Therapeutic Use of Cloning: Osmangazi Turk Identical Embryonic Stem Cells and Embryonic Stem Cell Transfer to Diabetic Mice

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There are important problems in the therapeutic use of cloning which increase with the more advanced differentiation of somatic cells. Problems include DNA reprogramming, tissue rejection, and chromatin remodeling. This study presents the first use in somatic cell nuclear transfer (SCNT) of tooth pulp cells containing progenitor cells that resemble the nonaging embryonic connective tissue in adults. Using tooth pulp cells in SCNT increased the first cleavage rates compared with adult somatic cells, leading to a higher rate of DNA reprogramming and increased production of an identical embryonic stem cells (ESCs) line. ESCs derived from tooth pulp cells using this method were observed to transfer into pancreatic β cells using transmission electron microscopy. These cells were named Osmangazi Turk Identical ESCs since this was the first use in SCNT of tooth pulp cells to demonstrate a decrease in glucose levels following administration of these cells. Furthermore, nonidentical ESC perform tissue repair by decreasing glucose levels; however, the renewed tissue was observed to undergo important alterations which affect its future. The prognosis of the therapy of mice with diabetes mellitus using Osmangazi Turk Identical ESC was good, with an 80% therapeutic efficiency. These outcomes are promising for regenerative medicine in the therapy of diabetes mellitus, and clarification of the importance of selecting appropriate adult somatic cells in deriving identical ESC lines is also a significant step.

Key words —— identical embryonic stem cells, therapeutic use of mouse cloning, streptozatocin-diabetes, β cells, glucose level, regenerative medicine

INTRODUCTION

The two different research topics of embryonic stem cells (ESCs) and cloning and reproductive cloning are addressed under the definition of therapeutic cloning. These techniques have been utilized to solve problems of tissue rejection and require the remodeling of chromatin. Developments in this field, combining *in vitro* embryogenesis and *in vivo* histogenesis and organogenesis, have been established the emerging techniques of *in vitro* histogenesis and organogenesis.[1)] Previous studies led numerous researchers to study the efficiency of repairing cells/tissues *in vivo* to obtain embryos and consequently derive ESCs from oocyte enucleation and somatic cell nuclear transfer (SCNT).[2,3)]

While the somatic cell differentiates, the nuclear DNA establishes memory; however, it cannot display embryonic behavior in its SCNT state without remembering its past in the embryonic stage. Deletion of the memory acquired during the process of differentiation and nuclear reprogramming must occur so that the somatic cell can differentiate as well. This molecular process in the oocyte cytoplasm is observed as nuclear aging. By deleting the memory of the related somatic cell established after the embryonic period and by reminding the somatic cell of its embryonic past, the possibility of DNA reprogramming can be ensured. However, the adult somatic cells used in cloning at present are differentiated cells, which is required to revert to the primitive

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form in the embryonic stage, *i.e.*, to dedifferentiate, to obtain a successful SCNT outcome, and therefore new techniques must be developed.[4–7)]

SCNT is unlike normal fertilization because heterogeneous molecular factors, H1 in the structure of chromatin which functions in histone modifications during oogenesis, the embryonic genome, and nuclear cloning may all cause incompletion and/or damaged completion of DNA methylation, which is significant in the establishment of retroscopic reprogramming. Thus nucleolar disappearance and difficulties in remodeling chromatin take place after traditional SCNT procedures, in which well-differentiated somatic cell DNA is used. For that reason, cloned embryos demonstrate developmental regressions and numerous chromosomal anomalies.[5–10)] This indicates that the solution of the problem should be the modification of the methods and/or cell types used in SCNT, since the benefit of using identical human ESCs seems to have initiated new approaches.[11–13)]

On the other hand, many factors affect or speed up aging by inducing methylation of the DNA helix of the nucleus. Consequently, beginning from the single-cell zygote stage, the aging of the nucleus or the cells is important both at more advanced embryonic stages and during fetal intrauterine growth.[14)] The undifferentiated stage, close to the totipotential embryonic stages, and the differentiated stages, occurring by departing from the undifferentiated stage, comprise important points in the reprogramming of DNA.[15)] The difficulties in DNA reprogramming during the course of aging and advanced differentiation may be elucidated with the use of adult cells bearing characteristics similar to embryonic-stage SCNTs. This study investigated possible solutions for some problems in the reprogramming of somatic cell DNA, which cause difficulties in the investigations carried out in the field of therapeutic cloning, by using adult cells showing a close resemblance embryonic tissue. As is known, the special connective tissue in the tooth pulp is the most analogous connective tissue type to the embryonic stage and it also contains progenitor cells, previously called adult stem cells.[16)] This study design used cloning both for therapeutic use and for ESC technology, and the choice of tooth pulp cells for the first time. ESCs can be obtained easily during in the treatment of tooth pulp by dentists. In the future, this method can be used routinely. The therapeutic effects of ESC transfer in diabetes were examined because of the differentiation of ESC to endodermal tissue, especially pancreatic cells. In this study, we chose the diabetes model. Diabetes mellitus is in the group of diseases which cannot be completely cured, along with cancer, myocardial ischemia, autoimmune diseases, stroke, Parkinson disease, and Alzheimer disease. The success of embryonic cell transfer in diabetic mice gives hope for further studies on the treatment of other diseases that cannot be easily treated. The diabetic mice model was also chosen because it is easy to follow the treatment process. For the first time, in the *in vivo* portion of this study, identical ESC transfer to diabetic mice and the efficacy of cell and tissue therapy were investigated biochemical, and using light and electron microscopy. Genetic analysis was used to determine whether the mouse ESC were genetically identical.

