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

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A B S T R A C T

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 (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
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 REXI (Whitworth et al., 2014a; Whitworth et al., 2012). Interestingly, our 2iL cESCs show markedly slower cell proliferation compared to naive (Chung et al., 2007). 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). Nevertheless, the fundamentals of pluripotent state progression appear to be conserved in placental mammals, making it a priority to de

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 × 10⁶ cells/cm² for ESC co-culture. Canine ESC lines (BES, I03) 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 (IGF1; 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 Geltrans™-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% CO₂ 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 Geltrans™-coated XF24 Seahorse plates (Agilent Technologies) at 3 × 10⁴ or 6 × 10⁴ 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-(triluromethoxy) 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% CO₂ 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).
2.4. Mitochondrial morphometric image analysis

Procedures for specimen preparation are detailed in Supplemental materials. At least 60 images containing 18 distinct cells were taken from coded LIF-FGF2 and 2iL cESC samples using a Philips CM10 transmission electron microscope at 34,000× magnification for observation of 380 total mitochondria. Mitochondrial counts, dimensions and cristae orientation were assessed by blinded investigators using ImageJ. The aspect ratio was calculated as the ratio between the unit length of major and minor axes for mitochondrial ellipsoids. The cristae density was calculated from the cristae number normalized to calculated mitochondrial surface area. To evaluate the degree of cristae alignment within mitochondria, cristae angles were recorded to calculate the mean incident angle between cristae.

2.5. Measurement of acute cESC expansion

20,000 cESCs were seeded into multiwell plates and total cell counts were performed every 24 h for 4 days. For inhibitor treatments, cells adhered overnight in the absence of inhibitors and growth in the presence of inhibitors was scored after 48 h. Data are presented as the fold change in total cell number relative to the initial cell number at the 0-h time point. 2-deoxy-D-glucose (2-DG) and Rotenone (RTN) were diluted into culture media to final concentrations of 1.25 mM and 250 nm, respectively. Concentrations used in our assays were determined by single inhibitor dose-titrations to find the minimum concentration affecting cell proliferation.

2.6. Determination of cellular ATP

LIF-FGF2 and 2iL cESCs were treated with 2-DG, RTN or vehicle and diluted to 100,000 cells/mL in cESC culture media. Cell suspensions were transferred to an opaque white 96-well microplate (10,000 cells/well) and ATP content was determined using the Luminescent ATP Detection Assay Kit (Abcam). Luminescence was quantified with the SpectroMax M5 (Molecular Devices, CA) instrument and ATP content (pM/cell) was interpolated from an eight-point standard curve.

2.7. Detection of dead and apoptotic cells

The incidence of cell death and apoptosis during steady-state growth and inhibitor assays was assessed by measuring the percentage of 7-aminoactinomycin D (7-AAD)/Annexin-V positive cells. Briefly, single cells suspended in Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) were stained with fluorescein isothiocyanate (FITC) Annexin-V conjugate (Thermo Fisher Scientific) for 20 min and 7-AAD Viability staining solution (eBioscience) for 5 min. 20,000 total events were recorded using an Accuri C6 flow cytometer (BD Biosciences). Spectral compensation was performed by subtracting 2% of FL1 from FL3 to adjust for minor FITC overlap into FL-3. Changes in cell viability was assessed as the mean proportion of Annexin-V-FITC−/7-AAD− (viable), Annexin-V-FITC+/7-AAD− (apoptotic) or Annexin-V-FITC+/7-AAD+ (dead) cells.

2.8. Statistics and reproducibility

Data were analyzed using R (version 3.2.3) by two-sample composite t-test or one-way analysis of variance (ANOVA) followed by Tukey’s HSD multiple comparison method as appropriate. P < 0.05 was considered statistically significant. Data were visualized using GraphPad Prism 6 and presented as mean ± standard error of the mean (SEM).

