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Positioning Canine Induced Pluripotent Stem Cells in the reprogramming landscape of naïve or primed state in comparison to Mouse and Human iPSCs

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Abstract:	This study aims to understand whether canine induced pluripotent stem cells (iPSCs) belong to naïve or prime state in comparison to mouse (m) iPSCs. Main Methods In the present study, we derived ciPSCs in presence of LIF and compared their state of pluripotency with that of mouse iPSCs by culturing them in the presence of LIF, bFGF, and LIF+bFGF. Gene expression level at transcript level was performed by RT-PCR and qRT-PCR and at the protein level was analyzed by immunofluorescence. We also attempted to understand the pluripotency state using lipid body analysis by bodipy staining and blue fluorescence emission. Key findings In contrast to miPSCs, ciPSCs culture in the presence of bFGF and LIF+bFGF showed enhanced expression of core pluripotent genes Oct4 , Nanog and Sox2 . However, these cells expressed naïve pluripotent marker SSEA1 and lacked the expression of primed state marker SSEA4. Interestingly, for the first time, we demonstrate the ciPSC pluripotency using lipid body analysis wherein ciPSCs showed enhanced bodipy staining and blue fluorescence emission, reflecting the primed state of pluripotency. As ciPSCs exhibit characteristic properties of both naïve and primed pluripotent state, it probably represents a unique intermediary state of pluripotency that is distinct from mice. Significance Elucidating the pluripotency state of ciPSCs assists in better understanding of the reprogramming events and development in different species. The study would provide the footprint of species-specific differences involved in the reprogramming and the potential implication of iPSCs as a tool to analyse the evolution.

Declaration of interests

☒ The authors declare that there is no conflict of interest that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Title:

Positioning Canine Induced Pluripotent Stem Cells in the reprogramming landscape of naïve or primed state in comparison to Mouse Induced Pluripotent Stem Cells

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Declarations Life Sciences require that the corresponding author, signs on behalf of all authors, a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated. Conflict of Interest A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

The authors declare that there are no conflicts of interests.

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Author Contribution to Study

All authors listed on your paper must have made significant contributions to the study. To ensure clarity, you are required to enter the specific details of each author’s contribution, which must substantiate the inclusion of each person on the manuscript. Please detail this information below (submit additional sheets as necessary):

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October 25nd , 2020

Dear Editor,

As suggested by the reviewer, we have reframed the sentence. Our responses, point by point, to the questions raised by the reviewers are in "Response to Reviewers" file.

The revised text in the main manuscript is marked by the track changes (Red font) throughout the manuscript for easy tracking. We hope for a positive evaluation of our responses. Once again thank you for considering our manuscript in your esteemed journal "***Life Sciences".***

Sincerely,

Anujith

Anujith Kumar, PhD

Dear Editor,

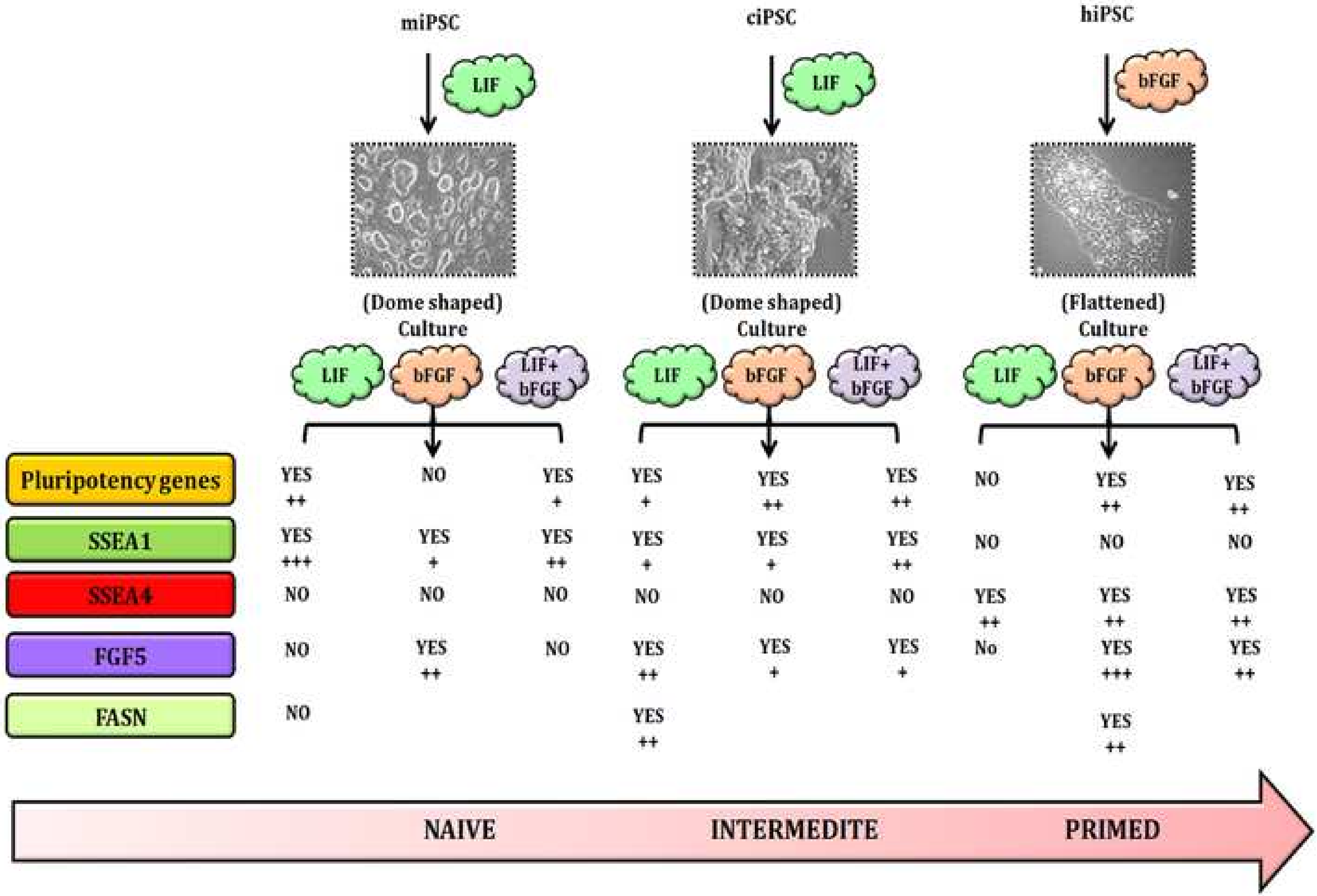
As per the suggestions of the reviewers, we have reframed the sentence. Hope the revised manuscript satisfies and finds it suitable for publication. Changes incorporated in the revised version are indicated by highlighting the sentences. Please find below the response for each comment provided in a point wise and italicized format. The text which is included in the main text are marked by red font for easy tracking.

