1 **Original Article**
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5 # 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 many serious complications that may arise in the patient. 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 that allows the epigenetic conversion of canine dermal fibroblasts, obtained from a skin biopsy, into epigenetically converted 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 contained insulin mRNA and protein. Furthermore, EpiCC were able to release insulin into the medium in response to an increased glucose concentration. To our knowledge, this is the first demonstration that it is possible to generate a renewable autologous, functional source of insulin-secreting cells in the dog. This procedure represents a novel and promising potential therapy of diabetes in the dog.

Keywords: Diabetes; Dog; Epigenetic conversion; Fibroblasts; Pancreatic β cells

Introduction

The growing diabetes epidemic is not limited to humans. In fact, 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 one in 200 dogs will develop the pathology. During the last 10 years, a large number of studies have focused on the aetiology of dog 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, to 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 canine diabetics 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 dog (Ciobotaru, 2013). At present, two forms of diabetes have been described in the dog (Catchpole, 2005): an insulin-deficiency diabetes (IDD) and 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 dog (Vrabelova et al., 2014). Therefore these patients would benefit from an innovative therapy able to simplify their management.

64 Furthermore, despite the high prevalence of the disease, its therapy in this species has
65 evolved very little and diabetic dogs today are mainly treated as they were 50 years ago. It is
66 evident that these patients would greatly benefit from an alternative source of viable insulin-
67 producing cells to replace their lost β cell mass.
68
69 In other species, mostly human and mouse, studies are progressing to achieve this
70 result through the derivation of autologous induced pluripotent cells (iPS) that are then
71 converted into insulin producing cells (Tateishi et al., 2008; Hanna et al., 2009; Maehr et al.,
72 2009; Zhang et al., 2009; Nostro et al., 2011; Thatava et al., 2011, 2013).
73
74 Canine iPS have been recently derived from adult and embryonic fibroblasts (Koh and
75 Piedrahita, 2014), by either retro- or lentiviral transduction of dog (Shimada et al., 2010),
76 human (Lee et al., 2011; Luo et al., 2011), or mouse (Koh et al., 2013) pluripotency factors.
77 However, in this species, the directed conversion of iPS into a specific cell type has proved to
78 be very challenging and has described only in two cases obtaining platelets (Nishimura et al.,
79 2013) or mesenchymal stromal cells (Whitworth et al., 2014) respectively.
80
81 Therefore, aim of the present study is to determine if it is possible to efficiently convert
82 canine fibroblasts, obtained from a skin biopsy, into insulin-producing cells using an
83 innovative method based on epigenetic cell conversion that we recently developed in human
84 and pig.
85
86 **Materials and methods**
87 *Chemicals*
88 All chemicals were purchased from Life Technologies unless otherwise indicated.

89

90 *Sample collection*

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

97

98 *Skin fibroblasts isolation*

99 Small skin fragments were plated in a 0.1% gelatin (Sigma) pre-coated Petri dish
100 (Sarstedt) and cultured in in Dulbecco's Modified Eagle's Medium (DMEM) supplemented
101 with 20% fetal bovine serum (FBS), 1% glutamine, and 1% antibiotics (fibroblast culture
102 medium) (Farese et al., 2004). After 4 days, fibroblasts started to grow out and fragments
103 were removed. Cells were propagated in 25-cm T-flasks (Sarstedt) using fibroblast culture
104 medium. For passaging, they were trypsinsed by adding 600 μL of trypsin-EDTA solution
105 and incubated for 5 min at 37 °C until cells began to detach from the bottom of the culture
106 dishes and to dissociate from one another. Cell suspension was diluted with 9 parts of
107 fibroblast culture medium in order to neutralize trypsin action and plated in new culture
108 dishes in a 1:3 ratio. Fibroblasts were passaged twice a week.

109

110 The three primary cell cultures obtained from the 3 dogs were used for all experiment
111 described below, and each experiment was run in triplicates.

