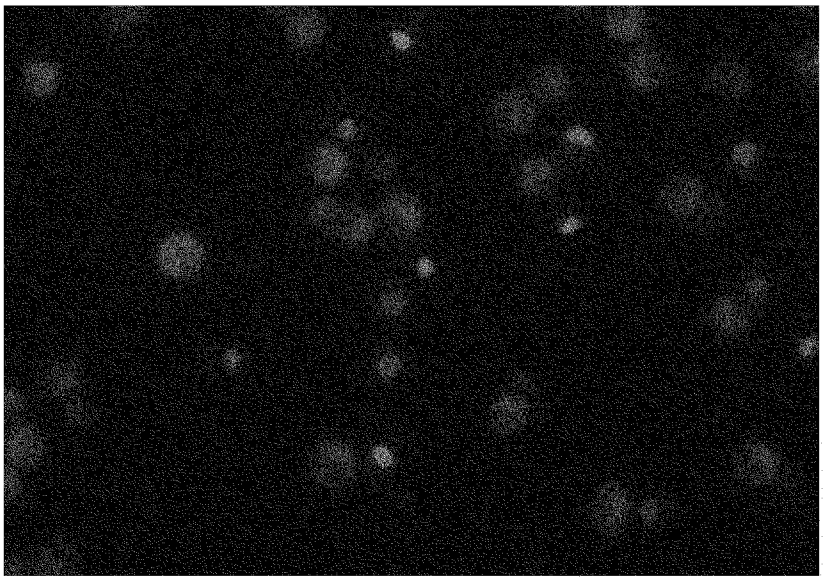
[Fig. 2F]



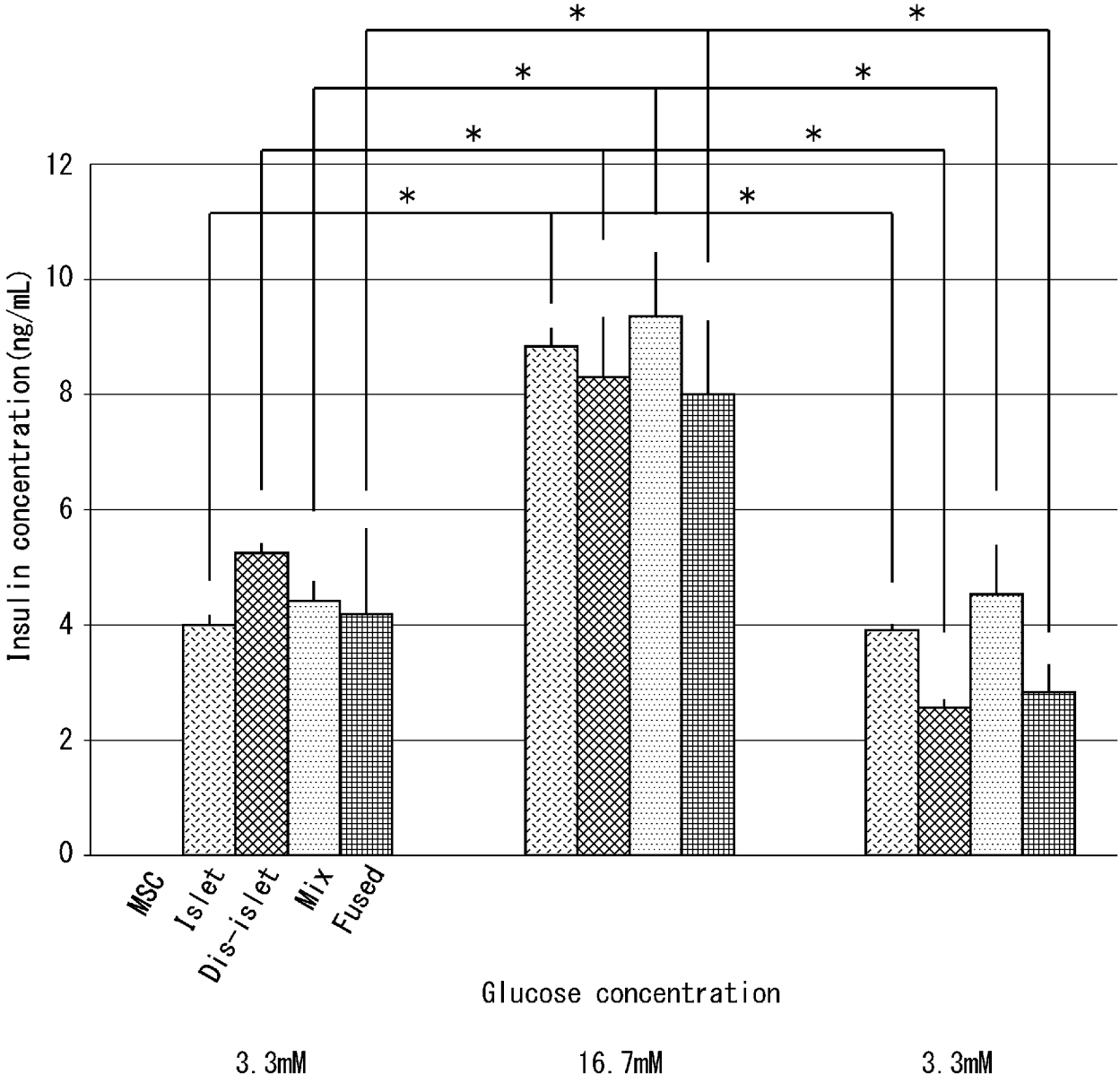
[Fig. 2G]



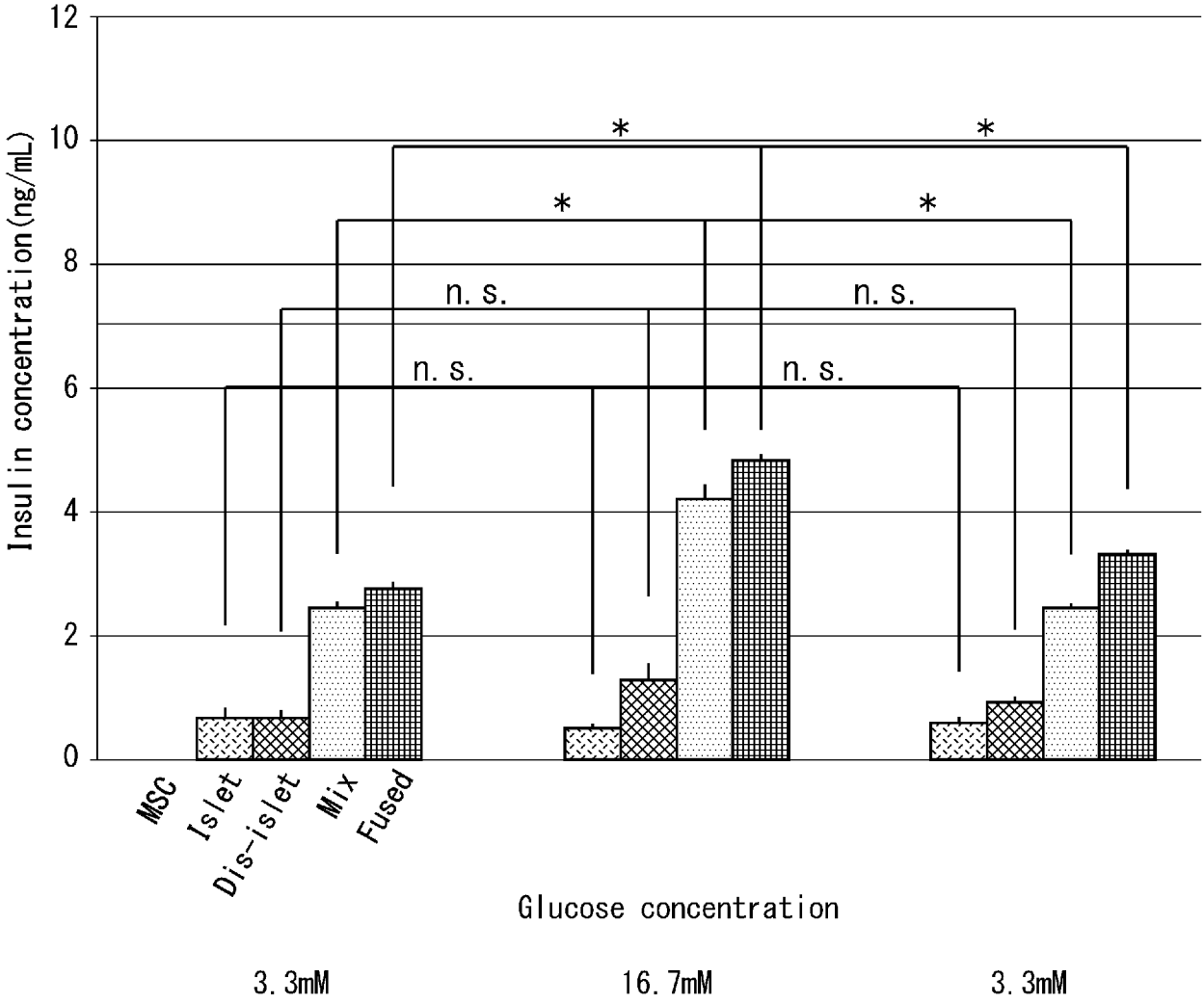
[Fig. 2H]



