General and Comparative Endocrinology 189 (2013) 1–6

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Mechanism of insulin production in canine bone marrow derived mesenchymal stem cells

Hiroshi Takemitsu [a], Dongwei Zhao [a], Shingo Ishikawa [a], Masaki Michishita [b], Toshiro Arai [a], Ichiro Yamamoto [a,*]

[a] Department of Veterinary Science, School of Veterinary Medicine, Nippon Veterinary and Life Science University, 1-7-1 Kyonancho, Musashino, Tokyo 180-8602, Japan
[b] Department of Veterinary Pathology, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180-8602, Japan

ARTICLE INFO

Article history:
Received 18 December 2012
Revised 20 March 2013
Accepted 11 April 2013
Available online 25 April 2013

Keywords:
Canine
Insulin
Pdx1
Beta2
Mafa

ABSTRACT

Insulin is a critical hormone in the regulation of blood glucose levels and is produced exclusively by pancreatic islet beta-cells. Insulin deficiency due to reduced pancreatic islet beta-cell number underlies the progression of diabetes mellitus, prompting efforts to develop beta-cell replacement therapies. However, precise information on beta-cell replacement and differentiation in canines is limited. In this study, we established insulin-producing cells from bone marrow derived mesenchymal stem cells transiently expressing canine pancreatic and duodenal homeobox 1 (Pdx1), beta cell transactivator 2 (Beta2) and V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (Mafa) using a gene transfer technique. Real-time PCR analysis revealed an increase in insulin mRNA expression of transfected cells. And ELISA revealed that insulin protein expressed was detected in cytoplasmic fraction. Insulin immunostaining analysis was performed and observed in cytoplasmic fraction. These results suggest that co-transfection of Pdx1, Beta2 and Mafa induce insulin production in canine BMSCs. Our findings provide a clue to basic research into the mechanisms underlying insulin production in the canines.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent and can differentiate not only into cells of the mesodermal lineage, such as osteoblasts (Dennis and Caplan, 1996), chondrocytes (Johnstone et al., 1998), and adipocytes (Dennis et al., 1999), but also into neurocytes (Kopen et al., 1999), and cardiomyocytes (Toma et al., 2002). Given the appropriate microenvironment, MSCs can differentiate into various tissues. In addition, recent studies suggest that MSCs derived from adipose-tissue (Timper et al., 2006), bone marrow (Moriscot et al., 2005), and pancreatic beta-cell stem cells (Seeberger et al., 2006) have the potential to differentiate and adopt a pancreatic phenotype in humans and mice. However, it is not clear whether canine bone marrow derived stem cells (cBMSCs) have the potential to differentiate into insulin-producing cells.

The pancreatic and duodenal homeobox 1 (Pdx1), beta cell transactivator 2 (Beta2) and V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (Mafa) proteins play important roles in activation of the insulin gene promoter establishment of beta-cell specific insulin expression, and regulation of beta-cell differentiation (Zhao et al., 2005). We recently studied the molecular mechanism underlying the effects of these transcription factors on the canine insulin promoter (Takemitsu et al., 2012), and found that like in humans, they were important for insulin gene expression in canines. Moreover, the Pdx1, Beta2 and Mafa genes play an essential role in differentiation and maturation of the pancreas. A deficit in Pdx1 at the early post-bud stage resulted in pancreatic agenesis in mice (Scott et al., 1989). Naya et al. (1997) demonstrated that mice homozygous for a targeted disruption of Beta2 has abnormal pancreatic islet morphogenesis. In contrast, the role of Mafa in beta-cell development has not been defined. However, the expression of the Mafa gene in beta-cells and its involvement in insulin gene transcription suggest that it contributes to beta-cell development (Matsuoka et al., 2003).

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the development of autoantibodies and destructive T-cell infiltration of insulin-producing islet beta-cells (Gillespie, 2006). Insulin deficiency due to a reduced number of pancreatic islet beta-cells underlies the progression of both T1DM and T2DM. Recently, DM has become a health issue for both humans and in canines. T1DM appears to be the most common form of canine DM (Rand et al., 2004). However the main veterinary clinical protocol for the management of canine DM is based only on supplementation of insulin via injection at the owner's expense. The aim of this study was to generate insulin-producing cells for transplantation.

* Corresponding author. Fax: +81 422 31 7841.
E-mail address: ichiroy@nvlu.ac.jp (I. Yamamoto).

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.04.009

2. Materials and methods

2.1. BMSC isolation and culture

Canine BMSCs were obtained from four young healthy female beagle dogs. They were isolated and cultured essentially as described previously (Takemitsu et al., 2012). To obtain BMSC enriched mono-nucleated cells, 5 ml of bone marrow was aspirated into a syringe and density separation (1.077 g/ml) was performed using Lymphoprep (Axis-Shield, Oslo, Norway). The cells were resuspended in Dulbecco's modified Eagle's medium (D-MEM: Invitrogen Carlsbad, CA, USA) with 10% fetal bovine serum (FBS: HyClone Laboratories, Utah, USA) and 1% antibiotic–antimycotic solution (Invitrogen). The cells were incubated at 37 °C in a humidified 5% CO_2 incubator with 5% CO_2, and the medium was changed twice weekly. When primary cultures reached 70–80% confluence, attached cells were passaged by exposure to 0.25% trypsin and 1 mM EDTA (Invitrogen) for 3 min, and replated at a density of 8.0×10^3 cells/cm^2 for subsequent passage.

