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Metabolic plasticity during transition to naïve-like pluripotency in canine embryo-derived stem cells

Tobias I.C.[a], Isaac R.R.[b], Dierolf J.G.[a], Khazaee R.[a,c,d], Cumming R.C.[b], Betts D.H.[a,e,*]

[a] Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada
[b] Department of Biology, Faculty of Science, Western University, London, Ontario, Canada
[c] Lawson Health Research Institute, Western University, London, Ontario, Canada
[d] Biotron Research Centre, Faculty of Science, Western University, London, Ontario, Canada
[e] Children's Health Research Institute, Lawson Health Research Institute, London, Ontario, Canada

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ABSTRACT

Pluripotent stem cells (PSCs) have been described in naïve or primed pluripotent states. Domestic dogs are useful translational models in regenerative medicine, but their embryonic stem cells (cESCs) remain narrowly investigated. Primed-like cESCs expanded in the presence of leukemia inhibitory factor and fibroblast growth factor 2 (LIF-FGF2) acquire features of naïve pluripotency when exposed to chemical inhibitors and LIF (2iL). However, proliferation of cESCs is influenced by the pluripotent state and is comparatively slower than human or mouse PSCs. We propose that different metabolic pathway activities support ATP generation and biomass accumulation necessary for LIF-FGF2 and 2iL cESC proliferation. We found that 2iL cESCs have greater respiratory capacity, altered mitochondrial chain complex stoichiometry and elevated mitochondrial polarization state. Yet, 2iL-enriched cESCs exhibited immature ultrastructure, including previously unrecognized changes to cristae organization. Enhanced ATP level in 2iL cESCs is associated with altered retrograde signalling, whereas LIF-FGF2 cESCs exhibit a lipogenic phenotype. Inhibition of oxidative phosphorylation impaired proliferation and ATP production in 2iL cESCs but not LIF-FGF2 cESCs, which remained sensitive to glycolysis inhibition. Our study reveals distinct bioenergetic mechanisms contributing to steady-state expansion of distinct canine pluripotent states that can be exploited to improve derivation and culture of canine PSCs.

1. Background and rationale

Pluripotency, a defining feature of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), describes the capacity to differentiate into cell types of the three germ lineages (Takahashi and Yamanaka, 2006; Thomson et al., 1998). Pluripotency is not a unitary state but defines at least two populations of pluripotent stem cells (PSCs). Naïve and primed PSCs are metastable and occasionally interconvertible cell lines, which are derived from temporally discrete cellular compartments within the pre-implantation embryo (Brons et al., 2007; Nichols and Smith, 2009; Tesar et al., 2007). The naïve pluripotent state may be established directly from cells of the inner cell mass (ICM) or induced by pharmacological manipulation of primed PSCs, with or without the assistance of transgenes favouring naïve pluripotency (Bao et al., 2009; Guo et al., 2009; Nichols et al., 2009). Tolerance to partial repression of mitogen-activated protein kinase kinase (MEK) signalling and/or abrogation of T-cell factor 3 (TCF3) signalling by chemical activation of the Wnt pathway appear to be conserved features of mammalian naïve pluripotency (Meek et al., 2013; Wray et al., 2011; Zhou et al., 2015). The molecular and functional characteristics of naïve and primed PSCs and the inductive inputs that support these phenotypes have been studied extensively (reviewed in (Hackett and Surani, 2014; Martello and Smith, 2014; Weinberger et al., 2016)). Briefly, naïve PSCs are characterized by global epigenomic de-repression (Ficz et al., 2013; Leitch et al., 2013), reactivation of the silent X-chromosome in female cells (Gafni et al., 2013; Ware et al., 2014), ease of clonogenic selection and contribution to embryonic lineages of pre-gastrulation embryos (Buecker et al., 2010; Gafni et al., 2013).