112

113 *Fibroblast Growth Curve*

114 Growth curve assessment was carried out by plating 1.5×10^5 cells/well in 24-well
115 multidishes (Nunc). Cell number was counted using Hycor KOVA Glasstic (Fisher) at 24 h,
116 36 h, 48 h, 72 h, and 96 h from plating. Cell viability was determined by trypan blue dye
117 exclusion assay. Cells for each point of each biological replicate were assessed in triplicate.
118
119 *5-aza-CR treatment*
120 Fibroblasts were plated in 4-well multidish (Nunc) previously treated with 0.1%
121 gelatin (Sigma) at a concentration of $7.8 \text{ X } 10^4$ fibroblasts/cm^2. They were then incubated
122 with 1 μM 5-aza-CR (Sigma) dissolved in fibroblast culture medium for 18 h. Concentration
123 and time of exposure were selected according to our previous work (Pennarossa et al., 2013;
124 Brevini et al., 2014; Pennarossa et al., 2014). Cells were then cultured for 3 h with embryonic
125 stem cell (ESC) culture medium (Vaags et al., 2009; Brevini et al., 2010).
126
127 *Pancreatic induction*
128 Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
129 (DMEM/F12) supplemented with 1% B27, 1% N2, 0.1 mM β-mercaptoetanol (Sigma), 2 mM
130 glutamine (Sigma), 1 mM MEM Non-essential amino acids and 0.05% bovine serum albumin
131 (BSA, Sigma). Between day 1 and day 6, medium was enriched with 30 ng/mL activin A. The
132 following 2 days, 10 μM retinoic acid (Sigma) was added. From day 9 onward, a medium
133 containing 1% B27, 20 ng/mL basic fibroblast growth factor and 1% insulin–transferrin–
134 selenium was used and changed daily. Cells were cultured for a total of 36 days.
135
136 *Karyotype*
137 Cells were treated with colcemid. Chromosomes were prepared on slides and stained
138 in Giemsa (KaryoMAX Giemsa stain solution). Samples were observed under a Leica HC

139 microscope and images were analysed using a CW4000 Karyo software (Leica). A total of 30
140 metaphases were analysed in triplicate.
141
142 *Immunocytochemistry*
143 Fibroblasts and epigenetically converted insulin-producing cells (EpiCC) were fixed,
144 permeabilized and blocked with PBS containing 5% normal goat serum. They were then
145 incubated with primary antibodies (Table 1), that have been shown to react with canine
146 antigens or tested on their canine positive control tissues. Subsequently, cells were incubated
147 with secondary antibodies (Alexa Fluor) and 4',6-diamidino-2-phenylindole (DAPI, Sigma)
148 for 45 min and analysed under a Nikon Eclipse TE200 microscope. Samples incubated with
149 primary isotypic antibody were used as a control.
150
151 Three-dimensional spherical structures were dissociated and deposited onto glass
152 slides by cytospin centrifugation (Cytospin 4, Thermo Shandon).
153
154 *Western blot*
155 Constitutive proteins were extracted using ReadyPrep protein extraction kit (Bio-Rad)
156 and quantified by Coomassie blu-G dye-binding methods (Read and Northcote, 1981). One
157 hundred micrograms of proteins were diluted in Laemmli sample buffer (Sigma). Proteins
158 were separated on a sodium dodecyl sulfate (SDS) -polyacrylamide gel (20% for low
159 molecular weight proteins and 10% for β-actin) and blotted onto 0.2 μm and 0.45 μm pore
160 size nitrocellulose filters (Hybond-C Extra, GE Healthcare), for low molecular weight
161 proteins and β-actin respectively. Membranes were blocked with saline solution containing
162 detergent and casein, and then probed with primary antibodies (Table 1) diluted in Western
163 Breeze antibody diluent according to the manufacturer's recommendations. Bands were

164 visualised using WesternBreeze chemiluminescent kit following the manufacturer's
165 instructions. Samples from each dog were tested separately and the experiment was run in
166 triplicates.
167
168 *Assessment of insulin release*
169 Untreated fibrobalsts and EpiCC were subjected to glucose stimulation as previously
170 described (Josefsen et al., 1998). To avoid possible confounding effects by the insulin content
171 of the culture medium, cells were rinsed five times with PBS before being exposed for 1 h to
172 5 mM D-glucose or 20 mM D-glucose in Nutrient Mixture F-12 (DMEM/F12) without ITS.
173 Insulin release in cell supernatants was analysed using canine insulin ELISA kit (Mercodia)
174 and following the manufacturer's instruction. Since etection limit of the ELISA kit was 0.23
175 µU/ml , and its dynamic range was0.46 µU/ml – 0.034 mU/ml, samples were diluted 1:100
176 before analysis. The intra- and inter- assay CV were ≤ 5%. ,
177 Samples from each dog were tested separately and experiments were run in triplicates.
178
179 *DNA methylation analysis*
180 DNA was extracted with Trizol and its concentration was assessed using
181 NanoDrop8000 (Theromoscientific). Extracted DNA (0.8 ng) was resuspended in a total
182 volume of 2 µL and spotted onto nylon membranes (Hybond-N+, GE Heathcare). The latter
183 was incubated with the anti- 5-Methylcytidine antibody (Table 1) and dots were visualised by
184 WesternBreeze chemioluminescence kit. Densitomentric analyses were performed using the
185 Image J analysis software (National Institutes of Health). Tests were carried out in triplicate
186 for each sample.
187
188 **Results**