MATERIALS AND METHODS

Animals and Experimental Protocol —— Thirty-one inbred Swiss albino mice were used in the study. They comprised 8 females and 23 males, 10–12 weeks of age, each weighing approximately 30 g (Table 1). All animal experimental and care protocols were approved (protocol no PR-05-11-01-5) by the guidelines of the Animal Use and Care Ethics Committee, Faculty of Medicine, Eskisehir Osmangazi University. In accordance with the ethical principles of using animals for experimental purposes and the animal rights law of the Republic of Turkey, ketalar anesthesia was administered for tooth extraction through an invasive procedure. Only one tooth was extracted so as not to impair food intake.

Female mice were used only for receiving oocytes, and only male mice were cloned to verify genetic identity using the Y chromosome. SCNT methods were implemented using an inverted mi-

Table 1. Distribution of the Groups and Subjects Used in the Study

Groups	Gender	Number of mice
Receiver Enuclear Oocyte Group	Female	8
Donor STZ Diabetic Experiment Group	Male	21
STZ Control Group (Diabetic Control Group 1)	Male	1
Normal Control Group (Non-diabetic Control Group 2)	Male	1

croscope with an electronic micromanipulator system equipped with electrofusion. A glucometer was used during the diabetes follow-up studies.

Groups —— The 23 male mice were divided into three groups: control group 1 consisted of diabetes-induced mice without stem cell transplantation; control group two consisted of noninduced mice without transplantation; and the streptozatocin (STZ) experimental group. A total of 22 mice, including 21 constituting the experimental group, underwent stem root transplantation after diabetes induction, and one mouse. In control group 1 was administered STZ in multiple low doses to induce diabetes. Eight female mice were used to obtain oocytes after ovulation induction. One out of the 23 adult male mice constituted the STZ-untreated control group, and one other mouse constituted the STZ-treated diabetic control group without stem cell transplantation (Table 1). Five mice were treated with STZ, since nonidentical transfer was planned but not all mice were able to develop diabetes. A total of 22 mice received STZ although only 14 of 22 were able to develop diabetes via this method. Since immediate postdiabetes death occured in 2 of the STZ-treated mice, the development of diabetes could not be determined. Tooth extraction was carried out in 16 of all STZ-induced diabetic mice to obtain tooth pulp cells. After the STZ administration to 22 male mice, one of which was an STZ control, initially 2 and then a total of 10 STZ-induced diabetic mice did at various stages of diabetes because they did not receive any traditional diabetes treatment. The blood glucose levels of the first 2 dead mice could not be measured; however, their tooth pulp cells, obtained at the beginning of the experiment, were used in SCNT applications for nonidentical ESC transplantation. The cleavage, blastocyst, and ESC formation values of these 2 mice following SCNT were also used when calculating the means of the related parameters. Consequently, in 14 of 20 male mice, except for 2 of the STZ-induced mice, the development of diabetes was confirmed. In 6 mice, the development of diabetes failed with the method used here. However, the cause of the inability to develop diabetes was considered to be due to a deficiency in the activity of STZ, since previously opened STZ was injected into 5 mice for the induction of diabetes.

Somatic Cell Culture of the Tooth Pulp —— The tooth pulp of Swiss albino male mice was placed into the medium, preheated and balanced in an incubator overnight, and cultured under aseptic conditions. Rinsing and culturing processes were implemented in the essential blastocyst culture media with a high glucose content, 10% serum, 1% nonessential amino acids, and antibiotic. Tooth pulp (approximately 2 mm^3) was mechanically broken up with a scalpel blade, and 0.05% (v/v) trypsin was added. The pulp was incubated for almost 2–7 days, and the medium was replenished each day.

After the cultures were trypsinized with 0.05% (v/v) trypsin and washed with the same medium, they were incubated in the same medium at 37°C with 5% CO_2 until SCNT. Since the cells are similar to embryonic-stage cells, their synchronization was not required. Additionally, serum-starved culture media were not used in this study to avoid damage induced by the apoptotic pathway of serum-free media. Tooth pulp cells, detached from each other for SCNT after an incubation of 30 sec at 37°C in 0.025% (v/v) trypsin solution, were placed in polyvinylpyrolidone (PVP) where sperm were placed in intracytoplasmic sperm injection (ICSI) applications and used for nuclear transfer.[17, 18]

Fluorescent Microscopic Observation of DNA Using Bisbenzimide and Enucleation —— A fluorescent dye method was used in the SCNT applications in this study, which makes all material visible, to identify the genetic material and to prevent parthenogenesis. Stock solution I, 5000 μg/ml (8.9×10^{-5} M) was prepared by dissolving 1 mg of bisbenzimide (Hoechst 33342, Sigma-Aldrich Corp., Frankfurt, Germany) in 20 ml of sterile distilled water. Stock solution II 50 μg/ml (8.9×10^{-7} M) was prepared by diluting 0.2 ml of stock solution I to 20 ml with sterile water. By diluting 0.2 ml (200 μl) of stock solution II to 2 ml by adding 1, 8 ml of sterile water, the final study concentration of 5 μg/ml (8.9×10^{-8} molar) was obtained. Enucleation was achieved by staining oocytes for 20 min in 5 μg/ml of bisbenzimide study solution, and aspirating the first polar body (1st PB) cytoplasm containing metaphase II chromosomes nearby, as observed with an inverted epifluorescent microscope. The one-step enucleation and nuclear transfer method (one-step SCNT) was preferred since these stages were invasive and long-lasting for the oocytes. While the tooth pulp somatic cells, aspirated into the micropipette were being transferred to the perivitellin space, the oocyte cytoplasm located nearby, containing the 1st of the oocytes and the mitosis shuttles (Fig. 1) were aspirated in the same step without requiring a second procedure, thereby combining enucleation with nu-