3. Results

3.1. Cultivation in 2iL enhances glucose oxidation and respiratory capacity of cESCs

Glycolytic and oxidative metabolism in ESCs were assessed by
measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively (Zhang et al., 2012b). To explore if activation of mitochondrial respiration is a feature conserved in naïve-like cESCs, we compared the metabolic rate measurements of BE5 cESCs maintained in LIF-FGF2 or 2iL, HES-2 human (hESCs) and R1 murine (mESCs). OCR was monitored after cells were successively exposed to oligomycin (OLIGO), 4-(trifluoromethoxy)-phenylhydrazone (FCCP) and antimycin A/rotenone (Fig. 1A).

In the presence of glucose, both R1 mESCs (P = 1.03 × 10^{-4}) and 2iL cESCs (P = 0.025) have greater OCR: ECAR ratio compared to primed-like LIF-FGF2 cESCs and hESCs (Fig. 1B). The respiration attributable to ATP-synthase activity did not differ between hESCs, LIF-FGF2 or 2iL cESCs (P = 0.25) but was markedly greater in mESCs (P = 6.8 × 10^{-6}) (Fig. 1C). The fold change in OCR following FCCP-stimulated maximal respiration (a measure of mitochondrial spare capacity) is significantly elevated in 2iL cESCs (P = 1.40 × 10^{-3}) and R1 mESCs (P = 7.2 × 10^{-6}) in comparison to primed human ESCs and LIF-FGF2 cESCs (Fig. 1D). However, rate measurements correlating to basal oxygen consumption (P = 0.024), ATP synthase-linked respiration and mitochondrial spare capacity (P = 0.013) in 2iL cESCs all remain below naïve mESCs.

To assess if enhanced 2iL cESC respiration is reproducible in a different genetic background, we examined OCR responses to chemical manipulation of mitochondrial function in IO3 cESCs (Fig. 1E). Similar to BE5 cESCs, we observed a statistically significant increase in basal OCR: ECAR ratio (P = 1.94 × 10^{-5}) and mitochondrial spare capacity (P = 0.042), but not ATP coupled respiration (P = 0.30) in IO3 2iL cESCs compared to those maintained in LIF-FGF2 (Fig. 1F–H).

3.2. Maintenance of mitochondrial mass and remodeling of mitochondrial ultrastructure in 2iL-enriched cESCs

We next sought to quantify mitochondrial content to determine if altered mitochondrial biomass contributed to cESC metabolic function. Lipophilic dialkylcarbocyanine dyes were applied to co-cultured MEFs to discriminate cESC-specific signals by flow cytometry (Fig. 2A–B). Surprisingly, the geometric mean of MitoTracker Green FM fluorescence intensity was significantly lower in the 2iL cESC population (Fig. 2C–D, P = 4.40 × 10^{-3}). Whereas, the geometric mean of forward scatter (FSC) intensity, an estimate of cell size, was not different between LIF-FGF2 and 2iL cESCs (Supplemental Fig. 1, P = 0.97). Therefore, it is unlikely that altered cell volume contributed to the observed change in MitoTracker Green FM accumulation in cESCs.

