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Safety and immune regulatory properties of canine induced pluripotent stem cell-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) exhibit broad immune modulatory activity *in vivo* and can suppress T cell proliferation and dendritic cell activation *in vitro*. Currently, most MSC for clinical usage are derived from younger donors, due to ease of procurement and to the superior immune modulatory activity. However, the use of MSC from multiple unrelated donors makes it difficult to standardize study results and compare outcomes between different clinical trials. One solution is the use of MSC derived from induced pluripotent stem cells (iPSC); as iPSC-derived MSC have nearly unlimited proliferative potential and exhibit *in vitro* phenotypic stability. Given the value of dogs as a spontaneous disease model for pre-clinical evaluation of stem cell therapeutics, we investigated the functional properties of canine iPSC-derived MSC (iMSC), including immune modulatory properties and potential for teratoma formation. We found that canine iMSC downregulated expression of pluripotency genes and appeared morphologically similar to conventional MSC. Importantly, iMSC retained a stable phenotype after multiple passages, did not form teratomas in immune deficient mice, and did not induce tumor formation in dogs following systemic injection. We concluded therefore that iMSC were phenotypically stable, immunologically potent, safe with respect to tumor formation, and represented an important new source of cells for therapeutic modulation of inflammatory disorders.

1. Introduction

Induced pluripotent stem cells (iPSCs) are stem cells derived from adult somatic cells by reprogramming through transient expression of specific transcription factors (Oct3/4, SOX2, Klf4, and c-Myc) (Takahashi and Yamanaka, 2016). Like embryonic stem cells, iPSC are capable of unlimited expansion, and can potentially differentiate into any cell type in the body. At present, most clinical studies of stem cell therapy utilize MSC derived from adipose tissues or bone marrow (Squillaro et al., 2016). However, the use of MSC for cellular therapy poses several challenges. For one, autologous MSC are difficult to generate from older patients, and their functionality is often impaired compared to MSC generated from young individuals (Choudhery Ms Fau - Badowski et al., 2014; Mohd Ali et al., 2016). Use of allogeneic MSC derived from young donors offers a means of overcoming the limitations of autologous MSC, but introduces new problems, including donor-to-donor variability, risk of iatrogenically introduced infectious agents, and the potential for alloimmune rejection (Reinders, et al., 2015, Zhang et al., 2015a, 2015b).

One means of overcoming the limitations inherent to the use of primary cultured MSC for clinical studies is to use MSC derived from iPSC (Jung et al., 2012; Kimbrel and Lanza, 2015). For example, MSC generated from iPSC offer several advantages, including unlimited passage potential, uniform cell sourcing and phenotyping, and the ability to select and/or modify iPSC-derived MSC for specific desirable properties (Jeon et al., 2016, Zhang et al., 2015a, 2015b). Therefore, there is increasing interest in the use of uniform source MSC such as iPSC-derived MSC for evaluation in clinical settings.

As noted in a recent review, domestic dogs offer several important advantages as animal models for stem cell therapy evaluation, including the spontaneous development of diseases that closely mimic human disease (*e.g.* autoimmune diseases, inflammatory bowel disease, neurological disease, and cancer), a shared environment with humans, and the availability of an outbred population with robust immune systems and lifelong exposure to diverse pathogens (Hoffman and Dow, 2016). Induced pluripotent stem cells have been generated from adult dog fibroblasts, but thus far have only been produced using integrating retroviral gene transduction methodologies (Baird et al., 2015; Koh et al.,

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2013; Koh et al., 2011; Kwon et al., 2012; Lee et al., 2011; Luo et al., 2011; Nishimura et al., 2013; Shimada et al., 2010; Whitworth et al., 2012). There are risks however to the use of retroviral gene transduction methodologies, including alterations of the host genome, or neoplasm formation (Herberts et al., 2011).

In previous studies using rodent models, iMSC have been evaluated as cellular therapy for suppression of inflammatory diseases (Lian et al., 2010, Zhang et al., 2015a, 2015b). The evaluation of human iPSC derived cells for therapy has been subjected to extremely stringent government regulations, and only one study has been conducted so far worldwide (Takahashi, 2016). Thus, the evaluation of iPSC cellular therapeutics in a large animal model to assess safety and efficacy could significantly advance the field of stem cell therapy and iPS cell based treatments.

Therefore in the current report we evaluated the immune modulatory properties of canine iMSC and compared their efficacy to that of conventional adipose and bone marrow-derived MSC. In addition, we assessed iMSC safety in terms of teratoma and tumor formation, using both immune deficient mouse models and purpose-bred dogs. We report here that canine iMSC were functionally equivalent to or superior to conventional Ad-MSC and BM-MSC in terms of their *in vitro* immune suppressive potency, for both T cell and DC suppression. In addition, while canine iPSC readily induced teratomas in immune deficient mice, canine iMSC did not induce teratoma formation. Most importantly, dogs injected i.v. with canine iMSC did not develop detectable tumors over a 1-year period of observation and imaging. Therefore, we conclude that cellular therapy with allogeneic iMSC holds promise as a well-tolerated and potentially effective new cellular therapy for treatment of inflammatory disorders.

