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Generation of LIF-independent induced pluripotent stem cells from canine fetal fibroblasts

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ABSTRACT

Takahashi and Yamanaka established the first technique in which transcription factors related to pluripotency are incorporated into the genome of somatic cells to enable reprogramming of these cells. The expression of these transcription factors enables a differentiated somatic cell to reverse its phenotype to an embryonic state, generating induced pluripotent stem cells (iPSCs). iPSCs from canine fetal fibroblasts were produced through lentiviral polycistronic human and mouse vectors (hOSKM/mOSKM), aiming to obtain pluripotent stem cells with similar features to embryonic stem cells (ESC) in this animal model. The cell lines obtained in this study were independent of LIF or any other supplemental inhibitors, resistant to enzymatic procedure (TrypLE Express Enzyme), and dependent on bFGF. Clonal lines were obtained from slightly different protocols with maximum reprogramming efficiency of 0.001%. All colonies were positive for alkaline phosphatase, embryoid body formation, and spontaneous differentiation and expressed high levels of endogenous *OCT4* and *SOX2*. Canine iPSCs developed tumors at 120 days post-injection in vivo. Preliminary chromosomal evaluations were performed by FISH hybridization, revealing no chromosomal abnormality. To the best of our knowledge, this report is the first to describe the ability to reprogram canine somatic cells via lentiviral vectors without supplementation and with resistance to enzymatic action, thereby demonstrating the pluripotency of these cell lines.

1. Introduction

The isolation and derivation of embryonic canine stem cells lines (cESCs) can be performed only with leukemia inhibitory factor (LIF) and fibroblast growth factor 2 (FGF2), but full characterization and in vitro maintenance of ESCs have not been fully described [1]. The production of induced pluripotent stem cells (iPSCs) [2–4] created a new way for obtaining pluripotent cells and studying their applicability in clinical trials and therapies. In veterinary medicine, there have been few previous reports describing and characterizing canine somatic cell-derived iPSCs [5–9,22]. However, only two reports have evaluated tumor formation of these cells [8,22].

Animal models represent a valuable tool in the field of translational research and may assist in the development of new therapeutic strategies for human regenerative medicine. In addition, animal models may provide support and acceptance in the field of stem cell research and therapy [10]. The canine model (*Canis lupus familiaris*) shares at least half of the more than 400 hereditary canine diseases with humans [11], thus representing an acceptable translational research model. For example, the IGF1R-overexpressing mammary carcinoma canine model [12] has been demonstrated as a new model to study new therapies targeting breast cancer in humans. In addition, the canine model also represents a unique naturally occurring model of genetic diseases such as myelopathy (CDM) for amyotropic lateral sclerosis (ALS) in

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humans [13].

However, derivation of cESCs for therapeutic approaches is still difficult, and the isolated cESCs are not fully characterized to ensure their safety. Hence, the development of canine iPSCs holds a great potential as an alternative option for the development of effective therapeutic treatments and pre-clinical trials.

In this context, this study tested two alternative reprogramming factors isolated from mouse and human to optimize an appropriate reprogramming method to produce canine iPSCs. These experiments aimed at increasing knowledge of the factors required in the reprogramming process of canine cells and the production of stable canine iPSCs with complete characterization.

2. Materials and methods

2.1. Cell culture

Canine fetal fibroblasts (CFFs) were derived from one 15-day-old gestational embryo. The somites were dissected, washed in saline solution (phosphate buffer saline, PBS), fragmented and disaggregated by pipetting, and the resulting cells were cultured in IMDM medium (Gibco, Life Science) supplemented with 10% fetal bovine serum (Gibco, Life Science) and 0.1% penicillin/streptomycin (Gibco, Life Science). The same medium was used to maintain 293 FT packaging cells (Life Technologies) and mouse embryonic fibroblasts (MEFs). Culture medium was changed every 48 h. All canine iPSCs were generated from CFFs between passage 2 or 3 and maintained in iPSC media, consisting of DMEM/F12 Knockout (Gibco), 20% Knockout Serum Replacement (Invitrogen), 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Sigma Aldrich), 0.1 mM b-mercaptoethanol (Gibco) and bFGF (10 ng/mL; BD Bioscience). All experiments performed were approved by the Ethics Committee of Animal Use (Protocol No. 2377/2011).

2.2. Feeder cells

MEFs were isolated from 13- to 14-day-old Swiss mouse fetuses, and cells at passages 1–3 were used as a feeder monolayer at a concentration of 1×10^5 cells in 6 well dishes. MEFs were treated with mitomycin before being used as monolayers.