We probed cESC lysates for TOMM20, an integral component of the outer mitochondrial membrane (Rassow and Pfanner, 2000), as a surrogate measure of mitochondrial content (Supplemental Fig. 2A). TOMM20 abundance was not different in 2iL cESCs compared to originating LIF-FGF2 cESCs (Fig. 2E, P = 0.21). Similarly, we observed no change in the relative mitochondrial DNA (mtDNA) quantity between LIF-FGF2 and 2iL cESCs (Supplemental Fig. 1, P = 0.97). The level of Mitofillin (MIC60), an inner mitochondrial membrane protein associated with tubular cristae formation (Hessenberger et al., 2017), was significantly reduced in 2iL cESCs compared to LIF-FGF2 cESCs (Fig. 2F, G, P = 9.0 × 10^{-4}, Supplemental Fig. 2B).
Alterations to 2iL cESC mitochondrial ultrastructure may affect MitoTracker Green FM accumulation despite no difference in overall mitochondrial biomass. Using transmission electron microscopy, we observed over 150 mitochondria from 18 individual cells per cESC population for morphometric analysis (Fig. 3A–B). The mitochondria of both LIF-FGF2 and 2iL cESCs were typically arranged in perinuclear clusters (Supplemental Fig. 2C). Mitochondria visualized in LIF-FGF2 cESCs tended to have lower overall abundance and larger surface area compared to mitochondria within 2iL cESCs, but these metrics were not considered statistically significant (Fig. 3C, P = 0.1403 and P = 0.1866). 2iL cESCs contained shorter and more rounded mitochondria with respect to mean aspect ratio compared to mitochondria within 2iL cESCs (Fig. 3D, P = 0.1866). Mitochondria from LIF-FGF2 and 2iL cESCs exhibited a similar density of cristae membranes (Fig. 3E, P = 0.861). Interestingly, the mitochondria in 2iL cESCs exhibited poorer cristae alignment with a mean incident cristae angle of 58.02° compared to 38.07° in LIF-FGF2 cESCs (Fig. 3F, P = 0.019).

3.3. Altered mitochondrial protonic accumulation, respiratory chain complex stoichiometry and retrograde signalling in 2iL cESCs

Cellular ATP production is regulated by mitochondrial membrane potential (ΔΨm), which is established by electron transport and the coincident transport of protons to the intermembrane space (Ehrenberg et al., 1988; Kamo et al., 1979). We assessed mitochondrial polarization state using the ΔΨm-sensitive dyes tetramethylrhodamine, methyl ester (TMRM) and JC-1 to explore the relationship between mitochondrial respiration and proton-motive force in cESCs.

Contrary to the anticipated result, quantification of cells stained with JC-1 (Supplemental Fig. 3A, P = 4.60 × 10⁻³) or TMRM (Supplemental Fig. 3B, P = 1.30 × 10⁻³) by flow cytometry showed significant reductions in the relative ΔΨm of 2iL cESCs compared to those expanded in LIF-FGF2. However, quantification of fluorescent intensity per cell may not be a reliable comparison if ultrastructural changes in mitochondria affect probe uptake and retention kinetics between LIF-FGF2 and 2iL cESCs. Rather than performing a static comparison between whole cell mitochondrial polarization states, we sought to quantify the dynamic re-distribution of TMRM upon selective depolarization of ΔΨm by live cell fluorescent microscopy (Fig. 4A). 2iL cESCs exhibited a significantly greater decrease in TMRM fluorescence intensity following FCCP-mediated depolarization compared to LIF-FGF2 cESCs (Fig. 4B, P = 0.0106). Calcein Green AM fluorescence intensity was maintained after FCCP treatment (Supplemental Fig. 4), suggesting that viability or plasma membrane integrity do not confound TMRM re-distribution.

We next investigated the abundance of mitochondrial chain complex subunits by immunoblot analysis (Supplemental Fig. 5A). 2iL cESCs exhibited a significant increase (P = 1.5 × 10⁻³) in succinate dehydrogenase iron sulfur subunit B (SDHB; complex II) and lower (P = 0.026) F1-ATP synthase subunit 5 alpha (ATP5A; complex V) (Fig. 4C–D) abundance compared to LIF-FGF2 cESCs. Ubiquinol-cytochrome c reductase core protein 2 (UQCR2; complex III) did not significantly differ between LIF-FGF2 and 2iL cESCs (Fig. 4E, P = 0.53). Furthermore, steady-state ATP levels were greater in 2iL cESCs compared to LIF-FGF2 cESCs (Fig. 4F, P = 1.0 × 10⁻³). This result is corroborated by enhanced phosphorylation of AMP-activated protein kinase (AMPK) at Thr172 (Fig. 4G, P = 0.044) as well as increased Ser79 phosphorylation of acetyl-CoA carboxylase (ACC1), a canonical AMPK target enzyme, in LIF-FGF2 cESCs compared to 2iL cESCs (Fig. 4G, P = 0.020, Supplemental Fig. 5B). Reliable signals could not be obtained for antibodies directed against human cytochrome C oxidase 2 (MTCOX2; complex IV) and NADH: ubiquinone oxidoreductase subunit B6 (NDUFB8; complex I) due to poor amino acid conservation in the canine homologs (< 85% similarity by blastp algorithm).