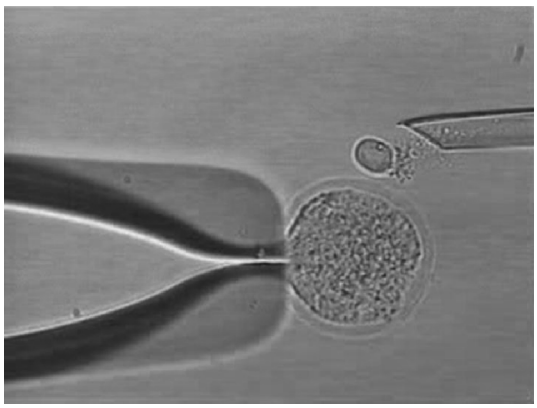

Fig. 1. Inverted Microscope Image of One-step Enucleation of the 1st PB in Metaphase II Mouse Oocytes, Tooth Pulp Somatic Cells Transferred into the Perivitellin Space, and Closely Located Cytoplasm Performed by Aspiration in One-step Enucleation
Original magnification × 20.

clear transfer in a one-step procedure.[13, 18)]

Tooth Pulp SCNT and Activation of Reconstructed Oocytes —— Forty-eight hours after the administration of follicle-stimulating hormone (FSH) 50–75 İU Swiss albino female mice received FSH at the same dose with intraperitoneal human chorionic gonodotropin (hCG). Thirteen to seventeen hours after hCG injection, tubal oocytes were collected postdecapitation. Enucleation of metaphase-II (MII) oocytes, cumulus-corona complexes were separated with 40 İU/ml hyaluronidase enzyme and pipetting, and the somatic cell transfer of the enucleated mouse oocytes to the perivitellin space using a micromanipulation technique was achieved in a one-step procedure. Electrical activation was applied with an electric current fusion/activation protocol. Oocytes were washed in the early embryonic-stage improvement medium and incubated for 5 min, incubated again in the same medium at 37°C and relative humidity of 5% CO_2 until their first cleavage.

Oocytes displaying cleavage were cultured until the 4 blastomer stage using the P1 (Irvine Scientifice®) micro drop method with liquid paraffin oil in early embryonic-stage improvement medium. They were then developed in blastocyst improvement medium (Complete Blastocyst Medium-Irvine Scientifice®) until the blastocyst stage. The oocytes reaching the early or late blastocyst stage on approximately the days 3–5 were generally hatched from the zona pellucida and placed in 0.1% gelatin (w/v)-coated new culture medium. Embryo culture for the receiver enucleated oocytes occurred under normal in vitro fertilization (IVF) embryo culture conditions after the SCNT procedures.[12, 13, 18)]

Electrical Activation Procedure —— Oocyte retrieval, enucleation and SCNT were performed via a one-step procedure, transferred between the two electrodes of the electrofusion apparatus (maximum 1 amp), (Eppendorf multiporator → cat no 4308000040), and exposed to a one-shot electric current for 75 μsec with 40-V direct current (DC) flow, (0.4 kV/cm, field strength).[5)]

Preparation of 0.1% Gelatin-Coated Culture Flasks or Dishes —— Initially, 2 g of powdered gelatin was dissolved in 100 ml of sterile water. A 2% jelly-like stock solution was prepared by autoclaving in a glass serum bottle. A 0.1% gelatin solution was made up by dissolving 50 ml of stock solution in 950 ml of the water was first filtreted (pore size 0.22 μm) sterile water to ensure sterility. Gelatin solution 0.5 ml was added to each well of a 4-well dish and 2 ml of the gelatin solution was added to each well of a 6-well dish according to the dimensions of the culture dishes used, and the dishes were left at room temperature for 30 min. The remaining unused solution was stored in a refrigeratus. For later use, the gelatin was aspirated and then dried in a sterile safety cabinet, and it was finally replaced with ESC medium.[19, 20)]

ESC Culture and ESC Medium —— Mouse fetal fibroblasts were not used as supporting layers in the present study to prevent different mouse cell proteins and DNA from contaminating identical stem cell lines. Instead, acellular gelatin and high-dose β fibroblast growth factor-basic (bFGF) was used as a substitute for the nutritive supporting layer to inhibit differentiation. In addition, leukemia inhibitor factor (LIF) was also used, as it is the basic factor that protects pluripotency.[21–25)] 10% fetal bovine serum (FBS) was added to 250 ml of basal medium (ES-Cult® Basal Medium-A, Stem cell, cat no. 05801) along with 30 μl of 1000 units/ml LIF and bFGF solution 0.5 ml. bFGF solution was prepared by adding bFGF 10 μg to 5 ml of 0.1% FBS in phosphate-buffered saline (PBS). The ESC cultures were incubated at 37°C in sterile conditions with a high relative humidity and 5% CO_2.[19)]

Mechanical Isolation and Passaging of Inner Cell Mass Cells —— After the spontaneous blastocyst hatching from the zona pellucida, blastocysts were stored for implantation in 0.1% gelatin-coated culture dishes. Blastocysts were allowed to attach to the gelatin coated dishes overnight. The trophectoderm (TE) layers of these blastocyts implanted into

the gelatin in 2–4 days and became invisible. Their inner cell masses (ICMs) gradually became apparent and expanded spherically toward the exterior of the implanted blastocyts.