2. Materials and methods

2.1. Generation of canine induced pluripotent stem cells

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee at Colorado State University. Canine iPSC were generated by the Colorado University Denver, Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology iPSC Core. Transgene integration-free iPS cells were generated from canine skin fibroblast using a CytoTune iPS Reprograming kit (Life Technologies Corp. Grand Island NY). Donor skin biopsy was collected using 6 mm skin biopsy punch (Miltex, York, PA) from a 6-year old male standard poodle. Donor dog was screened using a complete blood count and serum biochemistry panel, tested negative for Hemoplasma species, Ehrlichia species, Rickettsial species, Bartonella species using PCR, and negative for vector borne diseases using IDEXX 4DX – snap test for companion animals (IDEXX Laboratories, Inc. Westbrook, ME).

Skin fibroblasts were incubated overnight with CytoTune reprogramming vectors, and cultured 7 days before transferring to irradiated MEF (mouse embryonic fibroblasts) feeder cells (Global Stem, Gaithersburg, MD). Flat multinucleated iPSC colonies were observed approximately 14 days after transfection, and each colony was picked manually and expanded individually in a single well on MEF. Only a single colony was viable upon further passaging. The iPSC colonies so derived were maintained in iPSC medium and cultured on MEFs.

2.2. Generation of iPS-derived mesenchymal stem cells (iMSC)

Detached canine iPS colonies cells were collected and plated on Matrigel (Corning Inc. Corning, NY) coated plates in iPS maintenance media with addition of 10 μM Rock Inhibitor (Y-27632) (Tocris Bristol, UK). When plates reached 70% confluency, culture conditions were changed to generate iMSC, following a previously published protocol (Chen et al., 2012). Briefly, the iPSC culture medium was changed to MSC medium with addition of 10 uM TGF-β inhibitor (SB 431542) (Tocris Bristol, UK). The cells were then allowed to differentiate for 10 days with medium changes daily and addition of fresh SB431542. After 10 days, cells were detached and re-plated without SB 431542. Cells were grown to confluency and passaged (P1) at 20,000 cells/cm^2. At P2, the cell number was decreased to 10,000 cells/cm^2, and at P3 and subsequent passages, the cell number was decreased to 4000 cells/cm^2. The iMSC line generated was verified by QC procedures standard to cellular therapies, and tested for sterility by aerobic bacterial and mycoplasma, and fungal culture. 3 different passages of iPS cells were used for differentiation and experimental replicates.

2.3. Generation of canine adipose-derived MSC (Ad-MSC) and bone marrow derived MSC (BM-MSC)

Canine Ad-MSC and BM-MSC were generated as previously described (Chow et al., 2016). 3 biopsies were collected from a single donor, and passaged independently for experimental replicates. Bone marrow aspirates were collected from the proximal humerus. 3 bone marrow aspirates were collected from a single donor, and passaged independently for experimental replicates.

2.4. Tri-lineage differentiation

Tri-lineage differentiation of iMSC was performed according to manufacturer's instructions, using the StemPro Adipogenesis Differentiation Kit, the Chondrogenesis Differentiation Kit, and the Osteogenesis Differentiation Kit (Life Technologies Corp. Grand Island NY).

2.5. Immunocytochemical evaluation of iPS cells

iPS cells were seeded in chamber slides overnight, and the cells were fixed and permeabilized. Slides were incubated with primary antibodies overnight: anti-Oct3/4 (clone H134, Santa Cruz Biotechnology, Inc. Dallas, TX), anti-Nanog (Clone H-155 Santa Cruz Biotechnology Inc. Dallas, TX), and anti-CD105 (clone 8A1 Abcam, Cambridge, MA). Then washed and incubated with secondary antibodies conjugated to biotin (donkey anti mouse IgG or donkey anti rabbit IgG; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and counter stained with DAPI. Visualization of florescence staining was performed on Olympus IX83 spinning disk confocal microscope.

2.6. Teratoma assay for pluripotency verification of iPSC and evaluation of iMSC

iPS cells at a dose of 2×10^6 cells per injection site were delivered s.c. into the flank of NOD/SCID mice ($n = 4$ mice per group). After 20 days, when tumor growth exceeded 10 mm diameter, mice were euthanized and tumors were excised. Tumors were fixed by immersion in 10% neutral buffered formalin and processed for standard hematoxylin and eosin (H&E) staining. iMSC teratoma formation was evaluated using the same procedure.

2.7. Measurement of proliferation

Mesenchymal stem cells were seeded in 12-well culture plates and placed in an IncuCyte® instrument (Essen BioScience Inc. Ann Arbor, MI) to assess cell proliferation.

2.8. Assessment of tumor formation by iMSC injection in dogs

Three healthy, purpose-bred, adult Beagle dogs were injected i.v. with 2×10^6 iMSC per kg body weight. Prior to injection and again 3 months later, the animals were subjected to whole body CT evaluation to identify possible tumor formation. In addition, each animals was evaluated by physical examination monthly during the 6-month evaluation period. At the completion of the 6-month study period, the dogs were adopted to homes locally, and the animals were monitored by periodic physical examinations thereafter to detect possible tumor formation.