Fig. 3. Remodeling of mitochondrial ultrastructure in 2iL-enriched cESCs. Transmission electron micrograph at 34,000 × magnification of mitochondrial within (A) LIF-FGF2 cESCs and (B) 2iL cESCs. Over 150 mitochondria from at least 18 different cells per cESC population were observed for morphometric analysis by blinded investigators. (C) Quantification of mitochondrial surface area and relative mitochondrial abundance in LIF-FGF2 and 2iL cESCs. (D) Calculation of the mean aspect ratio (major axis length: minor axis length) of mitochondria. (E) Quantification of the mean cristae density estimated as the cristae number normalized to surface area. (F) Calculation of the mean incident angle between cristae. Scale bar is 500 nm. Data are presented as the mean of three biological replicates. Error bars represent standard error of the mean. Means with *P < 0.05, **P < 0.01, ***P < 0.001 from LIF-FGF2 cESCs were considered statistically significant.
3.4. Differential regulation of pyruvate metabolizing enzymes in cESCs is associated with altered lipogenic properties

Multiple enzymes responsible for pyruvate flux into the TCA cycle are differentially regulated in naïve versus primed PSC populations (Zhang et al., 2011; Zhou et al., 2012). Therefore, we assessed the levels of proteins involved in pyruvate handling by immunoblot analysis (Supplemental Fig. 6). LIF-FGF2 cESCs exhibited increased levels of lactate dehydrogenase isoform A (LDHA), a key enzyme in maintaining high glycolytic flux, compared to 2iL cESCs (Fig. 5A, \( P = 0.01 \)). Whereas levels of M2 isoform of pyruvate kinase (PKM2), an isoenzyme associated with elevated aerobic glycolysis in transformed cells, exhibited similar levels in LIF-FGF2 and 2iL cESCs (Fig. 5B, \( P = 0.65 \)). However, the M1 isoform of pyruvate kinase (PKM1), which is suggested to promote mitochondrial pyruvate oxidation (Taniguchi et al., 2016), was significantly enriched in 2iL cESCs (Fig. 5C, \( P = 0.013 \)). Surprisingly, levels of pyruvate dehydrogenase (PDH) kinase 1 (Fig. 5D, \( P = 0.045 \)) and the ratio phosphorylated PDH to total PDH (Fig. 5E, \( P = 7.0 \times 10^{-3} \)) were both elevated in 2iL cESCs.

Incomplete oxidation of glucose may favour the redirection of intermediate metabolites towards anabolic processes, such as lipogenesis (Tohyama et al., 2016). We therefore investigated the level of neutral lipids within LIF-FGF2 and 2iL cESCs using Nile Red. Image particle count and size analyses revealed no significant differences in the mean size or mean count per cell (Fig. 5F, \( P = 0.566 \) and \( P = 0.122 \)). However, flow cytometric analysis revealed greater mean fluorescence intensity associated with neutral lipids in LIF-FGF2 cESCs compared to 2iL cESCs (Fig. 5G, \( P = 3.0 \times 10^{-4} \)). Nile Red fluorescence attributable to polar lipids, such as phospholipids, did not significantly differ between LIF-FGF2 and 2iL cESCs (Supplemental Fig. 7, \( P = 0.78 \)). Indeed, fatty acid synthase (FASN), a key enzyme in de novo lipogenesis, was significantly more abundant in LIF-FGF2 cESC than 2iL cESC protein.