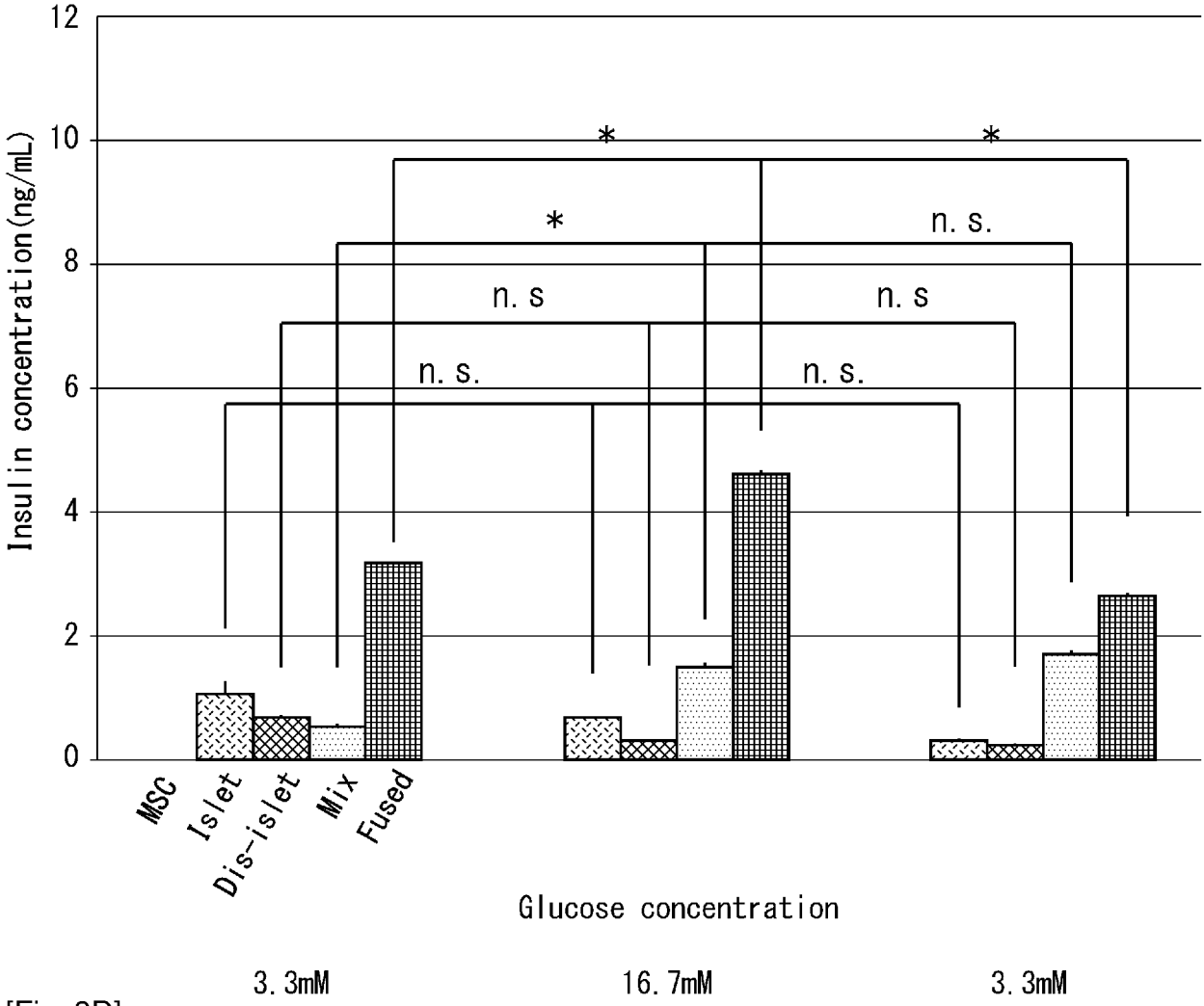
[Fig. 3A]


Insulin concentration(ng/mL)
0
2
4
6
8
10
12
*
MSC
Islet
Dis-islet
Mix
Fused
Glucose concentration
3.3mM
16.7mM
3.3mM

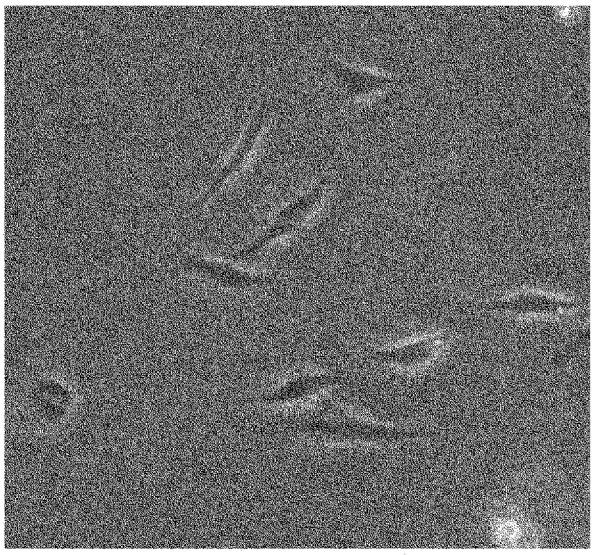
[Fig. 3B]


12
10
8
6
4
2
0
Insulin concentration(ng/mL)
*
*
*
*
n. s.
n. s.
n. s.
n. s.
MSC
Islet
Dis-islet
Mix
Fused
Glucose concentration
3.3mM
16.7mM
3.3mM

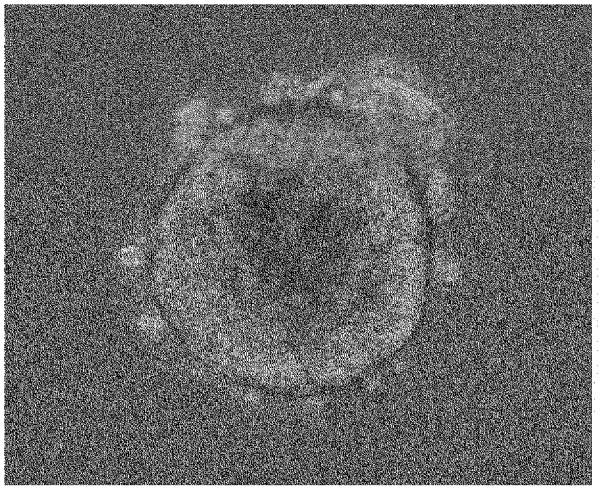
[Fig. 3C]


12
10
8
6
4
2
0
Insulin concentration(ng/mL)
*
*
*
n. s.
n. s
n. s
n. s.
n. s.
MSC
Islet
Dis-islet
Mix
Fused
Glucose concentration
3.3mM
16.7mM
3.3mM

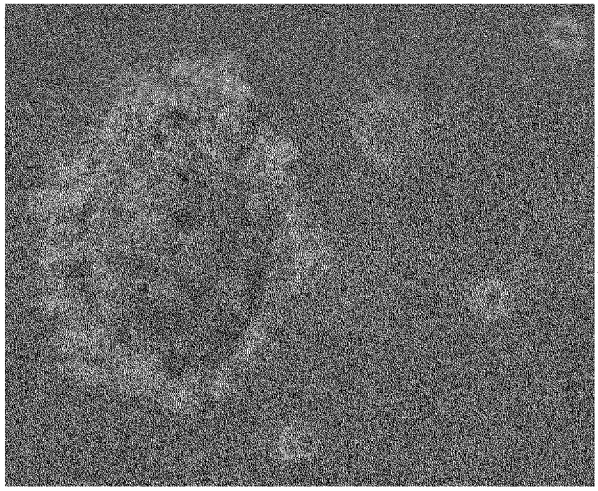
[Fig. 3D]