ESC Cultures and Plates —— Plating of ICMs isolated from the ICM structures with a clustered, three-dimensional, swollen appearance were collected with pipettes and placed in fresh medium ESCM to establish primary ESC colonies. This initiated the passaging of ESC colonies, established by the incubation of ICM mechanically separated from TE cells. The attachments were loosened during passaging either by pipetting mechanically with rapid movements and foam production, or by aspirating the medium and incubating for 2 min in 1–2 ml of medium with 0.05% trypsin added. Fresh medium 10 ml was added onto the detached colony cells and placed in a flask. Trypsin was removed by repetitive aspiration. Five milliliters of fresh ESCM was added, and a single-cell suspension was obtained by rapid pipetting. ESCM was distributed into gelatin-coated 6-well dishes in the form of circular drops. Since the cells could possibly collect on the tip of the pipette, the last small portion of media was pulled into the pipette and distributed to all wells. During passaging of ESC colonies, expandable ESCs were cut mechanically and distributed to new culture flasks. Since the small colonies were capable of maintaining pluripotentiality, none of the colonies was allowed to reach a bulky size. In an improving colony, the first cluster was observed to locate in the middle and was dark in color, and the cells differentiating with a tendency of flattening/elongating were located on the external surface of the colony. Large colonies had the tendency to flatten and differentiate. Morphologically large, bright cells with large nuclei and extensive cytoplasm existed in undifferentiated regions.

While passaging, the cells preferred the space between the two regions. The differentiation and karyotypes of the colonies were protected in this way. The colonies with undifferentiated-stage morphology were placed in fresh ESCM, and the medium was separated from the attachedment cells to the greatest possible degree by rapid pipetting.[19, 26)]

Osmangazi Turk Identical ESC (Tooth Pulp ESCs) Transplantation to Mice with Diabetes Mellitus and Differentiation *In Vivo* —— Osmangazi Turk Identical ESCs (tooth pulp ESCs) were transferred to *in vivo* medium by dilution with 50% sterile serum (v/v). Of the mice with STZ-induced experimental diabetes, those that could developed ESCs from the blastocysts established by cloning constituted the identical ESC transfer group. Stem cell lines were established for those living mice from which stem cells could not be developed from their blastocysts by cloning. The stem cells of the mice not surviving due to diabetes constituted the nonidentical ESC transfer group. For both groups, ESC transfers were transported to the pancreatic region via the intraperitoneal method using insulin injection, thus testing the power of the therapy. The diabetic mice, the blood glucose levels of which were measured pre and post ESC transfer, were killed by decapitation, and the Langerhans islets in the pancreas were compared at light and transmission electron microscopic (TEM) levels (Table 2). The study was carried out via routine light and TEM tissue processing methods for this purpose. Additionally, since they were being used for the first time, the Osmangazi Turk Identical ESCs, were genetically analyzed to determine whether they were identical.

Genotyping of ESCs obtained after SCNT —— Genotyping analysis was performed on nine ESC lines (3 of 9 lines were repeated). Six of these nine lines were transferred *in vivo*. Mouse ESC genomic DNA was extracted using a Qiagen DNA extraction kit. Y-chromosome specific DNA sequence (DYS) 212 and deleted in azoospermia (DAZ) sequence tagged-site (STS) locuses were amplified. As a control, the selected mouse cDNA on the X (SMCX) locuses were also amplified. Amplified samples were subjected to gel electrophoresis in a 3% NuSieve GTG agarose (Cambrex®) gel and analyzed under a gel screening and documentation system (Fig. 2 and Table 3).

Method of Inducing Type I Diabetes in Mice and Histological Scoring for Diabetes —— Swiss albino male mice (10–12 weeks of age, weighing approximately 30 g) were given STZ to induce type I diabetes. STZ was dissolved just prior to use in a citrate tampon (pH 4.5, citrate buffer) and injected intaperitoneally for 4 days at a dose of 50 mg/kg day.[27, 28)] Induction of diabetes was ascertained 2 days after STZ injection using a glucometer (Prestige®) and blood glucose test strips (Glucotrend Plus, Roche®) and by measuring the blood glucose levels obtained by tail-vein injection after overnight food deprivation. The diagnosis of diabetes induction was determined according to the classical diabetes blood level of > 250 mg/dl (13.9 mmol/l). Induction and therapy of diabetes

Table 2. SCNT Cleavage and ESC Line Development Rates Using Tooth Pulp Cell and Effects of Osmangazi Turk Identical ESCs and Non-identical ESCs Transplantation on Diabetes in the STZ-induced Diabetic Mouse

Number and Rate of Diabetes InducedMice	Number and Rate of Mice with Tooth Pulp Cell Production	Total Number of SCNTs	Number and Rate of Post-SCNT Cleavage and Embryo Development	Number of Blastocysts Initiated in ESC line (Blastocysts DevelopmentRate)	Number and Rate of Line Derivation at the ESC Transfer Level	Complete β cell Repair with Good Prognosis
14	16	82	23/82	13	8/23	Identical: 80%
63.6%	100%		28%	56.5%	34.7%	Non-Identical: 0%