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Fig. 4. 2iL cESCs exhibit altered mitochondrial protonic accumulation, respiratory chain complex stoichiometry and retrograde signalling compared to LEF-FGF2 cESCs. (A) Time course of tetramethylrhodamine, methyl ester (TMRM) mean fluorescence intensity before (baseline) and after selective depolarization of mitochondrial potential with FCCP. Calcein Green AM mean fluorescence intensity was also recorded as an indicator of plasma membrane integrity. (B) Quantification of the magnitude of FCCP-mediated reduction in TMRM staining in redistribution conditions (non-quenching). Densitometric quantification of protein levels for (C) ATP synthase alpha subunit (ATP5A), (D) succinate dehydrogenase beta (SDHB) and (E) ubiquinol-cytochrome c reductase core protein 2 (UQCRC2) in LIF-FGF2 and 2iL cESCs. (F) Calculation of cellular ATP by interpolating standard curve values in a luminescent microplate assay and normalized to cell number. Protein quantification by densitometry for (G) ratio of phosphorylated to total AMP-activated protein kinase (AMPK) and (H) ratio of phosphorylated to total acetyl-CoA carboxylase (ACC). Means are expressed relative ratios of arbitrary units normalized to internal standard beta-actin. Data are presented as the mean of four to seven biological replicates. Error bars represent standard error of the mean. Means with *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) from LIF-FGF2 cESCs were considered statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
lysates (Fig. 5H, P = 0.015).

3.5. HXK2 protein level correlates with glycolytic capacity in mammalian ESC lines

To examine glycolytic activity in PSC lines, ECAR was monitored after the sequential addition of glucose, OLIGO and 2-deoxy-D-glucose (2-DG) (Fig. 6A). ECAR measurements in the absence of glucose, or after 2-DG exposure, did not differ between all lines tested, implying that non-glycolytic acidification mechanisms did not substantially contribute to ECAR observations. Glucose-induced ECAR (i.e. glycolytic acidification) in LIF-FGF2 cESCs were equivalent to those recorded from naïve-like R1 mESCs or 2iL cESCs (P = 0.88) and lower than HES2 hESCs (P = 0.046) (Fig. 6B). Oligomycin-stimulated ECAR (i.e. maximum glycolytic capacity) was equivalent between LIF-FGF2 and 2iL cESCs (P = 0.98), but significantly lower than R1 mESCs (P = 6.3 × 10⁻³) and HES2 hESC lines (P = 9.25 × 10⁻³) (Fig. 6C). However, the quotient of oligomycin and glucose stimulated ECAR (i.e. glycolytic reserve capacity) was augmented in 2iL cESCs (P = 0.017), HES2 hESCs (P = 4.59 × 10⁻³) and R1 mESCs (P = 1.0 × 10⁻⁷) compared to LIF-FGF2 cESCs (Fig. 6D). Interestingly, LIF-FGF2 cESCs, 2iL cESCs, HES2 hESC and R1 mESC did not show any difference in constitutive glucose unipporter GLUT1 quantity (Fig. 6E, Supplemental Fig. 8, F = 0.20456, P = 0.89). However, rate-limiting glycolytic enzyme Hexokinase-2 (HXK2) (Bissonnette et al., 1996) differed among ESC populations (Fig. 6F, F = 11.38, P = 3.0 × 10⁻⁴). HXK2 level was significantly greater in HES2 (P = 3.02 × 10⁻²) and R1 (P = 2.0 × 10⁻³) compared to LIF-FGF2 cESCs, which exhibited similar HXK2 levels to 2iL cESCs (P = 0.68).

3.6. Chemical inhibition of bioenergetic pathways differentially affects acute expansion and ATP level of LIF-FGF2 and 2iL cESCs

We used inhibitors of the electron transport chain (ETC) and glycolysis to dissect the contributions of these metabolic pathways to acute LIF-FGF2 and 2iL cESC growth. Given that 2iL cESCs have greater respiratory and glycolytic reserve capacities than LIF-FGF2 cESCs, we posited that 2iL cESC expansion would be less sensitive to bioenergetic pathway antagonism.