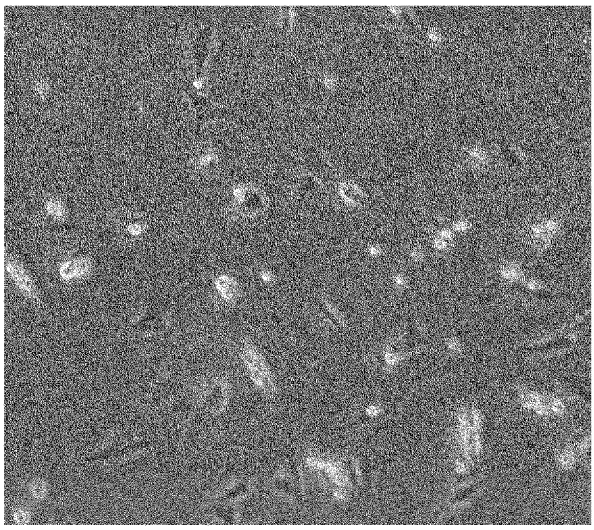
[Fig. 3E]



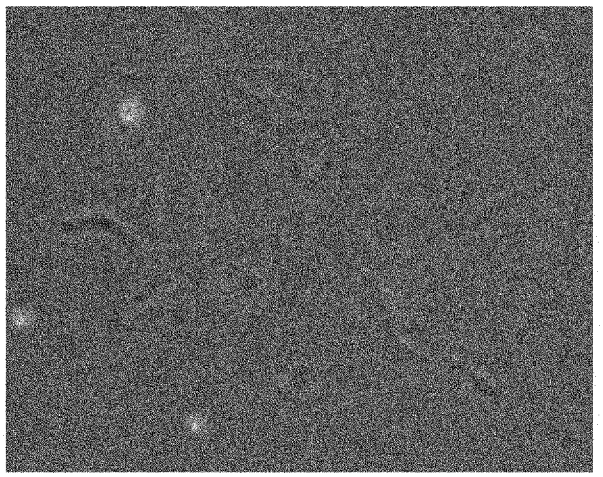
[Fig. 3F]



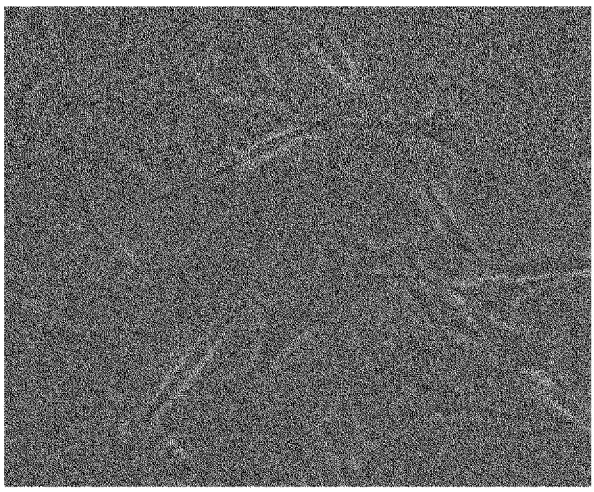
[Fig. 3G]



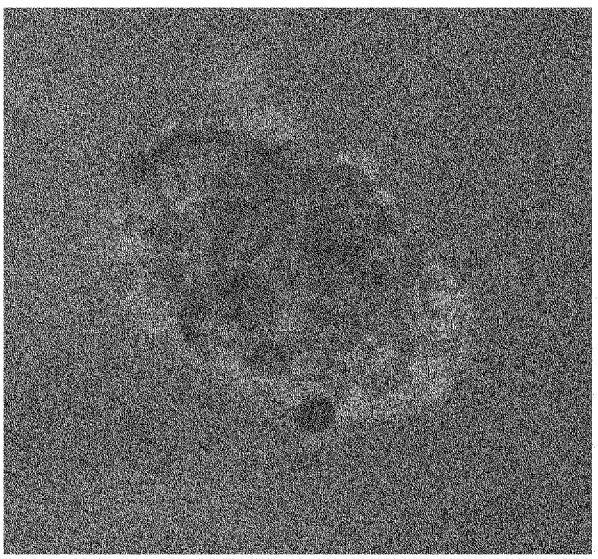
[Fig. 3H]



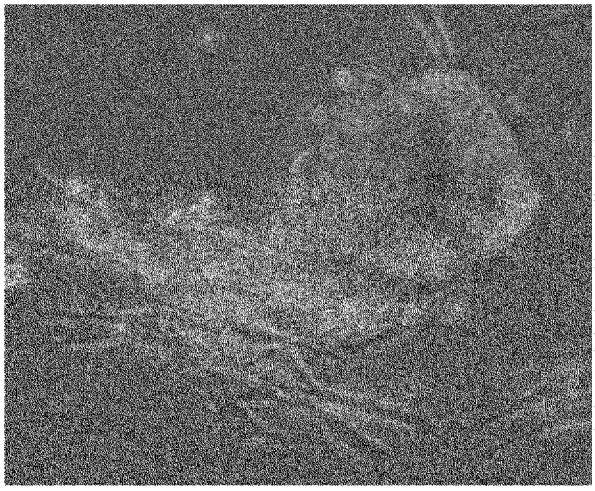
[Fig. 3I]



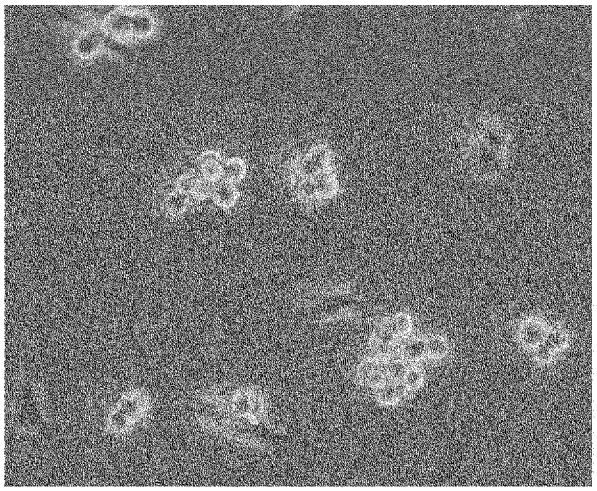
[Fig. 3J]



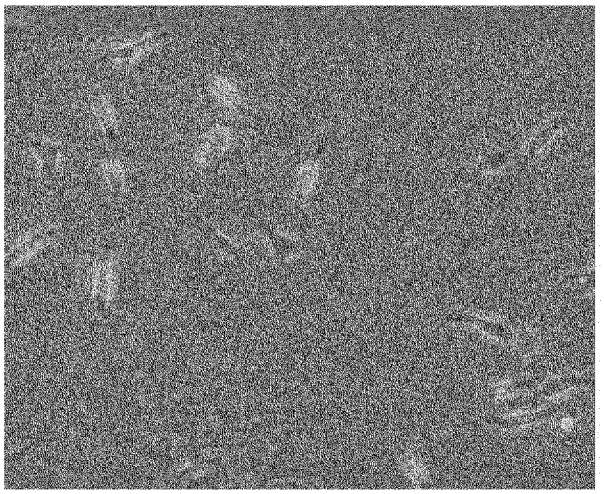
[Fig. 3K]



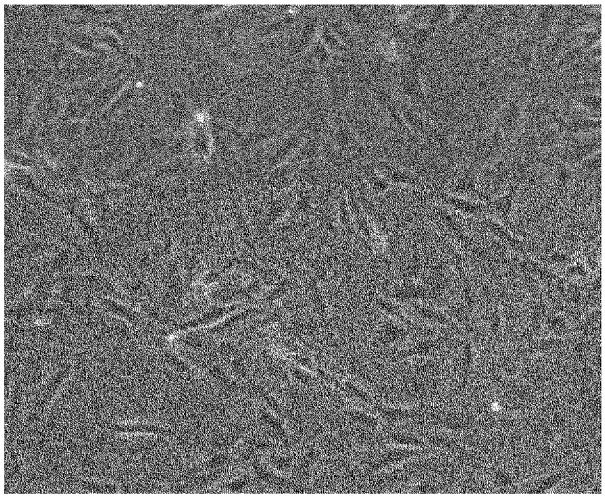
[Fig. 3L]