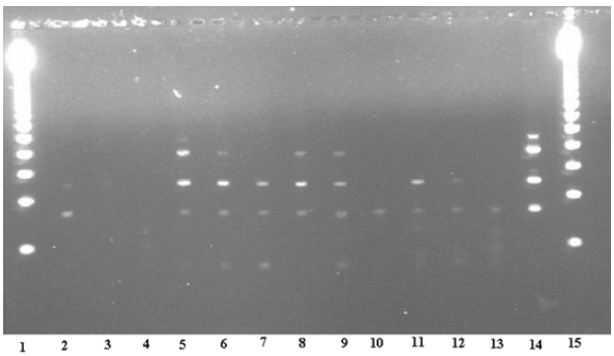

Fig. 2. Genetic Analysis. Amplified Osmangazi Turk Identical ESC DNA. The Outcomes of the Genetic Analysis Showing that the Transferred Osmangazi Turk Identical ESC Lines did not Develop from Parthogenetic Embryos, but were Genetically Identifical to the Cloned Male Mouse. The Expansion of the Numbers between 1 and 15 were Displayed in Table 3

1, Marker; 2, sample 1; 3, sample 3; 4, sample 5; 5, sample 8; 6, sample 10; 7, sample 14; 8, sample 16; 9, sample 9; 10, sample 10 (repeated); 11, sample 12; 12, sample 14 (repeated); 13, sample 16 (repeated); 14, control; 15, marker (Y chromosome content of the 9 Lines analyzed was confirmed).

were observed by the light and electron microscopic methods of histological scoring of diabetes. The blood glucose level was ascertained in decapitated mice to compare with the TEM results and between groups. Routine light and TEM tissue-processing methods were used for this purpose. Paraffin sections were stained using the hematoxylin and eosin method, semithin araldite sections were stained with toluidine blue, thin sections were stained with lead acetate, and all stained sections were scored according to the same criteria. Histological scoring was performed as described[27,28] after the routine procedures of light and TE microscopy (TEM; JEOL 1220 TEM at 80 kV JEOL, Tokyo, Japan).

The Degree of Insulitis or Diabetes was Classified into Four Categories —— *Score 0, No Insulitis* (*Nondiabetic*): There was no damage to the pancreatic islets of Langerhans.

Score 1, Peri-insulitis (*Mild Diabetes*): The cell diameter was normal, there was very little cell damage, and there was little or no lymphocyte infiltration of the pancreatic islets of Langerhans.

Score 2, Moderately Invasive Insulitis (*Moderate Diabetes*): The diameters of the pancreatic islets were reduced, they were surrounded by lymphocyte infiltration, and cell destruction was observed.

Score 3, Severely Invasive Insulitis (*Severe Diabetes = Islet Destruction*): The diameters of the pancreatic islets were severely reduced, and the cellular structure was completely destroyed.[27,28]

RESULTS

Diabetes Mellitus and the Somatic Cells of Tooth Pulp

In this study, a total of 22 mice were treated with STZ. Diabetes was diagnosed in 14 of 20 male mice, except for two STZ-induced subjects (Table 3). The amount of tooth substance obtained in mouse cells was sufficient for SCNT although less than 100% of mice had tooth pulp cell production (Table 2). In light of the above, the rate of failure with this method[13,17–28] to develop diabetes was 36.4%, and the rate of diabetes development was 63.6% (Table 2).

Blood Glucose Values Pre- and Post-STZ-Induced Diabetes

Glucose level measurements were performed using the blood samples taken from the tails of 21 mice pre-STZ. Two control and 5 nonidentical transfer planned mice were included in this number, but the values from the 2 mice that died immediately post-STZ were excluded. Glucometric blood glucose levels were found to be a mean of 79.2 mg/dl pre-STZ injection. Post-STZ blood glucose values were obtained from 14 STZ-treated mice at devel-

Table 3. Glucose Averages Pre- and Post-STZ Diabetes and Post ESC Transfer and Genetic Analysis

Mouse No:	Pre-STZ (mg/dl)	Post-STZ Pretransfer (mg/dl)	Post ESC Transfer (mg/dl)	DYS 212 and DAZ STS Locuses and Numbers in Fig. 2
1	68	300	81	+/2, Identical
2	—	—	Exitus	–
3	72	252	366	+/3, Identical
4	—	—	Exitus	–
5	70	260	137	+/4, Identical
6	76	252	Exitus	–
7	68	250	Exitus	–
8	70	261	Exitus	+/5
9	65	270	Exitus	+/9 (Not transferred)
10	80	256	116	+/6 and 10, Identical
11	75	253	76	–
12	68	—	Exitus	+/11 (Not transferred)
13	78	251	Exitus	–
14	80	270	104	+/7 and 12, Identical
15	82	250	Exitus	–
16	75	254	Exitus	+/8 and 13 (Not transferred)
17	141	—	—	–
18	79	—	—	–
19	106	—	—	–
20	93	—	—	–
21	101	—	—	–
22	78	250	—	–
23	40	—	—	–
Mean Value	1665 : 21 = 79.2 mg/dl	3379 : 14 = 241.3 mg/dl		(+) Y Chromosome Content of the Analyzed 9 Lines was Approved

oped diabetes and remained alive. The values of the 2 mice that died in the beginning of the experiment, another mouse that died post-STZ without developing diabetes, and 5 STZ-treated mice that did not develop diabetes were excluded from the statistics. The mean increase in glucometric blood glucose levels was 241.3 mg/dl following STZ injection performed at the dosing level and schedule in this study. These results are summarized in Table 3.