Expansion of LIF-FGF2 cESCs was impaired by 2-DG, a competitive inhibitor of HXK (Fig. 7A, P = 2.24 × 10⁻³). 2-DG significantly prolonged LIF-FGF2 cESC steady-state population doubling interval (Fig. 7B, P = 0.041), without altering the proportion of live (Fig. 7C, P = 0.58), apoptotic or dead cells (Supplemental Fig. 9A & B, P = 0.84 &
levels within 2iL cESCs were sensitive to the presence of partial dual inhibition of MEK and GSK3β (P = 9.08 × 10^{-5}). In contrast, the mitochondrial complex I inhibitor RTN did not observe any significant changes (P = 1.0 × 10^{-2}). In contrast, the mitochondrial complex I inhibitor RTN did not alter LIF-FGF2 cESC proliferation (Fig. 7A, P = 0.20). In contrast, the mitochondrial complex I inhibitor RTN did not alter LIF-FGF2 cESC proliferation (Fig. 7A, P = 0.67) or viability (Fig. 7F, P = 0.89).

2iL cESC expansion was unaffected by the presence 2-DG (Fig. 7D, P = 0.92), which was reflected in similar population doubling intervals to vehicle-treated controls (Fig. 7E, P = 0.93). However, RTN exposure decreased 2iL cESC expansion (Fig. 7D, P = 6.30 × 10^{-3}) and lengthened average population doubling time (Fig. 7E, P = 9.59 × 10^{-2}). We did not observe any significant changes in 2iL cESC viability (Fig. 7F, P = 0.67), death or apoptosis for the duration of RTN treatment (Supplemental Fig. 9C–D, P = 0.80 and P = 0.74). Exposure of LIF-FGF2 cESCs to 2-DG and RTN during steady-state growth did not significantly affect the ATP content (Fig. 7G, F = 3.185, P = 0.065). Whereas, ATP levels within 2iL cESCs were sensitive to the presence of partial blockade of bioenergetic pathways (Fig. 7H, F = 61.439, P = 9.08 × 10^{-9}). Interestingly, treatment of 2iL cESCs with RTN (P = 1.0 × 10^{-7}), but not with 2-DG (P = 0.93), significantly decreased ATP concentrations.

4. Discussion

We have previously shown that canine embryo-derived stem cells are pluripotent and amendable to pluripotent state manipulation by dual inhibition of MEK and GSK3β (2iL) (Tobias et al., 2016). Our data indicate that 2iL-enriched cESCs show enhanced mitochondrial respiration, increased mitochondrial intermembrane potential, remodeling of mitochondrial ultrastructure, and changes in the abundance of enzymes involved in pyruvate handling, electron transport and lipid synthesis. Pluripotent state-associated changes in cESC metabolic pathway activity and enzyme abundance have a functionally relevant impact on cESC ATP synthesis and proliferation. Our study provides the first evidence for suboptimal glycolytic flux in cESCs lines as well as extensive remodeling of the cristae membranes associated with a metabolic switch towards mitochondrial metabolism with naïve-like pluripotency induction.

The transition from a metabolically flexible ICM-like naïve pluripotent state to an epiblast-like primed pluripotent state that is reliant on aerobic glycolysis has been characterized in human and murine ESCs (Zhou et al., 2012). We found that 2iL subculture enriches for a cESC population with greater respiratory spare capacity and a preference for mitochondrial glucose oxidation under steady-state conditions compared to LIF-FGF2 cESCs. Augmented cellular respiration has also been reported upon induction of naïve-like hESCs (Takashima et al., 2014) and may represent a conserved attribute of naïve pluripotency in placental mammals. However, 2iL cESCs have not reached an energetic state equivalent to naïve mESCs with regards to multiple indices of mitochondrial function.