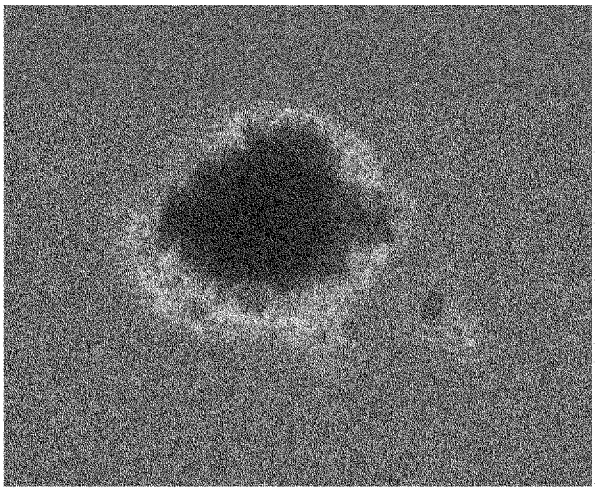
[Fig. 3M]



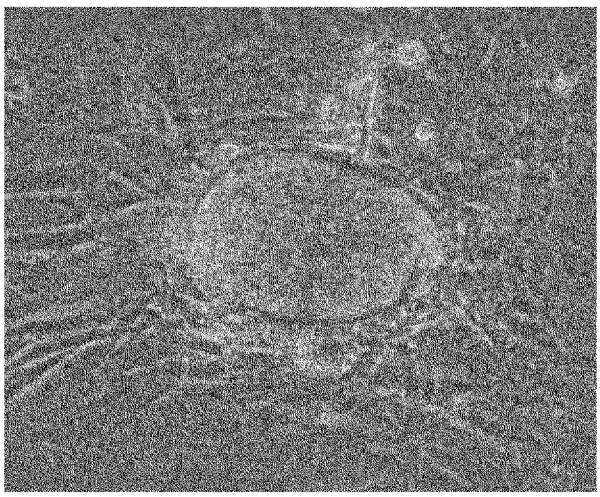
[Fig. 3N]



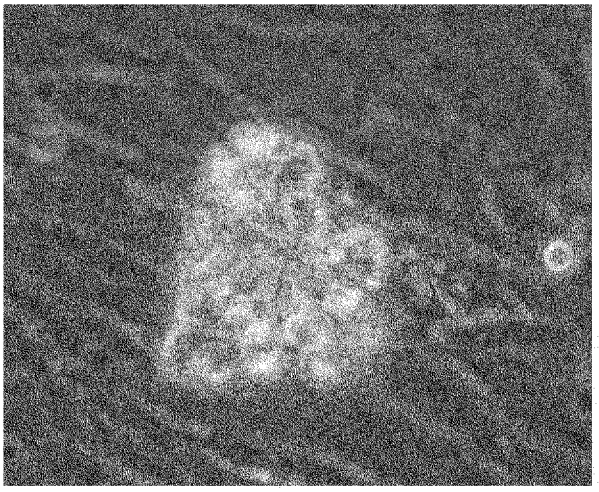
[Fig. 3O]



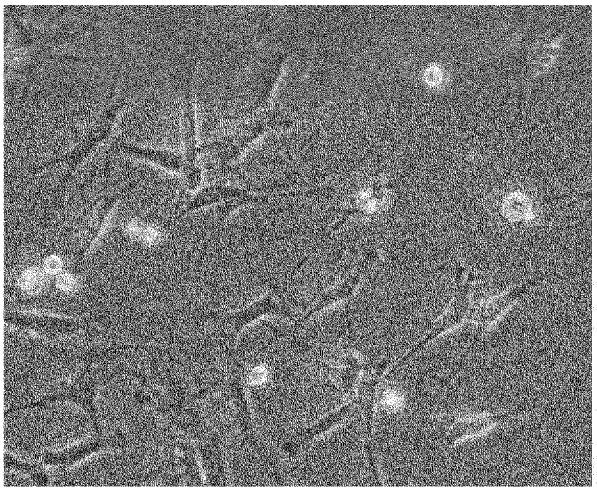
[Fig. 3P]



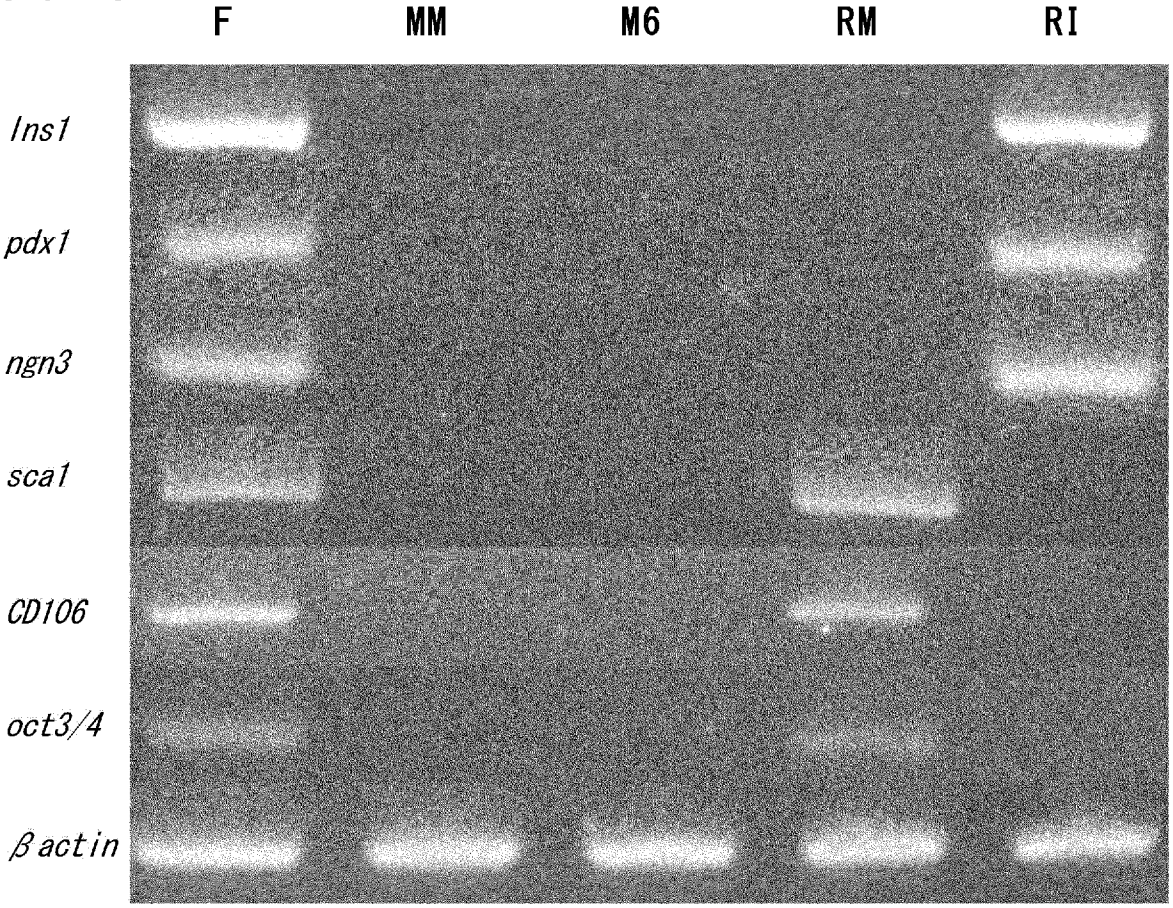
[Fig. 3Q]