Development Rates of SCNT Cleavage and ESC Lines Using Tooth Pulp Cells

In this study, among 23 cloned mouse embryos obtained by mouse tooth pulp somatic cell transplantation, 13 developed to the blastocyst stage, and five of these lines initiated development but then arrested. The blastocyst development rate was calculated as 56.5%. Eight lines developed to the transfer level. Six *in vivo* transfers were established, as five identical and one nonidentical transfers. Thus the development rate of ESC lines was determined to be 34.7% (Table 2). These findings, summarized in Table 4, show that the use of a few enzymes, enucleation of the oocyte in a less invasive one-step procedure, and the modified SCNT method contributed to increasing rates of cleavage and cell line formation. The transfer of the ESCs obtained, without allowing for likely abnormalities in their karyotypes *in vivo*, were determined to be useful for *in vivo* activity.

Genetic Analysis of Osmangazi Turk Identical ESCs (Tooth Pulp ESCs) Derived via the New Technique

ESCs derived via this method in mice were found to be genetically identical to the cloned male mouse. This genetic finding also demonstrated that the blastocysts obtained in our study were not parthenogenetic. Tooth pulp cells are the most similar cells to the embryonic stage, as opposed to known cells such as skin or breast cells.[16] According to the data on genotyping analysis, these ESC lines were derived from XY-male SCNT. The Y chromosome content of the nine lines analyzed was confirmed. This finding strongly supports the conclusion that the 23 cloned embryos were not parthenogenetic, since parthenote cells lack male chromosomes (Table 3 and Fig. 2).

Table 4. Development Rates of SCNT Cleavage and ESC Line Using Tooth Pulp Cells

Total Enucleation and Number of SCNT: 82	Total number of Initial Cleavage: 23	Initial Cleavage Rate %28
Number of Embryos Passaging ESC Lines: 23	Number of Developing ESC Lines at the transfer level: 8	ESC Line Development Rate:% 34.7

Table 5. Effects of Identical and Non-identical ESC Transfer on the Glucose Average and β-Cell Repair Post-STZ Diabetes

ESC Transfer	Mouse No	Pre-STZ mg/dl	Before post-STZ ESC Transfer (mg/dl)	Post-ESC Transfer mg/d	Decrease in Glucose Level	TEM β-cell Repair
Identical	1	68	300	81	+	+ (scor:0)
Identical	3	72	252	366	–	– (scor:3)
Identical	5	70	260	137	+	+ (scor:1)
Identical	10	80	256	116	+	+ (scor:0)
Non-Identical	11	75	253	76	+	– (scor:2)
Identical	14	80	270	104	+	+ (scor:0)
Decrease in the Glucose Level	Identical: 4/5 (80%)	Non-Identical: 1/1 (100%)	Recovery in Both			
TEM β-cell repair	Identical: 4/5 (80%)	Non-Identical: 0/1 (0%)	Good Prognosis in the Identical, Poor Prognosis in the Non-identical			

Activity of Identical ESC Transplantation in Cell and Tissue Therapy upon Light and Electron Microscopic Examination

In this study, the experimental occurrence of diabetes mellitus and rising glucose levels were observed to decrease after the transfer of both identical and nonidentical ESCs. We observed on improvement in glucose levels in both groups. Because the quality of the obtained ESCs are the same and ESCs obtained from SCNT tooth pulp cells have high therapeutic efficiency in diabetes-induced mice, nonidentical ESCs are not derived from dead animals. Tooth extraction was carried before creating diabetes. For this reason, tooth pulp cells were obtained before diabetes and cells of the Mouse that died from diabetes were not used. Therefore the quality of the obtained ESCs was the same in all groups. However, when this was assessed by light and TEM examination of the pancreas, β cells renewed by non identical transfers were determined to initiate immune rejection (Tables 3 and 5). According to the findings of the light and electron microscopic study, the pancreas was totally damaged (invasive insulitis, score 3), by STZ-induced diabetes (Fig. 3). Nonidentical transferred ESCs were seen as capable of repairing damaged pancreatic tissue and forming new β cells; however, they were observed to result once again in autoimmune destruction when they were assessed from the viewpoint of diabetes prognosis.[27, 28] On the contrary, light and

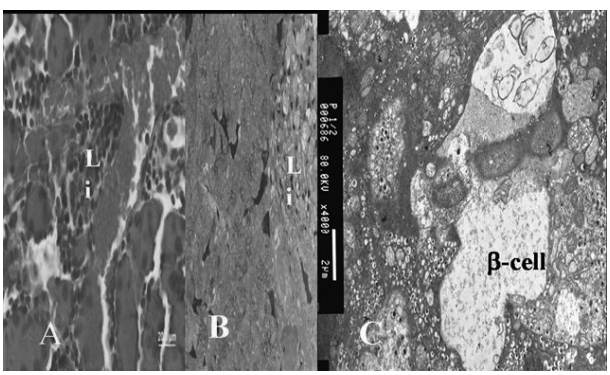

Fig. 3. In the Pancreas Destroyed by STZ-induced Diabetes. A. (H&E staining) and B. (toluidin blue staining) Showed Damaged, Shrunken Islets of Langerhans with Lymphocyte Infiltration (Li); C, Damaged β Cells. TEM micrograph

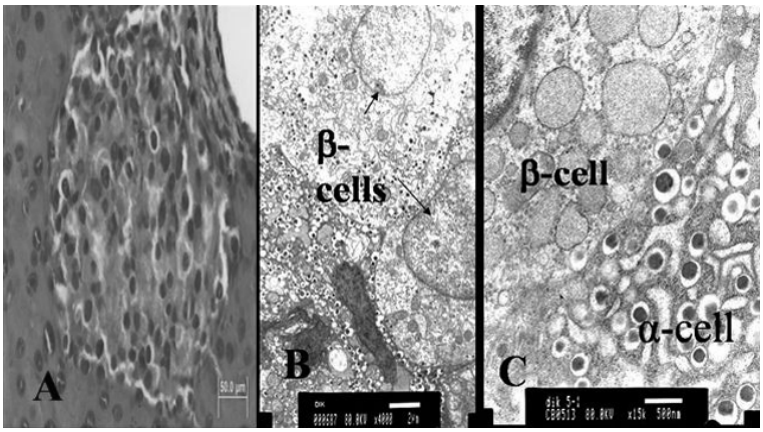

Fig. 4. Identical ESC A, Normal Structure in the Islets of Langerhans. H&E Staining Showed (B and C) Insulin Secreting β Cells with in Normal Fine Structure. TEM micrograph