189 Fibroblasts obtained from dorsal skin biopsies grew out of the original explants within
190 4 days and formed a monolayer (Fig. 1A), displaying a standard elongated morphology and a
191 vigorous growth in culture typical of this cell population (doubling time, 18-24 h; Fig. 1B).
192 Established primary cultures showed uniform immune-positivity for the fibroblast specific
193 marker vimentin and complete absence of pancreatic related markers C-peptide (C-PEP) and
194 insulin (INS, Fig. 1A).
195
196 Following the exposure to 5-aza-CR, cell phenotype changed and fibroblast elongated
197 morphology (T0) disappeared, with cells adopting an oval or round shape (Fig. 2A). Treated
198 cells appeared smaller with granular cytoplasm and vacuolated, larger nuclei (Post 5-aza-CR).
199 These nuclear changes were functionally accompanied by a decrease of global DNA
200 methylation, as demonstrated by a lower 5-Methylcytidine signal intensity that gradually
201 returned to the initial levels observed in untreated fibroblasts within 3 days of pancreatic
202 induction (Fig. 2B).
203
204 The changes observed in 5-aza-CR treated fibroblasts were reversible since cells re-
205 acquired their elongated morphology and resumed vimentin immunopositivity (Fig. 3A),
206 when they were returned to standard fibroblast culture medium. Cells maintained a normal
207 karyotype along the entire conversion process (Fig. 3B).
208 Cell differentiation was induced by a three-step protocol originally developed for the
209 pancreatic induction of mouse ESC (Shi et al., 2005) and subsequently adapted to 5-aza-CR
210 treated human (Pennarossa et al., 2013a) and porcine fibroblasts (Pennarossa et al., 2014).
211
212 During the first 7 days of culture, cells acquired a flat morphology and slowly
213 arranged in clusters. The use of retinoic acid in combination with activin A (second step)

214 drove cells to organise in a reticular pattern that evolved into distinct cell aggregates (10 day,
215 Fig. 2A). During the last step, the recruitment of cells increased forming large three-
216 dimensional colonies that tended to detach and float freely as individual spherical structures
217 around day 36 of culture (36 day, Fig. 2A).

218

219 In agreement with these morphological changes, we detected cell immune-positivity
220 for the pancreatic markers C-peptide and insulin (C-PEP and INS, Fig. 4A) that co-localized
221 in cells. Efficiency towards β cell differentiation was38 ± 6.1% as measured by counting cells
222 that co-expressed C-peptide and insulin. Pancreatic conversion was further confirmed by
223 western blotting analysis (Fig. 4B). Insulin and C-peptide, originally undetectable in untreated
224 fibroblasts (T0, Figs. 1A and 4B), were observed after 36 days of pancreatic induction (36
225 day, Fig. 4B). Cell functional conversion was demonstrated by their ability to respond to a
226 20mM D-glucose exposure. Insulin release in cell supernatants after 1 h exposure
227 demonstrated that glucose triggered a dynamic response in canine EpiCC, inducing active
228 insulin secretion in the culture medium (Fig. 4C). In contrast, untreated fibroblasts were not
229 able to respond to glucose challenge (Fig. 4C).