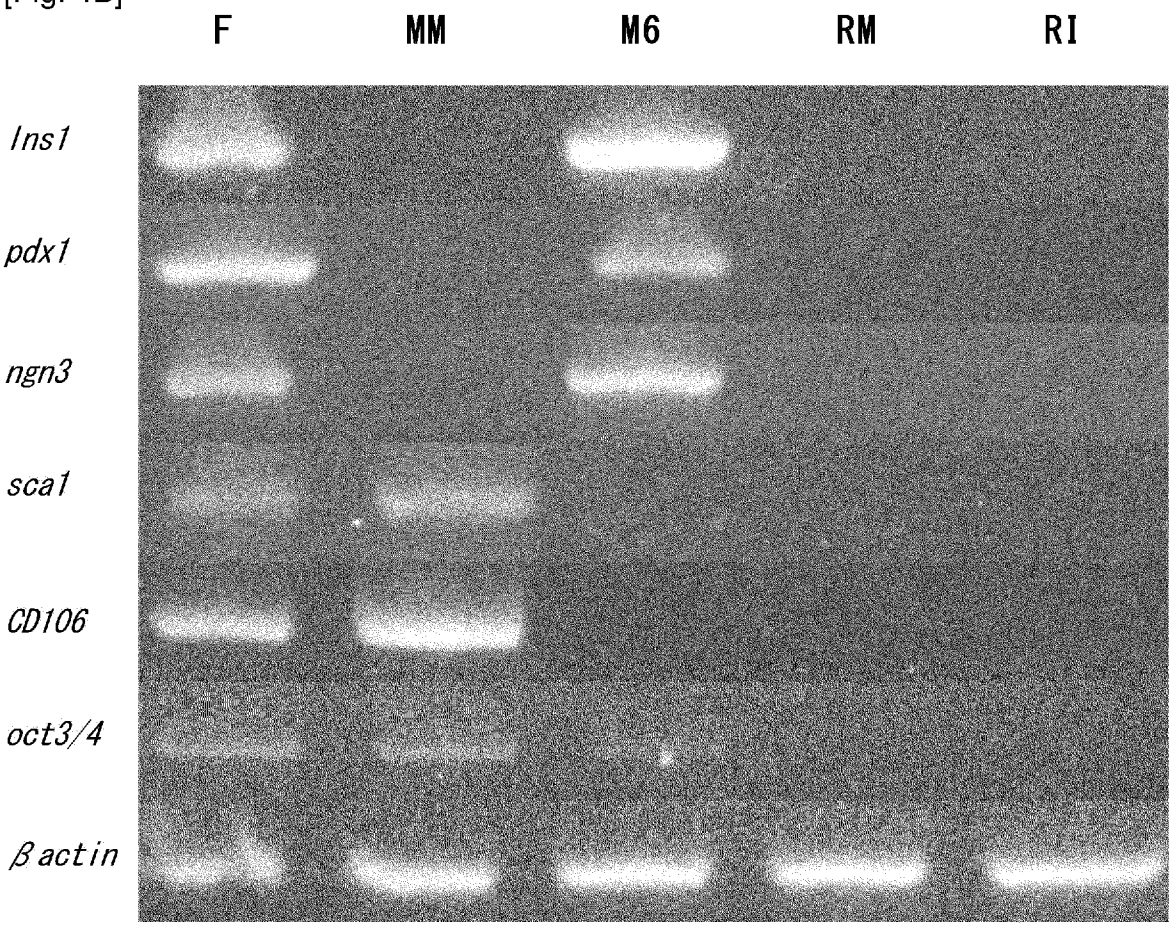
[Fig. 3R]



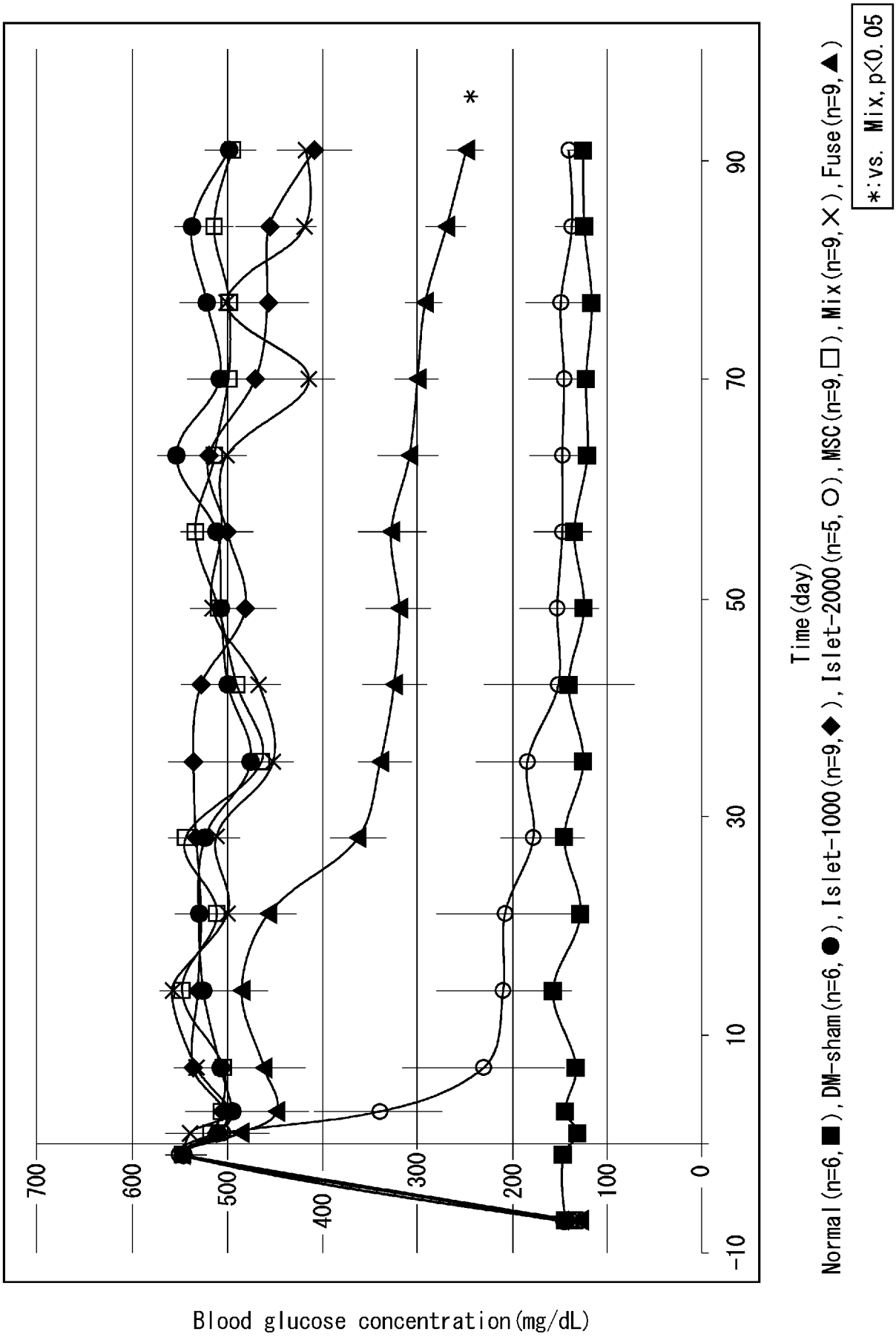
[Fig. 4A]