2.3. Lentiviral production and transduction of canine fibroblasts

STEMCCA polycistronic lentiviral vectors [14] expressing human OCT4, SOX2, KLF4, and c-MYC (hOSKM) or murine (mOSKM) were used to transfect 293 FT cells. The STEMCCA vector is comprised of human or mouse OCT4, KLF4, SOX2, and c-MYC (OKSM) transcription factors separated by the self-cleaving 2A peptide and IRES sequences driven by the EF-1alpha constitutive promoter (Millipore SCR 544 [14]). The following day after plating 5×10^6 293FT cells in 100 mm plates, the cells were transfected with 12 μg of STEMCCA vector, 1.2 μg of auxiliary vector and 2.4 μg of packaging VSVG vector using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The mixture of DNA/Lipofectamine was incubated overnight, and the supernatant (culture medium) containing viral particles was recovered after 24 and 48 h. The supernatant was filtered and used the following day. hOSKM or mOSKM was used separately or in combination to reprogram CFFs. CFFs were plated at 1.5×10^4 per well in a 6-well plate and transduced with 1 mL of lentiviral supernatant. The following day, the medium containing lentiviral particles was removed from the canine fibroblasts, and a second volume of supernatant from the transfected 293FT cells was added (two transductions). After 24 h, the medium was removed and replaced with fresh 293FT medium. After 5 days, transduced fibroblasts were passaged onto MEFs and cultured in iPS medium for a minimum of 14 days. IPSC colonies were observed as early as 11 days on MEFs, and clonal lines were manually isolated using a scalpel and transferred to a new feeder plate with MEFs. The following passages were successfully generated by the enzyme dissociation process (Tryple Express Enzyme, Thermo Fisher Scientific). The iPSCs were incubated with 250 μL/mL TrypLE for 1 min and then dissociated by pipetting, and the colonies were then transferred to new feeder plates.

2.4. Alkaline phosphatase detection and immunocytochemistry

Alkaline phosphatase staining was performed using the Alkaline Phosphatase (AP) Leukocyte Kit (Sigma -Aldrich) according to the manufacturer's instructions.

For immunocytochemistry, the cells were fixed in 4% paraformaldehyde (PFA) for 20 min, washed with phosphate-buffered saline (PBS) and incubated overnight with PBS supplemented with 3% bovine serum albumin (BSA) and 0.5% Triton X-100. Cells were washed and incubated in PBS with 3% BSA and 0.2% Tween-20 for 1 h at room temperature. The cells were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-OCT3/4 (IgG) diluted at 1:50 (Sigma-Aldrich C279); rabbit anti-SOX2 (IgG) diluted at 1:100 (Abcam ab97959); and rabbit anti-NANOG (IgG) diluted at 1:100 (Abcam ab80892). The cells were then washed three times with PBS and incubated with secondary antibodies diluted at 1:100 (goat anti-rabbit IgG Alexa Fluor 488, A-11008, Life Technologies). The nuclei were stained by Hoechst 33342 (1 mg/mL) for 15 min.

2.5. Embryoid body formation and in vitro differentiation

Canine IPS colonies were plated in agarose-coated tissue culture plates and maintained without bFGF. After 72 h, embryoid bodies were transferred to 0.1% gelatin-coated tissue culture plates. Differentiating EBs were maintained in IMDM media supplemented with 10% fetal bovine serum for approximately 5 days until morphological changes to fibroblast-like cells were observed.

Table 1
Primer sequences used for pluripotency analysis, exogenous factor expression and tumor origin as designed by Primer3 [30].

Gene	Sequence (5′- 3′)	Product size	Evaluation
*OCT4*_FWD	CAGGCCCGAAAGAGAAAGC	78bp	Pluripotency
*OCT4*_REV	CGGGCACTGCAGGAACA		Pluripotency
*SOX2*_FWD	TGCGAGCGCTGCACAT	72bp	Pluripotency
*SOX2*_REV	TCATGAGCGTCTTGGTTTTCC		Pluripotency
18S_FWD	CCTGCGGCTTAATTTGACTC	65bp	Housekeeping
18S_REV	CTGTCAATCCTGTCCGTGTC		Housekeeping
hOSKM_FWD	AAGAGGACTTGTTGCGGAAA	182bp	Exogenous factors/origin of tumor
hOSKM_REV	GGCATTAAAGCAGCGTATCC		HOSKM
mOSKM_FWD	ACGAGCACAAGCTCACCTCT	203bp	Exogenous factors/origin of tumor
mOSKM_REV	GGCATTAAAGCAGCGTATCC		MOSKM