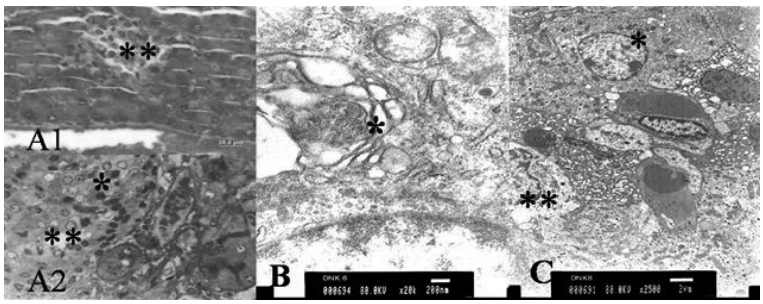

Fig. 5. Nonidentical ESC: A1 (H&E staining) and A2 (toluidine blue staining) Showed Damaged and Shrunken Islets of Langerhans with Lymphocyte Infiltration; B and C, β Cells with Poor Prognosis in the Endocrine Pancreas Secreting Insulin but Displaying Cell Infiltration ($*$). TEM micrograph

electron microscopic examination showed that the identical stem cells were in a healthy structure likely to be maintained and were able to repair (no insulitis, 80% score 0–1), damaged pancreatic tissue and form new β cells (Fig. 4).

Nonidentical ESC transplantations were considered to lead to inflammatory reactions (moderate insulitis, score 2) and to future complications (Fig. 5). Since the tooth pulp cells increased the first cleavage rates, the Osmangazi Turk Identical ESCs were observed to have 80% therapeutic activity (Tables 3 and 5) with good prognosis (Fig. 4) for the treatment of diabetes mellitus.

DISCUSSION

Although animal experiments have been yielding significant results since 1981, the derivation of the first ESCs from humans occurred in 1998.[29, 30] Optimization of culture media and the improvement of related methods are necessary to initiate treatment in humans.[20, 31, 32] In addition to efforts to increase the derivation rate of identical ESC lines, the suitability of the method in this study was tested via individual genetic analysis. To protect karyotypes and to put the outcomes into practice, methods were selected which avoid the use of enzymes as much as possible in an attempt to surmount the difficulties in the differentiation of ESCs, including the endodermic structures of three germ layers, especially for pancreatic cells.[20, 27] In addition, incomplete DNA methylation after SCNT has been considered to be responsible for low success rates in cloned mammals.[8, 11, 12, 14, 33–35] Therefore new cells must be discovered to replace cells such as skin fibroblasts, which are frequently used with traditional methods because their dedifferentiation potency decreases in the backward direction since they are differentiated in the forward direction. In the present study, as a solution to this problem, no aging tooth pulp cells were used in adults. The researchers demonstrated that mouse blastocysts, derived using traditional cloning techniques, cannot achieve the

delivery of living offspring due to the development of unhealthy blastocysts in addition to the poor rate of development.[12] In this study, the rate of development to the blastocyst stage of cloned mouse embryos derived from mouse tooth pulp somatic cell transplantation was found to be 56.5%. Therefore, the increasing success rate is considered to be associated with the use of young cells. Preceding reports stated that the preimplanted embryo development at the 4 and 8-cell stage was very limited, and the rate of blastocyst development was almost 17.9%. Another problem observed along with this low rate is Oct4 expression in the ICM of blastocysts derived using traditional methods. Cdx2 expression of trophoblasts does not have any noteworthy problems.[35] This information is important because the ICM of blastocysts derived via therapeutic cloning will be used, and it is interesting because the level of the problem changes according to the cell type used. The Oct4 expression of the cloned blastocysts was 50% when cloned with cumulus granulose cells, and 40% when cloned with tail-tip fibroblasts. The outcomes of this study qualitatively support the relationship between increasing success rates and younger cells, as this study used the youngest cell used so far in SCNT.[35] Likewise, in another study investigating the relationship between the factors affecting success and adult somatic cell types, very low live offspring birth rates according to the derived cell types (1.5–1.9%, 0–0.3%) were observed to increase by 2.2% with the use of fetal ovarian cells.[33] Additionally, in studies examining the relationship between nuclear remodeling and obtaining healthy blastocysts, it was demonstrated that the development rates of the preimplanted embryos with 2, 4, and 8 cells were limited and the normal diploid embryo development rate in these embryonic developments, most of which were tetraploid, was 37.5% even during the blastocyst stage.[5] Genetic analysis of the diploid stem cells via the technique used here in mice revealed that nonparthogenetic blastocyts can be obtained which are genetically identical to the cloned male mouse. Moreover, the line-forming rate of 34.7% suggested that there was neither DNA damage nor reprogramming deficiencies.