230

231 **Discussion**

232 The present experiments demonstrate, for the first time, the possibility to generate a
233 renewable autologous source of pancreatic endocrine cells in the dog, through a brief
234 exposure to a de-methylating agent and a subsequent tissue specific induction protocol,
235 avoiding the forced expression of transcription factors or microRNAs and the acquisition of a
236 stable pluripotent state (Pennarossa et al., 2013; Brevini et al., 2014; Pennarossa et al., 2014).
237 In order to induce a global hypomethylation of canine fibroblasts, we selected the
238 registered drug, 5-aza-CR, which is a direct inhibitor of methyltransferases. It was previously

239 used to reactivate silent genes and to alter differentiation states of eukaryotic cells (Taylor and
240 Jones, 1979; Glover et al., 1986). In agreement with this, our results demonstrate that 5-aza-
241 CR treated fibroblasts showed a global DNA demethylation, indicating that this compound
242 was able to induce a higher plasticity state. At the same time, it is interesting to note that this
243 'permissive' state was transient and reversible, since post 5-aza-CR skin fibroblasts reverted to
244 their original phenotype, when returned to their standard culture medium, after removal of the
245 drug. This data indicate that no toxic, irreversible effects are involved in the conversion
246 process and support the safety of epigenetic conversion.
247 The direct conversion of fibroblasts into insulin secreting cells has been obtained only
248 in the mouse (Li et al., 2014). However in this case the process required the use of a
249 transgenic cell line containing the doxycycline (Dox)-inducible form of the conventional four
250 iPS factors (Oct4, Sox2, Klf4, and c-Myc). Furthermore, the efficiency was limited to 2% and
251 the cells were unable to respond to a glucose challenge. Therefore this protocol is much less
252 efficient that the one described in the present paper and the resulting cells were not functional.
253 In addition, it is also important to note that the approach of Li et al. (2014) is only feasible
254 using genetically modified cells. This would not be allowed in any clinical application neither
255 in dogs nor in humans.
256
257 Dramatic changes in cell morphology paralleled the induction of a higher plasticity
258 state. Cells appeared smaller in size and displayed larger nuclei. Interestingly, nuclear
259 enlargement was previously associated by Niwa (2007) to increased plasticity and relaxed
260 chromatin. Previous work reported chromosomal abnormalities and found tetraploid cell
261 transformation after treatment of mouse fibroblasts with reversine (Anastasia et al., 2006).
262 Similar findings have been reported in dog iPS with evidence for the emergence of low-level
263 aneuploidy during prolonged culture or tumour formation (Koh et al., 2013). We thoroughly

264 investigated this aspect and our results indicate that 5-aza-CR did not alter cell karyotype. In
265 agreement with our findings, Davidson and coworkers previously demonstrated that 5-aza-CR
266 treated cells exhibited a normal karyotype (Davidson et al., 1992). In our understanding, these
267 diverging results can be attributed to a different susceptibility of chromosomes to the two
268 synthetic peptides, although species-related aspects may be involved and cannot be ruled out.
269
270 In the present manuscript we demonstrate that post 5-aza-CR dog fibroblasts can be
271 re-addressed to pancreatic β cell-like cells, in response to specific differentiation conditions.
272 This indicates that epigenetic conversion is able to switch canine cells from a mesoderm
273 derived population to an endoderm specific lineage. The result obtained in the dog, confirms
274 previous experiments carried out in human as well as in porcine (Pennarossa et al., 2013a,
275 2014). It also further supports the robustness of epigenetic cell conversion as an approach that
276 allows adult somatic cell phenotype switch through the use of epigenetic modifiers (Thoma et
277 al., 2014; Mirakhori et al., 2015).
278
279 Insulin, which was originally undetectable in untreated skin fibroblasts, is actively
280 produced at the end of pancreatic induction. Furthermore, canine EpiCC were able to respond
281 to a 20 mM D-glucose exposure. Indeed, ELISA results showed that epiCC actively release
282 insulin after 1 h challenge, demonstrating that changes in ambient glucose were able to trigger
283 a dynamic response. This suggests that EpiCC have the ability to respond to the primary and
284 physiological stimulus for insulin secretion and supports the achievement of a mature
285 differentiated and functional phenotype.
286
287 The extreme difficulty to obtain functional dog pancreatic islets made it so far
288 impossible, to directly compare the level of insulin secretion between natural beta cells and