2.2. In vitro differentiation

For osteogenic differentiation, passage 2 cBMSCs were plated on 6-well culture plates at a density of 5.0×10^3 cells/cm^2. The medium was changed to osteogenic medium after incubation in control medium for 24 h. The osteogenic medium used was Canine Osteoblast Differentiation Medium. (Cell Applications, San Diego, CA, USA). The medium was changed twice weekly. For osteogenic analysis, alkaline phosphatase was analyzed quantitatively using by Fast red (Sigma, St. Louis, MO, USA) staining at 14 days of cultured.

For adipogenic differentiation, passage 2 cBMSCs were plated on 6-well culture plates at a density of 8.0×10^3 cells/cm^2. The cells were cultured in control medium until confluence, and the medium was then changed to Canine Adipocyte Differentiation Medium (Cell Applications). The medium was changed twice weekly. Oil Red O (Sigma) staining was performed to analyze adipogenesis at 14 days of culture.

For chondrogenic differentiation, passage 2 cBMSCs were collected in 15 mL centrifuge tube (Nunc Roslilde, Denmark) at a density of 1.0×10^5 cells/tube. The suspension was centrifuged for 5 min at $300 \times g$ and collected as a pellet. The pellet was cultured in Canine Chondrocyte Differentiation Medium (Cell Applications). The medium was changed twice weekly. After 14 days of culture, pellet slice to thin sectioned. Toluidine blue (Sigma) staining was performed for proteoglycan analysis.

2.3. Construction and transfection of the expression vector for canine Pdx1, Beta2, and Mafa

Mammalian cell-based expression vectors for Pdx1, Beta2 and Mafa were generated using a pcDNA3.2/V5/GW/D-TOPO Expression Kit (Invitrogen), as described previously (10). In brief, subcloning fragments were generated using the gene specific RT-PCR primers, Pdx1-1 and Pdx1-2, beta2-1 and beta2-2, mafa-1 and mafa-2, respectively (Table 1). The canine Pdx1 (GenBank Accession No. HQ454506), Beta2 (GenBank Accession No. XM_545553) and Mafa (GenBank Accession No. AC197467) sequences were found in the canine genome database. Cloned canine Pdx-1, Beta2 and Mafa expression vectors were sequenced and their mRNA expression was confirmed in a cultured cell line.cBMSCs were plated at 1×10^4/cm^2 in 6-well plates and transfected with 1.6 μg/ml of DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated with D-MEM (Invitrogen) with, 10% FBS, 1% antibiotic–antimycotic solution and 100 μg/ml Geneticin (Invitrogen). Samples were collected 14 days after transfection.

2.4. Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was obtained from cBMSCs transfected with the three transcription factors and from non-transfected cBMSCs. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was measured by spectrophotometry. Total RNA (1 μg) was reverse-transcribed at 42 °C for 15 min in a final volume of 20 μl using the QuantiTect Reverse Transcription Kit (Qiagen, Düsseldorf, Germany). Then reverse transcription was inactivated by heating at 95 °C for 3 min.

The cDNA product was subjected to qRT-PCR according to the manufacturer's instructions for the Real-Time PCR System 7300 (Applied Biosystems, Foster City, CA). qRT-PCR was performed at 95 °C for 5 s and 60 °C for 34 s in 20 μl of buffer containing SYBR premix ExTaq II and ROX Reference Dye (Takara Bio, Shiga, Japan) and 0.2 μM each of the primers, RT-pdx-1-F and RT-pdx-1-R, RT-beta2-F and RT-beta2-R, RT-mafa-F and RT-mafa-R, RT-ins-F and RT-ins-R (Table 1). Quantitative measurement was performed by establishing a linear amplification curve from serial dilutions of plasmid DNA containing the cDNA of each of the transcription factor and insulin.

2.5. ELISA

Protein was obtained from cBMSCs transfected with the three transcription factors and from non-transfected cBMSCs. Protein extraction was performed using the M-PER Mammalian Protein Extraction Reagent (Thermo, Rockford, USA). The proteins were collected and frozen at −80 °C until assay. Insulin was determined using the Dog Insulin ELISA kit (SHIBAYAGI Co., Gunma, Japan) according to the manufacturer's protocol.

2.6. Immunocytochemistry

Immunofluorescent staining was used to assess the expression of the insulin protein in cBMSCs transfected with the three transcription factors and in non-transfected cBMSCs. Transfected cells were cultured in 4-well chamber slides (Nunc, Roskilde, Denmark) until 50% confluence. The cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After washing thrice with PBS, the cells were incubated with blocking solution containing 0.4% Triton X-100 and 4% Block Ace (DS Pharma Biomedical, Osaka, Japan) in PBS at room temperature for 1 h. The cells were incubated with mouse anti-human insulin antibody (Abcam, Tokyo, Japan) diluted in blocking solution at 4 °C for 16 h. Negative control cells were incubated without primary antibody and isotype control cells were incubated with a normal goat anti-mouse IgG antibody (Abdserotec, Oxford, UK). The cells were washed thrice with PBS and incubated with an FITC-labeled secondary anti-rabbit antibody diluted in blocking solution at room temperature for 1 h in the dark. The cells were then washed thrice with PBS and slides were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen). The cells were analyzed under a Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss MicroImaging, Jena, Germany), and image overlay was performed using the Axio Vision Rel.4.6 software (Carl Zeiss MicroImaging).

2.7. Statistical analysis

Data were analyzed using an independent samples *t*-test, and a value of $p < 0.05$ was considered significant. Statistical analyses were performed using the Prism software (GraphPad Software, San Diego, CA, USA).