F
MM
M6
RM
RI
Ins1
pdx1
ngn3
sca1
CD106
oct3/4
βactin

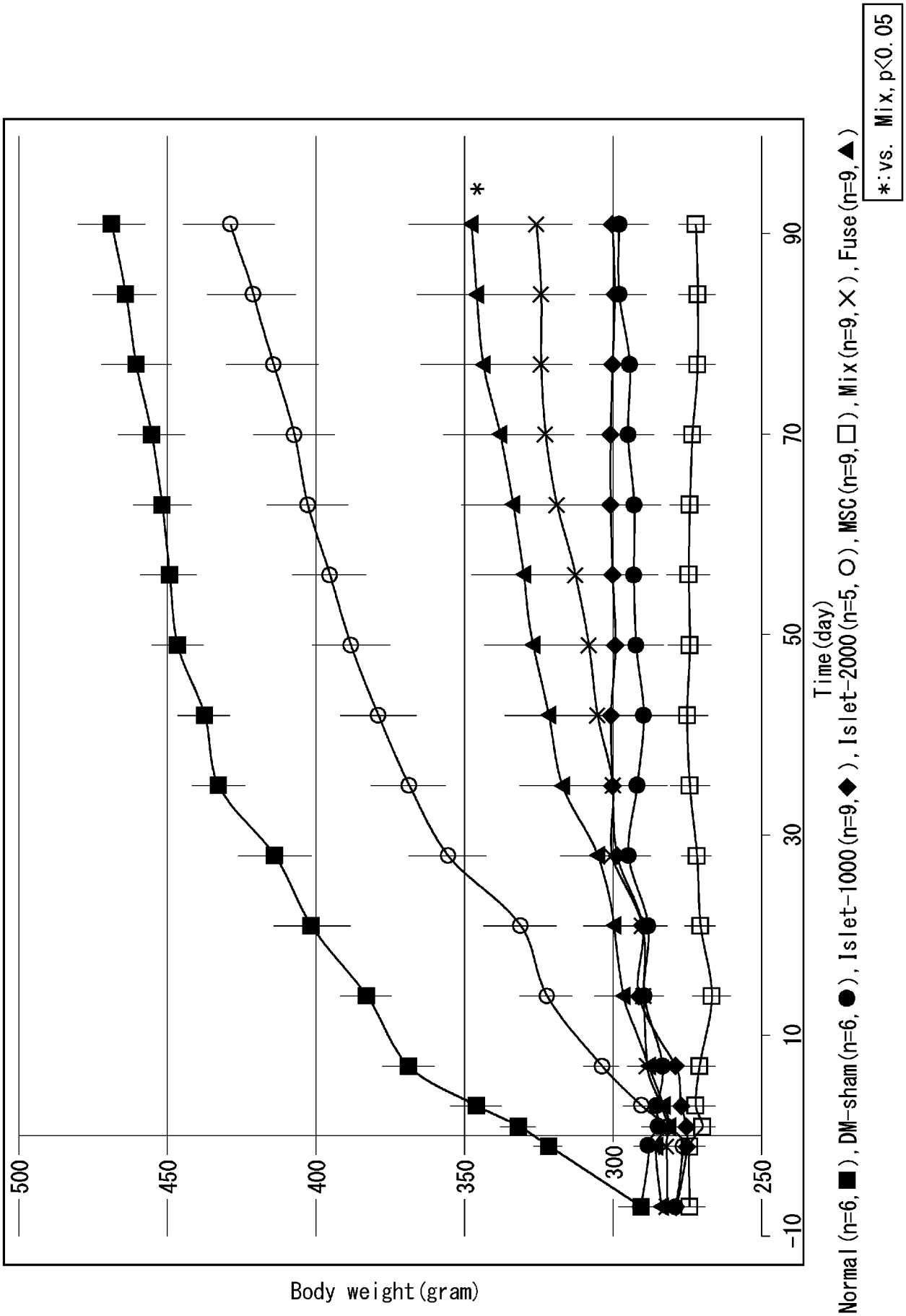
[Fig. 4B]


F
MM
M6
RM
RI
Ins1
pdx1
ngn3
sca1
CD106
oct3/4
βactin

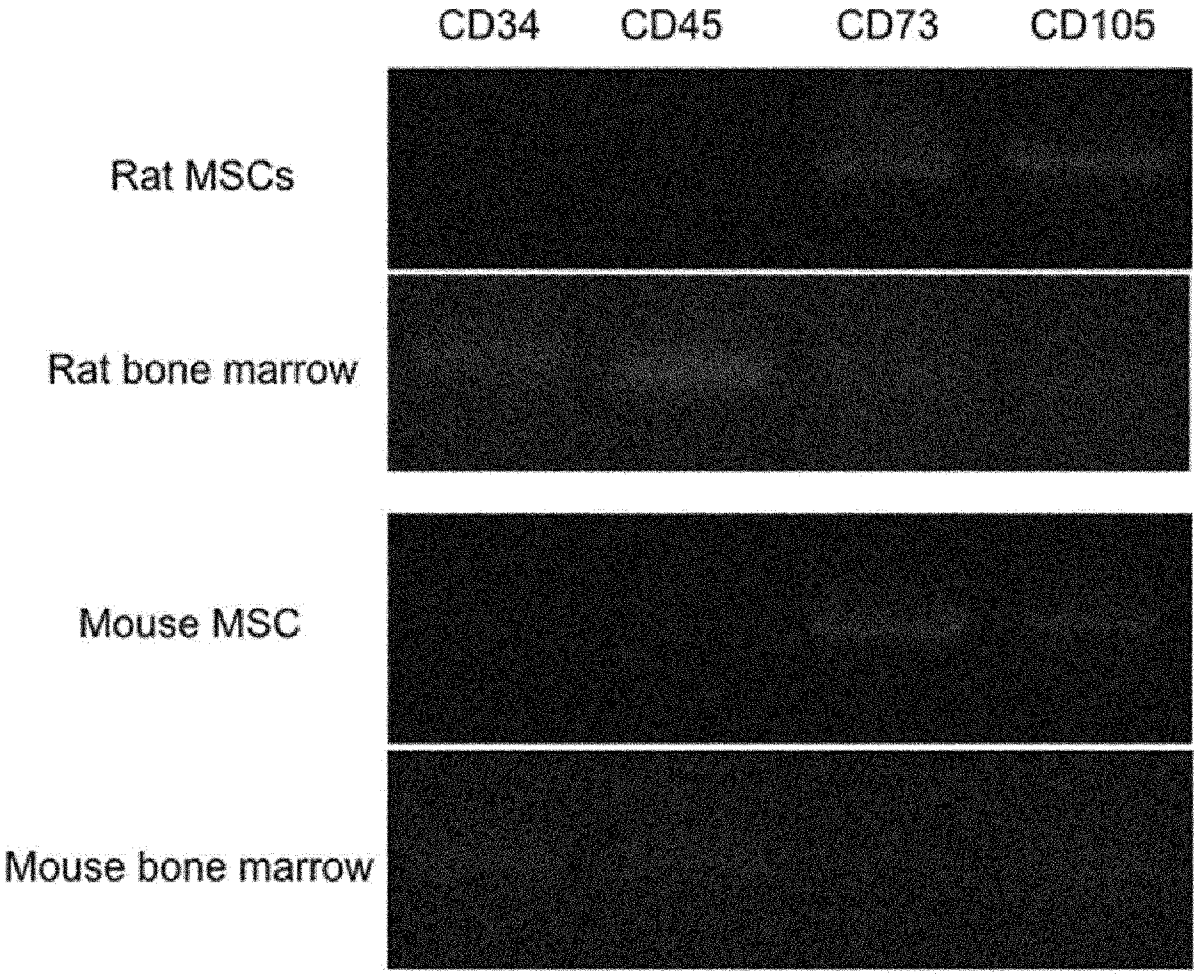
[Fig. 5A]


700
600
500
400
300
200
100
0
-10
10
30
50
70
90
*
Blood glucose concentration(mg/dL)
Time(day)
Normal(n=6,■),DM-sham(n=6,●),Islet-1000(n=9,◆),Islet-2000(n=5,○),MSC(n=9,□),Mix(n=9,×),Fuse(n=9,▲)
*:vs. Mix,p<0.05

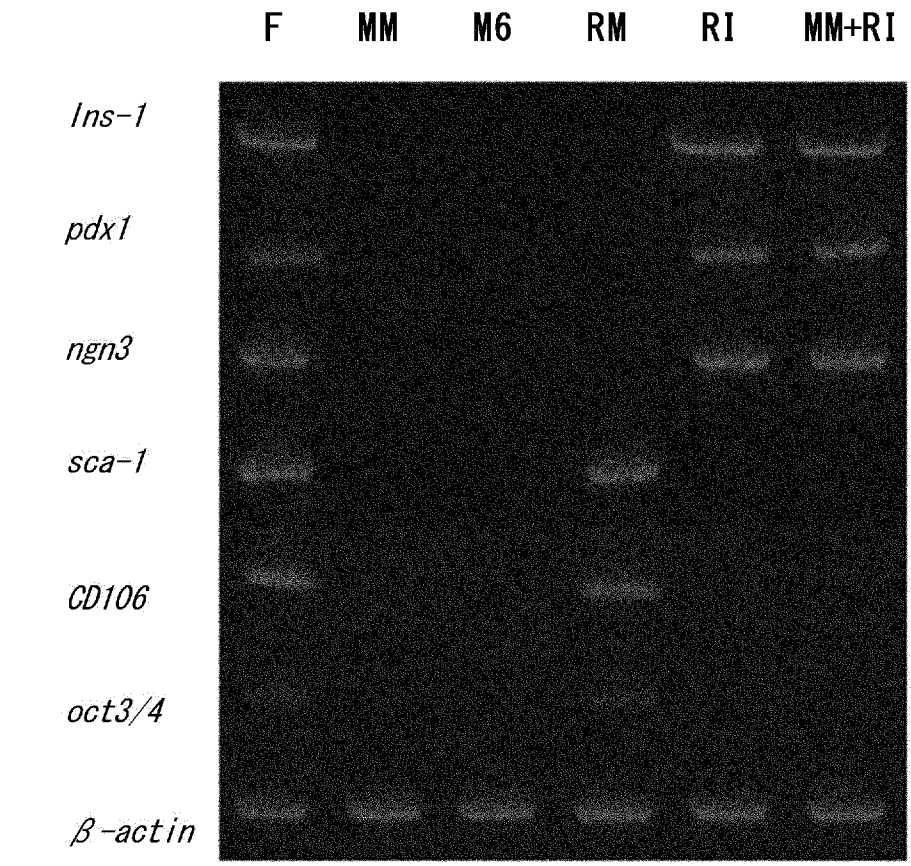
[Fig. 5B]


