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Epigenetic conversion of adult dog skin fibroblasts into insulin-secreting cells

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ABSTRACT

Diabetes is among the most frequently diagnosed endocrine disorder in dogs and its prevalence continues to increase. Medical management of this pathology is lifelong and challenging because of the numerous serious complications. A therapy based on the use of autologous viable insulin-producing cells to replace the lost β cell mass would be very advantageous.

A protocol to enable the epigenetic conversion of canine dermal fibroblasts, obtained from a skin biopsy, into insulin-producing cells (EpiCC) is described in the present manuscript. Cells were briefly exposed to the DNA methyltransferase inhibitor 5-azacytidine (5-aza-CR) in order to increase their plasticity. This was followed by a three-step differentiation protocol that directed the cells towards the pancreatic lineage. After 36 days, 38 ± 6.1% of the treated fibroblasts were converted into EpiCC that expressed insulin mRNA and protein. Furthermore, EpiCC were able to release insulin into the medium in response to an increased glucose concentration. This is the first evidence that generating a renewable autologous, functional source of insulin-secreting cells is possible in the dog. This procedure represents a novel and promising potential therapy for diabetes in dogs.

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Introduction

The prevalence of diabetes mellitus is increasing among animal species, becoming one of the most frequently diagnosed endocrinopathies in dogs. Prevalence of this disease continues to increase and it is estimated that 1/200 dogs will develop the pathology. During the last 10 years, a large number of studies have focused on the aetiology of canine diabetes and have concluded that the disease is diagnosed mainly in certain breeds; however, aetiopathogenesis of diabetes mellitus in dogs remains unclear for the majority of diagnosed cases (Davison, 2015). The progression from normal to glucose intolerant and then overt diabetes is generally slow, so that most islets (>90%) are lost before clinical signs of diabetes occur (Vrabelova et al., 2014).

The majority of dogs with diabetes suffer from a deficiency in insulin production (referred to as type I diabetes in humans) and are dependent on injections of exogenous insulin (Feldman and Nelson, 1996). Unfortunately, the human system of classification of diabetes is not entirely applicable to dogs (Ciobotaru, 2013). At present, two forms of diabetes have been described in the dog (Catchpole et al., 2005): an insulin-deficiency diabetes (IDD) and an insulin-resistance diabetes (IRD). Although some similarities exist, neither matches the human forms of the disease exactly.

Medical management of diabetes poses a number of challenges to the owner because of a vast array of complications (Labato and Manning, 1997; Fleeman and Rand, 2001) and up to 40% of owners elect to euthanase their dogs (Vrabelova et al., 2014). Therefore, these animals would benefit from an innovative therapy able to simplify their management. Furthermore, despite the high prevalence of the disease, its therapy in dogs has evolved very little and diabetic dogs today are mainly treated as they were 50 years ago. It is evident that an alternative source of viable insulin-producing cells to replace their lost β cell mass would be very useful for these dogs.

In other species, mostly humans and mice, studies are progressing to achieve this result through the derivation of autologous induced pluripotent cells (iPS) that are then converted into insulin producing cells (Tateishi et al., 2008; Hanna et al., 2009; Maehr et al., 2009; Nostro et al., 2011; Thatava et al., 2011). Canine iPS have been recently derived from adult and embryonic fibroblasts (Koh and Piedrahita, 2014), by either retro- or lentiviral transduction of dog (Shimada et al., 2010), human (Lee et al., 2011; Luo et al., 2011), or mouse (Koh et al., 2013) pluripotency factors. However, in dogs, the directed conversion of iPS into a specific cell type has proved to be very challenging and has been described only in two cases obtaining platelets (Nishimura et al., 2013) or mesenchymal stromal cells (Whitworth et al., 2014), respectively.

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Therefore, the aim of the present study was to determine if it is possible to efficiently convert canine fibroblasts, obtained from a skin biopsy, into insulin-producing cells using an innovative method based on epigenetic cell conversion that we recently developed in humans and pigs.