289 EpiCC. However, previous results, obtained in other species, showed that EpiCC, producing a
290 similar amount of insulin, were able to restore and maintain normoglycemia in diabetic mice
291 (Pennarossa et al., 2013, 2014). This led us to hypothesize that the level of insulin secreted by
292 dog EpiCC is likely to reach a functional level.
293
294 If these observation were confirmed by the engraftment of EpiCC in diabetic dogs, this
295 treatment would provide patients with an autologous self-regulating source of insulin that
296 would greatly increase the ease of maintaining glycaemia within its physiological range, with
297 no need for daily injections nor danger of hyper- or hypoglycaemic events.
298
299 **Conclusions**
300 This is the first report that describes the conversion of dog dermal fibroblasts into
301 insulin secreting cells, that may represent an alternative, functional and autologous source for
302 the production of this hormone. This work confirms and expands our previous results and
303 extends to the dog the advantages of an epigenetically-based cell conversion, laying the basis
304 for a promising therapeutic perspective for diabetes in this species. Since the same method is
305 effective in humans, its availability in the dog represents a useful tool for translational studies.
306
307 **Conflict of interest statement**
308 None of the authors of this paper have a financial or personal relationship with other
309 people or organizations that could inappropriately influence or bias the content of the paper.
310
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318

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473

474 **Table 1**

475 List of antibodies and working dilutions used for immunocytochemical and western blot

476 analysis.

477

478

Antibody	Host species	Company	ICC working dilution	WB working dilution
Anti- Ovalbumin-conjugated 5-Methylcytidine	Mouse	Eurogentec	-	1:500 (dot blot)
Anti-human vimentin	Mouse	Abcam	1:20	-
Anti-human insulin	Chicken	Abcam	1:100	1:500
Anti-human C-peptide	Rabbit	Abcam	1:100	1:100
Anti- β-actin N-terminal peptide	Mouse	Sigma	-	1:1000

479

480 **Figure legends**

481

482 Fig. 1 Characterization of skin-derived fibroblasts. (A) Fibroblasts obtained from dog dorsal
483 skin biopsies formed a monolayer and displayed a standard elongated morphology (T0).
484 Uniform positivity for vimentin (VIM), the typical fibroblast intermediate filament protein,
485 indicated a homogenous cell population at the onset of the experiments. Untreated fibroblasts
486 showed no signals of pancreatic endocrine markers C-peptide (C-PEP) and insulin (INS).
487 Nuclei were stained with DAPI. Scale bars = 100 μm. (B) Cells displayed a vigorous growth
488 in culture typical of this cell population with a doubling time of 18-24 h. Bars represent the
489 SD of three independent replicates.

490

491 Fig. 2 Morphological changes and DNA methylation pattern during epigenetic conversion of
492 dog skin fibroblasts into insulin-secreting cells. (A) Untreated cells (T0) underwent marked
493 morphological changes in response to an 18 h exposure to 5-aza-CR (Post 5-aza-CR).
494 Fibroblasts changed their typical elongated shape into a round epithelioid aspect. Cell size
495 was smaller, and nuclei became larger and more granular. In response to the addition of
496 activin A and retinoic acid, cells rearranged in a reticular pattern and clustered in
497 distinguishable aggregates (10 day). These formations progressed with time and were further
498 stimulated by B27/bFGF/ITS, which led to the recruitment of a growing number of cells,
499 aggregating in large three.dimensional spherical structures, reminiscent of typical pancreatic
500 islets in vitro (36 day). Scale bars = 100 μm. (B) Global methylation pattern of canine dermal
501 fibroblasts exposed to 5-aza-CR during their pancreatic differentiations. Histogram represents
502 dot-blot signal intensity quantified by densitometric analysis using Image J analysis software
503 (National Institutes of Health). Bars represent the mean ± SD of 9 independent replicates.

504

505 Fig. 3 The effect of 5-aza-CR is reversible. (A) When 5-aza-CR treated fibroblasts were
506 returned to standard culture medium, they reverted to the original morphology and resumed
507 vimentin expression (VIM). Nuclei were stained with DAPI. Scale bar = 100 μm. (B) Cells
508 maintained a normal karyotype throughout the entire length of the experiment.
509
510 Fig. 4 Morphological characterization of canine EpiCC. (A) Immunostaining of EpiCC after
511 36 days of culture reveals a clear signal of C-peptide (C-PEP) and insulin (INS). Scale bars =
512 100 μm.) (B) Representative western blot analysis of Insulin and C-peptide in untreated
513 fibroblasts (T0) and EpiCC after 36 days of culture (36 day). β actin was used to check that an
514 equal protein amount was loaded on each lane. (C) Quantification of insulin release in the
515 culture medium in response to 20 mM D-glucose and 5 mM D-glucose for 1 h. Bars represent
516 the mean ± SD of 9 independent replicates.