Both naïve and primed PSCs exhibit high rates of glycolysis in hyperoxic environments, which is similar to the aerobic glycolysis phenotype observed in certain transformed cells (Racker, 1972; Vander Heiden et al., 2009; Zhang et al., 2012a). The diversion of metabolic intermediates from complete mitochondrial oxidation supplies

* Corresponding author at: Collaborative Graduate Program in Developmental Biology, Children's Health Research Institute, Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, Western University, Dental Sciences Building, Room DSB 2022, London, ON N6A 5C1, Canada.
E-mail addresses: dean.betts@schulich.uwo.ca, dbetts@uwo.ca (D.H. Betts).

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intermediates to anabolic pathways, while mitigating the production of reactive oxygen species (ROS) (Folmes et al., 2011; Panopoulos et al., 2012; Varum et al., 2009). This metabolic state differs from specialized post-natal cell types, which prioritize efficient adenosine triphosphate (ATP) synthesis by oxidative phosphorylation (OXPHOS) (Chung et al., 2007). Interestingly, naïve PSCs have greater rates of respiration despite housing less mature mitochondria compared to their primed counterparts (Sperber et al., 2015; Zhou et al., 2012). Mitochondrial function is restrained in primed PSCs by uncoupling the oxidation of glycolysis-derived pyruvate (Samudio et al., 2009; Zhou et al., 2012). The progression from metabolically flexible naïve PSCs (ICM-like) to mainly anaerobic primed PSCs (late epiblast-like) is regulated by the complex interplay of intrinsic epigenetic and transcriptional programs as well as trophic input (Carbognin et al., 2016; Zhou et al., 2012).

The existence of a *de facto* naïve pluripotent state in non-rodent species is controversial. However, naïve-like PSC lines have been characterized in various mammalian species including porcine (Nakano et al., 2013), rabbit (Osteil et al., 2013), equine (Whitworth et al., 2014b and bovine (Verma et al., 2013). Canine ESCs (cESCs) can be derived and expanded on mouse embryonic fibroblast (MEF) feeder layers in the presence of leukemia inhibitory factor (LIF) and fibroblast growth factor 2 (FGF2) (Tobias et al., 2013). These LIF-FGF2 dependent cESCs exhibit features of primed pluripotency such as sensitivity to enzymatic passaging regimes, expression of primed pluripotency markers (*e.g.* OTX2, FGF5) and a neural lineage bias in minimal media (Wilcox et al., 2011; Wilcox et al., 2009). We have recently shown that dual inhibition of MEK and GSK3β along with LIF (2iL) conditionally stabilizes cESCs in a pluripotent state that shares several properties with canonical naïve PSCs (Tobias et al., 2016). Furthermore, Whitworth et al. has established canine iPSCs in the presence of LIF, 2i, valproic acid and the A83-01 inhibitor, which express OCT4, NANOG and REX1 (Whitworth et al., 2014a,; Whitworth et al., 2012). Interestingly, our 2iL cESCs show markedly slower *in vitro* growth compared to initiating LIF-FGF2 cESC populations (Tobias et al., 2016). Nevertheless, the fundamentals of pluripotent state progression appear to be conserved in placental mammals, making it a priority to define the divergent attributes and culture requirements of non-rodent equivalents to naïve and primed PSCs. iPSC lines from a marsupial species may provide novel insights into the evolution of multiple pluripotent states from a common therian ancestor (Weeratunga et al., 2018).

PSC metabolism is inherently flexible, permitting the maintenance of proliferation and differentiation capacity within a dynamic physicochemical environment (Harvey et al., 2016), but the persistence of suboptimal conditions would be expected to negatively impact PSC physiology. All canine ESC/iPSC lines established to date are maintained in media formulations previously optimized for the growth of human or mouse PSCs (reviewed in (Betts and Tobias, 2015)). Furthermore, imprecise definition of the extrinsic requirements for canine PSC self-renewal may hinder the ability of these lines to proliferate extensively *in vitro* or demonstrate pluripotency *in vivo* through teratoma formation. The goal of this work is to assess mitochondrial function and the contribution of bioenergetic pathways to proliferation in the undifferentiated state for primed-like LIF-FGF2 cESCs and naïve-like 2iL cESC populations. We propose that different metabolic pathway activities support ATP generation and biomass accumulation necessary for proliferation of cESCs.