Materials and methods

Chemicals

All chemicals were purchased from Life Technologies unless otherwise indicated.

Sample collection

Skin biopsies were collected from three adult healthy animals (two mixed breeds and one Labrador Retriever) under general anaesthesia for elective surgery and after signed informed consent of the owners and institutional ethical committee approval (The Ethics Committee of the University of Milano, approval number 65/13, 18 December 2013). The dogs were determined to be healthy based on physical examination, complete blood count analysis and serum biochemistry profile.

Skin fibroblasts isolation

Small skin fragments were plated in a 0.1% gelatin (Sigma) pre-coated Petri dish (Sarstedt) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% glutamine, and 1% antibiotics (fibroblast culture medium) (Farese et al., 2004). After 4 days, fibroblasts started to grow out and fragments were removed. Cells were propagated in T-25 flasks (Sarstedt) using fibroblast culture medium. For passaging, they were trypsinised by adding 600 µL of trypsin-EDTA solution and incubated for 5 min at 37 °C until cells began to detach from the bottom of the culture dishes and to dissociate from one another. Cell suspension was diluted with nine parts of fibroblast culture medium in order to neutralise trypsin action and plated in new culture dishes in a 1:3 ratio. Fibroblasts were passaged twice a week. The three primary cell cultures obtained from the three dogs were used for all experiments described below, and each experiment was run in triplicate.

Fibroblast growth curve

Growth curve assessment was carried out by plating 1.5×10^5 cells/well in 24-well multidishes (Nunc). Cell number was counted using KOVA Glasstic (Fisher Scientific) at 24, 36, 48, 72, and 96 h from plating. Cell viability was determined by trypan blue dye exclusion assay. Cells for each point of each biological replicate were assessed in triplicate.

5-aza-CR treatment

Fibroblasts were plated in 4-well multidish (Nunc) previously treated with 0.1% gelatin (Sigma) at a concentration of 7.8×10^4 fibroblasts/cm^2. They were then incubated with 1 µM 5-aza-CR (Sigma) dissolved in fibroblast culture medium for 18 h. Concentration and time of exposure were selected according to our previous work (Pennarossa et al., 2013a, 2014; Brevini et al., 2014). Cells were then cultured for 3 h with embryonic stem cell (ESC) culture medium (Vaags et al., 2009; Brevini et al., 2010).

Pancreatic induction

Cell differentiation was induced by a three-step protocol originally developed for the pancreatic induction of mouse ESC (Shi et al., 2005) and subsequently adapted to 5-aza-CR treated human (Pennarossa et al., 2013a) and porcine fibroblasts (Pennarossa et al., 2014). Briefly, cells were cultured in DMEM: Nutrient Mixture F-12 (DMEM/F12) supplemented with 1% B27, 1% N2, 0.1 mM β-mercaptoetanol (Sigma), 2 mM glutamine (Sigma), 1 mM MEM Non-essential amino acids and 0.05% bovine serum albumin (BSA, Sigma). Between day 1 and day 6, medium was enriched with 30 ng/mL activin A. On the following 2 days, 10 µM retinoic acid (Sigma) was added. From day 9 onwards, a medium containing 1% B27, 20 ng/mL basic fibroblast growth factor and 1% insulin–transferrin–selenium was used and changed daily. Cells were cultured for a total of 36 days.

Karyotype

Cells were treated with colcemid. Chromosomes were prepared on slides and stained in Giemsa (KaryoMAX Giemsa stain solution). Samples were observed under a Leica HC microscope and images were analysed using a CW4000 Karyo software (Leica). A total of 30 metaphases were analysed in triplicate.