On the other hand, studies investigating embryo development from the perspective of the nucleus and cytoplasm reported that the nuclear transfer technology used, the fusion parameters, and the cytoplasm were more indicative than the nucleus.[5, 11, 31] Even the types of protein supplements, method and timing used in the studies were observed to a effect the success of the results.[36, 37] Consequently, it appears that the electrical activation parameters (0.4 kV/cm 72 μsec) with short duration and low intensity; the one-step procedure[13] which decreases enucleation, nuclear transfer duration, and damage; and the modified serum containing development medium protecting the donor cytoplasm contributed to improved stem cell differentiation in this study.

In this study, high glucose levels with induced experimental diabetes were observed to decrease after the transfer of both identical and nonidentical ESCs. However, the joint assessment of these findings and outcomes of light and electron microscopic examination of the pancreas revealed that β cells renewed by nonidentical transfers were immunologically rejected. According to the light and electron microscopy results, nonidentical transferred ESCs were as capable of repairing the damaged pancreatic tissue and growing new β cells; however, they were determined to have led once more to autoimmune eradication, making them problematic for long-term diabetes therapy. On the other hand, identical ESCs were observed to provide healthy, reproducible light and electron microscopic results, in addition to their ability to repair damaged pancreatic tissue and grow new cells.[27, 28] The fine structure of the pancreas showed a possibility of temporary improvement despite the apparent decrease in glucose levels in the nonidentical stem cells, demonstrating that the future of ESCs lies in identical stem cells or cloning technology. The progress in technology appears to make joint planning possible. It is interesting that the same mechanisms are used simultaneously by the embryo and by all molecular procedures of both cloning and ESC technologies, showing a common denominator for embryo,[38–45] from organ repair in a lizard with a fractured tail[45] to cancer. ESCs have the ability to regenerate and/or repair; however, tumor growth must be inhibited for application to human treatment. One method is the transformation of ESCs into specific cells pretransplantation; another is the permanent inhibition of undifferentiated stages. The Wnt cascade and glycogen synthase kinase-3 (GSK-3) in particular play key roles in the mechanisms mentioned above[44] with respect to the common characteristics of ESCs such as dedifferentiation, proliferation, immortalization, self-renewal, and invasion.[46]

A decrease in blood glucose levels following the transfer of both identical and nonidentical ESCs does not seem surprising. However, establishing

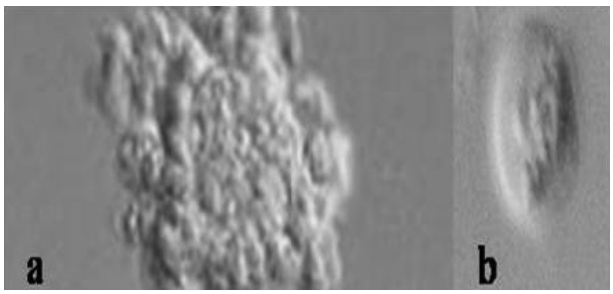

Fig. 6. Phase-contrast micrographs of an ESC colony and a single cell.

a) Phase-contrast micrograph of primary nonidentical ESC colony grown in feeder-free conditions. The small colony is capable of maintaining pluripotentiality. Original magnification × 20. b) High-magnification phase-contrast micrograph of a single cell after pipetting showing the typical morphology of an ESC. Morphologically undifferentiated ESCs are large, bright cells with large nuclei and extensive cytoplasm in the culture dish.

the initiation of immune rejection of β cells initially renewed by nonidentical transfer indicates that autoimmunity has separated the pathway of the two cell sources after repair.

In conclusion, the increase in cleavage rates and accordingly in the rates of ESC line development per unit of mouse oocyte after SCNT using tooth pulp cells was closely correlated with the young tooth pulp cells used in this study. Nonidentical ESC colonies are capable of maintaining their pluripotentiality. Morphologically of undifferentiated ESCs cells is large, bright cells with large nuclei and extensive cytoplasm in the culture dish (Fig. 6). These *in vitro* identical colonies are the same as *in vivo* ones, because ESCs used in transplantation of nonidentical is transfers used to obtain tooth pulp cells occur before diabetes was induced. As a result, the quality of nonidentical ESCs is the same as that of identical ESCs cells. The cells were transplanted into the pancreas region of mice, which induced diabetes, and our results showed that reductions in blood glucose levels from in mice and renewal of β cells in the pancreas occured in both groups. Finally, identical ESCs cells may have advantages over nonidentical ESCs cells for pancreas repair without immune rejection. Our results are evidence that identical ESCs containing the nuclear of tooth pulp cells have high therapeutic efficiency in diabetes-induced mice for the prevention of immune rejection and the demonstration that the grafts survive.

Hence, the ESCs derived were named Osmangazi Turk Identical ESCs instead of known names since tooth pulp cells, the cells most similar to the embryonic stage, were first used in this study. Osmangazi Turk Identical ESCs were effective the repair of the endodermic pancreas, an organ with very difficult differentiation. It is easier to the effects of ESCs on cardiomyocytes, which are easier for ESCs to differentiate into. Moreover, the quality of germ cells is an important factor in infertility and human reproduction.[47] When this factor is considered, identical ESCs derived from tooth pulp somatic cells can be used both in reproduction biotechnology and as a good-quality germ cell source for regenerative medicine. Therefore the outcomes of this study obtained with Osmangazi Turk Identical ESCs *in vivo* and *in vitro* may be a source of hope for other diseases. In accordance with our hypothesis, promising findings similar to the results of this study also seem possible with the nucleus pulpous of the intervertebral disks in the form of the remains of the notochord, the second most similar tissue to embryonic tissue in adults. Advanced studies on this subject may make vital contributions to the treatment of diseases that are currently impossible or intricate to treat.

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