Mitochondrial organization and membrane architecture is dynamic and specific to the cell phenotype, extracellular environment and developmental stage (Collins et al., 2002; Sathananthan and Trounson, 2000). In this work, we quantified the fine structure of mitochondria within cESCs that approximate distinct states of pluripotency. 2iL cESCs maintain overall mitochondrial biomass with respect to LIF-FGF2 cESCs despite an enrichment of smaller, rounded mitochondria. The mitochondria contained within 2iL are morphologically similar to those within mouse ESC and chemically induced naïve human ESC (Sperber et al., 2015; Zhou et al., 2012). It is not yet known whether structural remodeling of mitochondria during this primed-to-naïve conversion is due to altered mitochondrial fission-fusion homeostasis, mitochondrial biogenesis or autophagy (mitophagy). Mechanistic studies targeting the cellular machinery for mitochondrial fission-fusion, replication and clearance during pluripotent state transitions will improve our understanding of the mitochondrial rejuvenation process.

In contrast to previous reports that mitochondria within naïve-like
ESCs are qualitatively cristae-poor (Ware et al., 2014; Zhou et al., 2012), we observed that mitochondrial cristae density is preserved in 2iL cESCs. Furthermore, we quantified previously unrecognized changes to the degree of cristae organization between different states of pluripotency in vitro. 2iL cESCs contained mitochondria with disorganized cristae that project along the periphery of the organelle rather than assuming a lamellar, axis-spanning orientation. The arch-like inner mitochondrial membranes within 2iL cESCs are similar to those described in early preimplantation embryos (Baharvand and Matthaei, 2003; Sathananthan and Trounson, 2000), suggesting that immature mitochondrial morphology is not an artifact of in vitro culture. Altered cristae architecture is associated with changes to the abundance of MitoFlin/MIC60, a component of the mitochondrial contact site and cristae organizing system (MICOS) (Hessenberger et al., 2017). We also observed, but did not directly quantify, the widening of cristae, particularly at the tips and the cristae junctions. Conceivably, lower abundance of catalytic ATP synthase subunits in 2iL cESCs could limit the pool of available ATP synthase complexes, which are suggested to form oligomers that function to restrict the circumference of cristae rims and tips (Davies et al., 2012). Structural changes to the cristae alter the dimensions of the intracristal space and plausibly function as a reservoir for oxidative phosphorylation (OXPHOS) substrates (e.g. ADP), protons or ROS.

Enzymes which regulate the availability of pyruvate for mitochondrial import are expressed and tightly controlled in ESCs and iPSCs (Cao et al., 2015; Prigione et al., 2014; Varum et al., 2011; Zhang et al., 2011). We identified elevated levels PKM isoform 1 and concurrent repression of LDH isoform A level in 2iL cESCs. These observations are suggestive of increased mitochondrial pyruvate availability at the expense of LDH-mediated NAD+ regeneration. Elevated phosphorylation of PDH would be expected to limit pyruvate conversion to acetyl-CoA in 2iL cESCs, implicating reductive carboxylation of pyruvate as an alternative mechanism of TCA cycle replenishment. As a plausible consequence of greater pyruvate oxidation, fewer metabolic intermediates may be available for anabolic pathways such as de novo lipogenesis (Tohyama et al., 2016). We found that LIF-FGF2 cESCs exhibited greater FASN abundance and accumulated more intracellular neutral lipids than 2iL cESCs. However, differential activity of the β-oxidation pathway may also contribute to altered lipid biomass between LIF-FGF2 and 2iL cESCs.
The transition from OXPHOS to aerobic glycolysis in human and mouse ESCs is accompanied by coordinated changes in mitochondrial chain complex subunit expression (Zhou et al., 2012), electron transport chain (ETC) activity and ATP synthesis by OXPHOS. However, reconfiguration of membrane composition or chain complex stoichiometry can have a less obvious influence on mitochondrial function through the formation of supercomplexes that channel substrates and electron carriers (Dudek et al., 2013; Lapuente-Brun et al., 2013). We identified an enrichment of SDHB subunit and a reduction of ATP5A subunit, which may indicate that electron transport and OXPHOS kinetics have been altered. A reduction in overall catalytic ATP synthase complexes may explain the similar degree of oligomycin-sensitive respiration despite enhanced respiratory capacity in 2iL cESCs. Nevertheless, our data indicate that ΔΨm is elevated in 2iL cESCs, which is consistent with observations of elevated ETC activity in naïve murine ESCs compared to their primed counterparts (Zhou et al., 2012). Greater cellular ATP level may promote global changes in 2iL cESCs through retrograde signalling via the energy-sensing AMPK pathway (reviewed in (Herzig and Shaw, 2018)) or ATP-dependent enzymes (Zhang et al., 2018).