Body weight(gram)
500
450
400
350
300
250
-10
10
30
50
70
90
Time(day)
*
*:vs. Mix, p<0.05
Normal(n=6,■), DM-sham(n=6,●), Islet-1000(n=9,◆), Islet-2000(n=5,○), MSC(n=9,□), Mix(n=9,×), Fuse(n=9,▲)

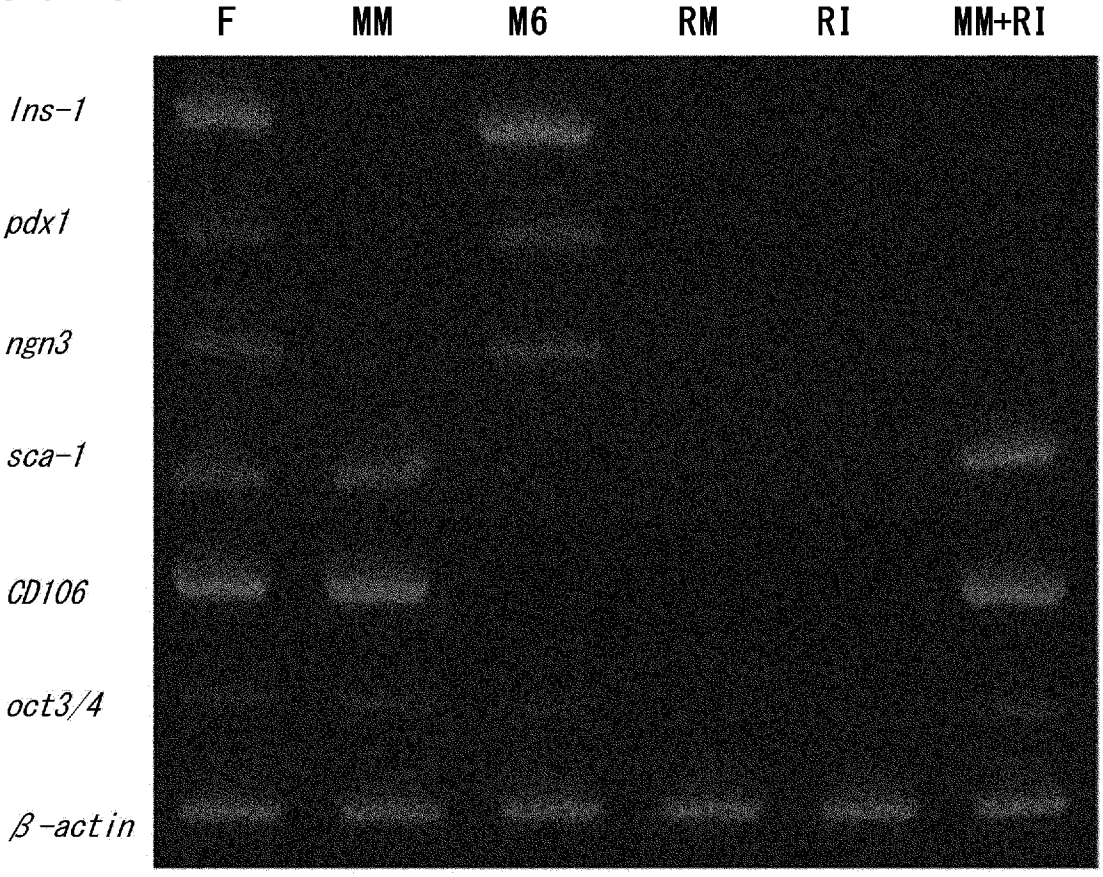
[Fig. 6]


CD34
CD45
CD73
CD105
Rat MSCs
Rat bone marrow
Mouse MSC
Mouse bone marrow

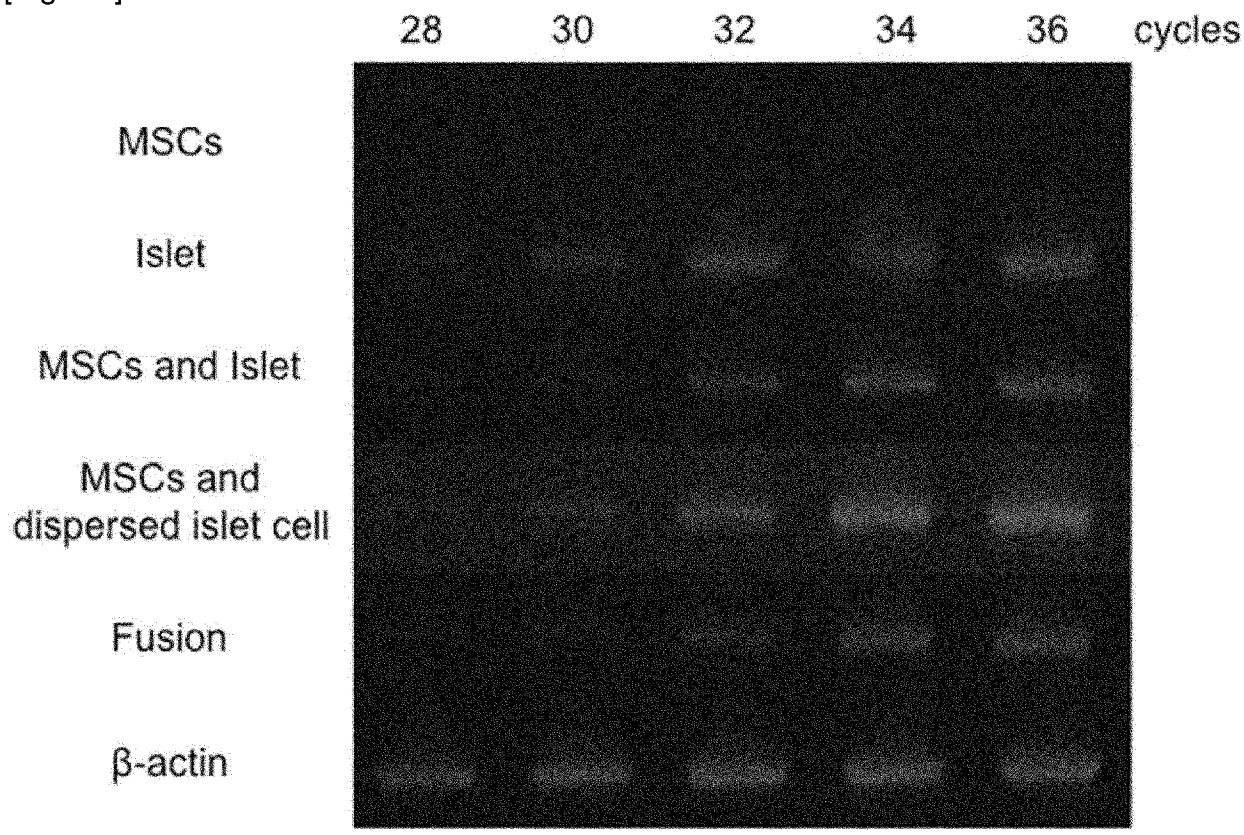
[Fig. 7A]