2. Materials and methods

2.1. Embryonic stem cell culture

Mouse embryonic fibroblast (MEF) monolayer preparation and culture of cESCs were conducted as previously described (Tobias et al., 2016). Briefly, E12.5 DR4 MEFs were mitotically arrested and seeded at 1.5×10^4 cells/cm^2 for ESC co-culture. Canine ESC lines (BE5, IO3) derived at the Ontario Veterinary College from embryo explants (OVC.EX) (Wilcox et al., 2009) were seeded onto growth-arrested MEFs and cultured in base media: KnockOut DMEM/F12, 15% KnockOut Serum Replacement (KOSR), 1× GlutaMAX, 1× non-essential amino acids, 10 ng/mL recombinant insulin-like growth factor (R_3IGF1; Sigma Aldrich) and 0.1 mM 2-mercaptoethanol. Base medium was supplemented with 10 ng/mL human LIF and 4 ng/mL human FGF2 (LIF-FGF2) for maintenance of control LIF-FGF2 cESCs; or 10 ng/mL human LIF, 0.5 μM MEK inhibitor PD0325901 and 3 μM GSK3β inhibitor CHIR99021 for establishment and culture of 2iL cESCs (Tobias et al., 2016).

To deplete MEF contamination prior to experiments, cESCs were transferred to Geltrex™-coated dishes and cultured with 70% MEF-conditioned medium balanced with non-conditioned base media. Control human ESC line HES-2 (Pera et al., 2000) and murine ESC line R1 (Nagy et al., 1993) were adapted and maintained in base medium containing 15% KOSR for at least three passages prior to analyses to standardize nutrient availability (Zhang et al., 2016). Incubators were maintained at 37 °C, 5% CO_2 and ambient oxygen. Unless otherwise stated, all cell culture reagents were obtained from Thermo Fisher Scientific (MA, USA). Inhibitors of metabolic enzymes were from Sigma Aldrich (MO, USA).

2.2. Oxygen consumption rate and extracellular acidification rate

Cells were seeded onto Geltrex™-coated XFe24 Seahorse plates (Agilent Technologies) at 3×10^4 or 6×10^4 cells per well. Culture media were exchanged for unbuffered media supplemented with either 1 mM sodium pyruvate and 10 mM glucose (Mitochondrial assay); or 2 mM glutamine (Glycolysis assay) one hour before the assay. After basal metabolic readings were recorded, substrates and selective inhibitors were injected to achieve final concentrations of: glucose (2.5 mM), 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 μM), oligomycin (1 μM), 2-deoxyglucose, (2-DG, 50 mM) antimycin A (2.5 μM) and rotenone (2.5 μM). Changes in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in response to the addition of substrates/inhibitors were described as the mean change after injection compared with the average OCR or ECAR before the injection. The OCR and ECAR values were normalized to the amount of protein isolated from each well.

2.3. Quantification of mitochondrial membrane potential

Cells were stained for 1 h with 1 μM Calcein Green AM and then 15 min with 25 nM TMRM diluted in Live Cell Imaging Solution (Thermo Fisher Scientific). Cells were imaged within a 5% CO_2 live cell imaging chamber mounted on the stage of a Leica DMI 6000B microscope. Digital images were captured with an Orca Flash camera (Hamamatsu Photonics) and Application Suite X software (Leica Microsystems). Exposure time, illumination intensity and experimental duration were minimized to preserve mitochondrial integrity and avoid artefactual changes in fluorescence intensity (Iannetti et al., 2016; Mitra and Lippincott-Schwartz, 2010).

Brightness and contrast were standardized to unstained control samples and the equivalent imaging parameters were applied to all other images using ImageJ. Membrane potential was calculated as the change in fluorescence intensity of TMRM in redistribution (non-quenching) mode after FCCP-induced mitochondrial depolarization. Calcein fluorescence was recorded to control for plasma membrane integrity. Fluorescent intensity was monitored for five minutes to establish baseline values (set to 100%) and an additional fifteen minutes after treatment with 250 nM FCCP or vehicle (DMSO). The mean fluorescent intensity of cytoplasmic foci corresponding to mitochondria in cESCs was measured using ImageJ (National Institutes of Health, MD).