Our data indicate that LIF-FGF2 and 2iL cESCs have equivalent basal rates of glycolysis, but lower maximal rates of acidification compared to hESCs and mESCs. The correlation between glycolytic capacity and prolonged population doubling intervals in cESCs compared to hESCs and mESCs. The correlation between glycolytic capacity and prolonged population doubling intervals in cESCs compared to hESCs and mESCs. The correlation between glycolytic capacity and prolonged population doubling intervals in cESCs compared to hESCs and mESCs.

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Others have demonstrated that complete blockade of glycolysis with 2-DG lowered the colony-forming ability of primed hESCs (Zhou et al., 2012). We find that LIF-FGF2 cESCs do not exhibit the mitochondrial reserve capacity to oxidize pyruvate thereby rendering LIF-FGF2 cESCs dependent on glycolysis for in vitro growth. Indeed, 2-DG-mediated reduction of glycolytic flux may limit anabolism to slow LIF-FGF2 cESC proliferation independent of ATP. Additionally, chemical inhibition of electron transport had no effect on the acute expansion of LIF-FGF2 cESCs. This is consistent with respirometry experiments with hESCs (Sperber et al., 2015) and reports that ETC activity is dispensable for maintenance of primed pluripotency (Varum et al., 2009). In contrast, 2iL cESCs showed both elevated respiratory and glycolytic reserve capacity and were tolerant to partial inhibition of glycolysis by 2-DG, implicating that divergent metabolic programs support proliferation in LIF-FGF2 and 2iL cESCs with regards to glucose utilization.

Enhanced glycolytic reserve capacity observed in oxidative 2iL cESCs suggests a degree metabolic pathway flexibility that is similar to the "bivalent" metabolic responses observed with naïve R1 mESCs than primed hES-2 hESCs. However, 2iL cESC proliferation and ATP generation was sensitive to RTN exposure. Lower ATP content of RTN-treated 2iL cESCs could signify that glycolysis alone cannot meet the bioenergetic demand for steady state growth of 2iL cESCs. Conversely, RTN treatment may be associated with ROS production (Li et al., 2003) and indirectly affecting cellular ATP through undefined retrograde ROS signalling mechanisms. Given the practical and possibly therapeutic advantages of naïve-like mammalian ESC and iPSC (reviewed in (Dodsworth et al., 2015)), it is critical that the influence of persistent oxidative metabolism on stress pathways and cellular damage be thoroughly explored.

Despite the interest in deriving and maintaining naïve-like PSCs from non-rodent species (Ware, 2017), little is known regarding the advantages of active mitochondrial metabolism for naïve PSC physiology. Nutrient utilization can have a global effect in cell phenotype by regulating the availability of reactants or cofactors needed for epigenetic modification enzymes, thereby altering the epigenetic landscape and transcriptional networks responsible for cell identity (Moussaieff et al., 2015; Sperber et al., 2015). Our laboratory is currently investigating whether this metabolic shift is a determinant for pluripotent state reversion in mammalian PSCs and if ectopic activation/inhibition of metabolic enzymes alters the pluripotent identity.

5. Conclusion

The described work represents the first evidence of suboptimal glycolytic flux in cESCs lines as well as quantifiable remodeling of the cristae membranes associated with a metabolic switch towards mitochondrial metabolism with naïve-like pluripotency induction in the dog. This metabolic plasticity between primed- and naïve-like cESCs is likely to impact the demand and utilization of nutrients that support self-renewal. This study lends additional support to the notion that cellular metabolism is a useful functional readout of pluripotent identity in placental mammals, but species-specific attributes are likely to exist.

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Disclosure of potential conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions


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