F
MM
M6
RM
RI
MM+RI
Ins-1
pdx1
ngn3
sca-1
CD106
oct3/4
β-actin

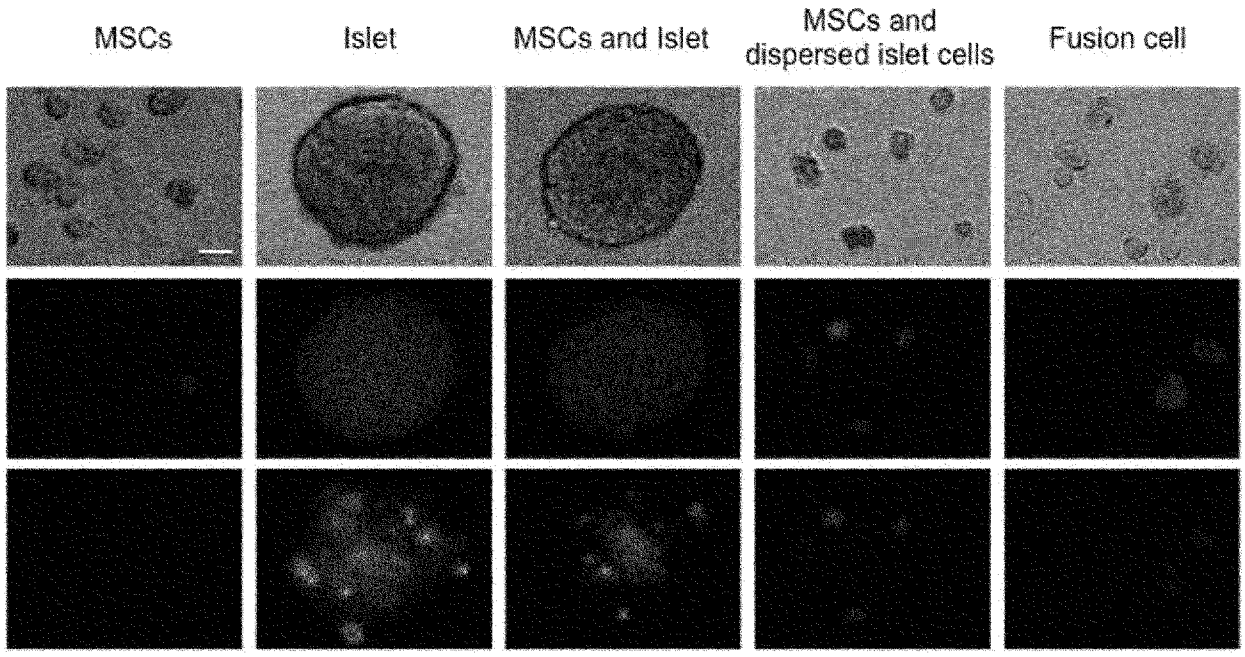
[Fig. 7B]


F
MM
M6
RM
RI
MM+RI
Ins-1
pdx1
ngn3
sca-1
CD106
oct3/4
β-actin

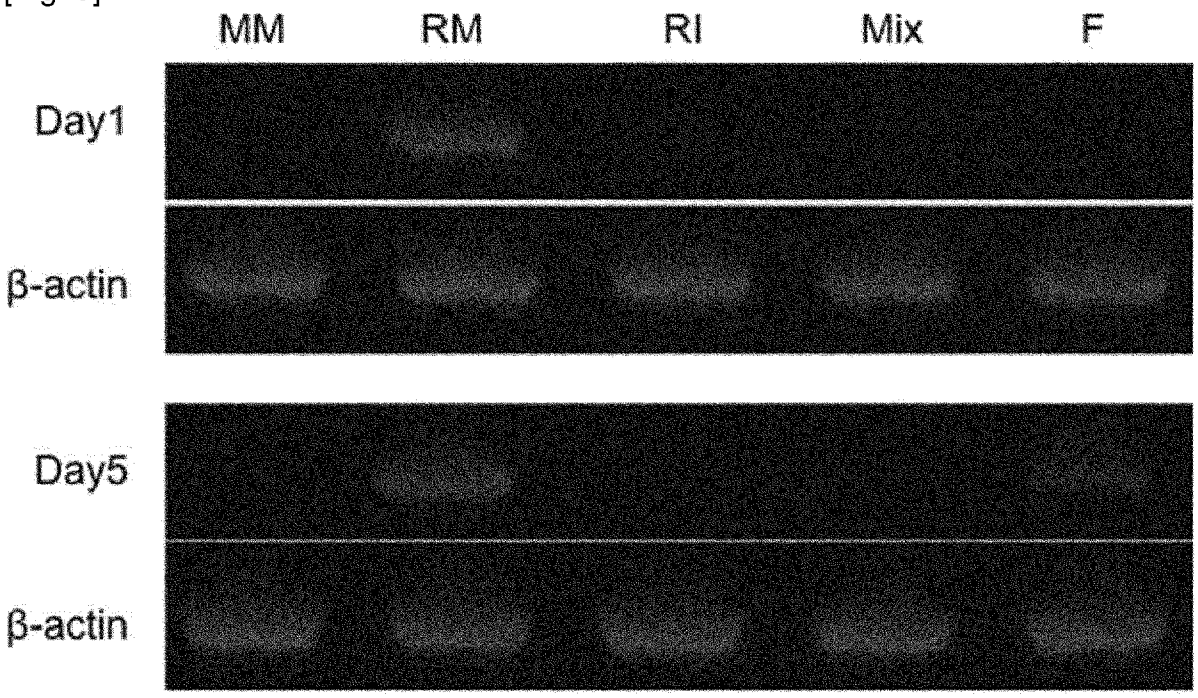
[Fig. 8A]


28
30
32
34
36
cycles
MSCs
Islet
MSCs and Islet
MSCs and dispersed islet cell
Fusion
β-actin

[Fig. 8B]


MSCs
Islet
MSCs and Islet
MSCs and dispersed islet cells
Fusion cell

[Fig. 9]


MM
RM
RI
Mix
F
Day1
β-actin
Day5
β-actin

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/007164

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12N5/0775(2010.01)i, C12N5/071(2010.01)i, C12N5/077(2010.01)i, C12N15/02(2006.01)i, A61K35/39(2006.01)n, A61P3/10(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N5/0775, C12N5/071, C12N5/077, C12N15/02, A61K35/39, A61P3/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2014
Registered utility model specifications of Japan 1996-2014
Published registered utility model applications of Japan 1994-2014

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAplus/REGISTRY/MEDLINE/BIOSIS(STN), JSTPlus/JMEDPlus/JST580(JDreamIII), WPIDS(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	角昭一郎他，間葉系幹細胞と膵島細胞の電気的細胞融合に関する研究，Journal of Japan Surgical Society, 2008.04.25, Vol. 109, Special Issue No. 2, p. 586, DP-121-5	1-4
X	柳井伍一，間葉系幹細胞と膵島細胞の電気的細胞融合に関する研究，Regenerative Medicine, 2008, Vol. 7, Suppl, p. 270, P-127	1-4
X	柳井伍一，ラット膵島と間葉細胞を用いた電気融合実験における基礎的研究，膵臓，2007, Vol. 22, No. 3, p. 403, P-6	1-4

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24.02.2014	04.03.2014

Name and mailing address of the ISA/JP	Authorized officer	
Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	HAMADA, Mitsuhiro Telephone No. +81-3-3581-1101 Ext. 3448	4B 3763

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/007164

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DING, Yunchuan et al., Mesenchymal Stem Cells Prevent the Rejection of Fully Allogenic Islet Grafts by the Immunosuppressive Activity of Matrix Metalloproteinase-2 and -9, Diabetes, 2009, Vol. 58, No. 8, p. 1797-1806	1-4
A	RACKHAM, C. L. et al., Co-transplantation of mesenchymal stem cells maintains islet organisation and morphology in mice, Diabetologia, 2011, Vol. 54, No. 5, p. 1127-1135	1-4
A	WO 2009/057537 A1 (JCR Pharmaceuticals CO., LTD.) 2009.05.07, & US 2011/0274663 A1 & EP 2210608 A1	1-4
A	WO 2009/111030 A1 (OSIRIS THERAPEUTICS, INC.) 2009.09.11, & JP 2011-514901 A & US 2008/0286249 A1 & US 2007/0253931 A1 & US 2010/0330052 A1 & US 2013/0121975 A1 & US 2010/0291047 A1 & EP 2262513 A	1-4
A	WO 2006/011354 A1 (Kowa Company, Ltd.) 2006.02.02, & JP 4294688 B & US 2008/0287391 A1 & US 2011/0110903 A1 & EP 1792985 A1 & WO 2006/011354 A1 & DE 602005027712 D & AT 507290 T & ES 2364563 T	1-4
T, X	YANAI, Goichi et al., Electrofusion of Mesenchymal Stem Cells and Islet Cells for Diabetes Therapy: A Rat Model, PLOS ONE, 2013.05, Vol. 8, No. 5, e64499	1-4