Immunocytochemistry

Fibroblasts and epigenetically converted insulin-producing cells (EpiCC) were fixed, permeabilised and blocked with PBS containing 5% normal goat serum. They were then incubated with primary antibodies (Appendix: Supplementary Table S1), which have been shown to react with canine antigens or tested on their canine positive control tissues. Subsequently, cells were incubated with secondary antibodies (Alexa Fluor) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 45 min and analysed under a Nikon Eclipse TE200 microscope. Samples incubated with primary isotypic antibody were used as a control. Three-dimensional spherical structures were dissociated and deposited onto glass slides by Cytospin centrifugation (Cytospin 4, Thermo Scientific).

Western blot

Constitutive proteins were extracted using ReadyPrep protein extraction kit (Bio-Rad) and quantified by Coomassie blue-G dye-binding methods (Read and Northcote, 1981). One hundred micrograms of proteins was diluted in Laemmli sample buffer (Sigma). Proteins were separated on a sodium dodecyl sulfate–polyacrylamide gel (20% for low molecular weight proteins and 10% for β-actin) and blotted onto 0.2-µm and 0.45-µm pore size nitrocellulose filters (Hybond-C Extra, GE Healthcare) for low molecular weight proteins and β-actin respectively. Membranes were blocked with saline solution containing detergent and casein, and then probed with primary antibodies (Appendix: Supplementary Table S1) diluted in WesternBreeze antibody diluent (ThermoFisher Scientific) according to the manufacturer's recommendations. Bands were visualised using WesternBreeze Chemiluminescent kit (ThermoFisher Scientific) following the manufacturer's instructions. Samples from each dog were tested separately and the experiment was run in triplicate.

Assessment of insulin release

Untreated fibroblasts and EpiCC were subjected to glucose stimulation as previously described (Pennarossa et al., 2013a). To avoid possible confounding effects by the insulin content of the culture medium, cells were rinsed five times with PBS before being exposed for 1 h to 5 mM D-glucose or 20 mM D-glucose in Nutrient Mixture F-12 (DMEM/F12) without ITS. Insulin release in cell supernatants was analysed using canine insulin ELISA kit (Mercodia) and following the manufacturer's instruction. Since detection limit of the ELISA kit was 0.23 µU/mL, and its dynamic range was 0.46–0.034 mU/mL, samples were diluted 1:100 before analysis. The intra- and inter-assay CV were ≤5%. Samples from each dog were tested separately and experiments were run in triplicate.

DNA methylation analysis

DNA was extracted with TRIzol (ThermoFisher Scientific) and its concentration was assessed using NanoDrop 8000 (Thermo Scientific). Extracted DNA (0.8 ng) was resuspended in a total volume of 2 µL and spotted onto nylon membranes (Hybond-N+, GE Healthcare). The latter was incubated with the anti-5-Methylcytidine antibody (Appendix: Supplementary Table S1) and dots were visualised by WesternBreeze Chemiluminescent kit. Densitometric analyses were performed using the Image J analysis software (National Institutes of Health). Tests were carried out in triplicate for each sample.

Results

Fibroblasts obtained from dorsal skin biopsies grew out of the original explants within 4 days and formed a monolayer (Fig. 1A), displaying a standard elongated morphology and a vigorous growth in culture typical of this cell population (doubling time, 18–24 h; Fig. 1B). Established primary cultures showed uniform immune-positivity for the fibroblast specific marker vimentin and complete absence of pancreatic related markers C-peptide (C-PEP) and insulin (INS, Fig. 1A).

Following the exposure to 5-aza-CR, cell phenotype changed and fibroblast elongated morphology (T0) disappeared, with cells adopting an oval or round shape (Fig. 2A). Treated cells appeared smaller with granular cytoplasm and vacuolated, larger nuclei (Post 5-aza-CR). These nuclear changes were functionally accompanied by a decrease of global DNA methylation, as demonstrated by a lower 5-methylcytidine signal intensity that gradually returned to the initial levels observed in untreated fibroblasts within 3 days of pancreatic induction (Fig. 2B).

The changes observed in 5-aza-CR treated fibroblasts were reversible since cells re-acquired their elongated morphology and resumed vimentin immune-positivity (Fig. 3A), when they were

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