Reviewer #1:

Comment 1: Thank you again for those additional revisions. Unfortunately, I strongly disagree with you regarding your response statement regarding the potential effect of residual transgene expression possibly affecting your observations: "As the present study is focusing on placing the ciPSCs in the landscape of naïve and primed PSCs, we believe that the residual transgene expression probably does not affect the inference of our observation". All I'm asking is for you to acknowledge this possibility.

Response: As suggested by the reviewer, we have reframed the sentence and included the possible consequence of residual transgene expression. Following sentence has been included in the discussion section, line 372-376 "*To authenticate the pluripotency of ciPSCs, we performed several pluripotent assays and found, except for the differential suppression of transgenes, ciPSCs fulfilled majority of the criteria required to be confirmed it as a bonafide iPSCs. However, we can't negate the residual transgene expression having the possibility of potentially affecting the pluripotent state and differentiation ability of the cells".*

Graphical Abstract



	miPSC			ciPSC			hiPSC		
	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF
Pluripotency genes	YES ++	NO	YES +	YES +	YES ++	YES ++	NO	YES ++	YES ++
SSEA1	YES +++	YES +	YES ++	YES +	YES +	YES ++	NO	NO	NO
SSEA4	NO	NO	NO	NO	NO	NO	YES ++	YES ++	YES ++
FGF5	NO	YES ++	NO	YES ++	YES +	YES +	No	YES +++	YES ++
FASN	NO			YES ++				YES ++	

Positioning Canine Induced Pluripotent Stem Cells (iPSCs) in the reprogramming landscape of naïve or primed state in comparison to Mouse and Human iPSCs

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ABSTRACT

Aims

Deriving canine-induced pluripotent stem cells (ciPSCs) have paved the way for developing novel cell-based disease models and transplantation therapies in the dog. Though ciPSCs have been derived in the presence of Leukemia inhibitory factor (LIF) as well in the presence of basic fibroblast growth factor (bFGF), the positioning of ciPSCs in the naïve or the primed state of pluripotency remains elusive. This study aims to understand whether canine iPSCs belong to naïve or prime state in comparison to mouse (m) iPSCs and human (h) iPSCs.

Main Methods

In the present study, we derived ciPSCs in presence of LIF and compared their state of pluripotency with that of miPSCs and hiPSCs by culturing them in the presence of LIF, bFGF, and LIF+bFGF. Gene expression level at transcript level was performed by RT-PCR and qRT-PCR and at the protein level was analysed by immunofluorescence. We also attempted to understand the pluripotency state using lipid body analysis by bodipy staining and blue fluorescence emission.

Key findings

In contrast to miPSCs, the naïve pluripotent stem cells, ciPSCs showed the expression of FGF5 similar to that of primed pluripotent stem cell, hiPSCs. Compared to miPSCs, ciPSCs cultured in presence of LIF showed enhanced expression of primed pluripotent marker FGF5, similar to hiPSCs cultured in presence of bFGF. Upon culturing in hiPSC culture condition, ciPSCs showed enhanced expression of core pluripotency genes compared to miPSCs cultured in similar condition. However, ciPSCs expressed naïve pluripotent marker SSEA1 similar to miPSCs and lacked the expression of primed state marker SSEA4 unlike hiPSCs. Interestingly, for the first time, we demonstrate the ciPSC pluripotency using lipid body analysis wherein ciPSCs showed enhanced bodipy staining and blue fluorescence emission, reflecting the primed state of pluripotency. ciPSCs expressed higher levels of fatty acid synthase (*FASN),* the enzyme involved in the synthesis of palmitate,similar to that of hiPSCs and higher than thatof miPSCs. As ciPSCs exhibit characteristic properties of both naïve and primed pluripotent state, it probably represents a unique intermediary state of pluripotency that is distinct from that of mice and human pluripotent stem cells.

Significance

Elucidating the pluripotent state of ciPSCs assists in better understanding of the reprogramming events and development in different species. The study would provide a footprint of species-specific differences involved in reprogramming and the potential implication of iPSCs as a tool to analyse evolution.

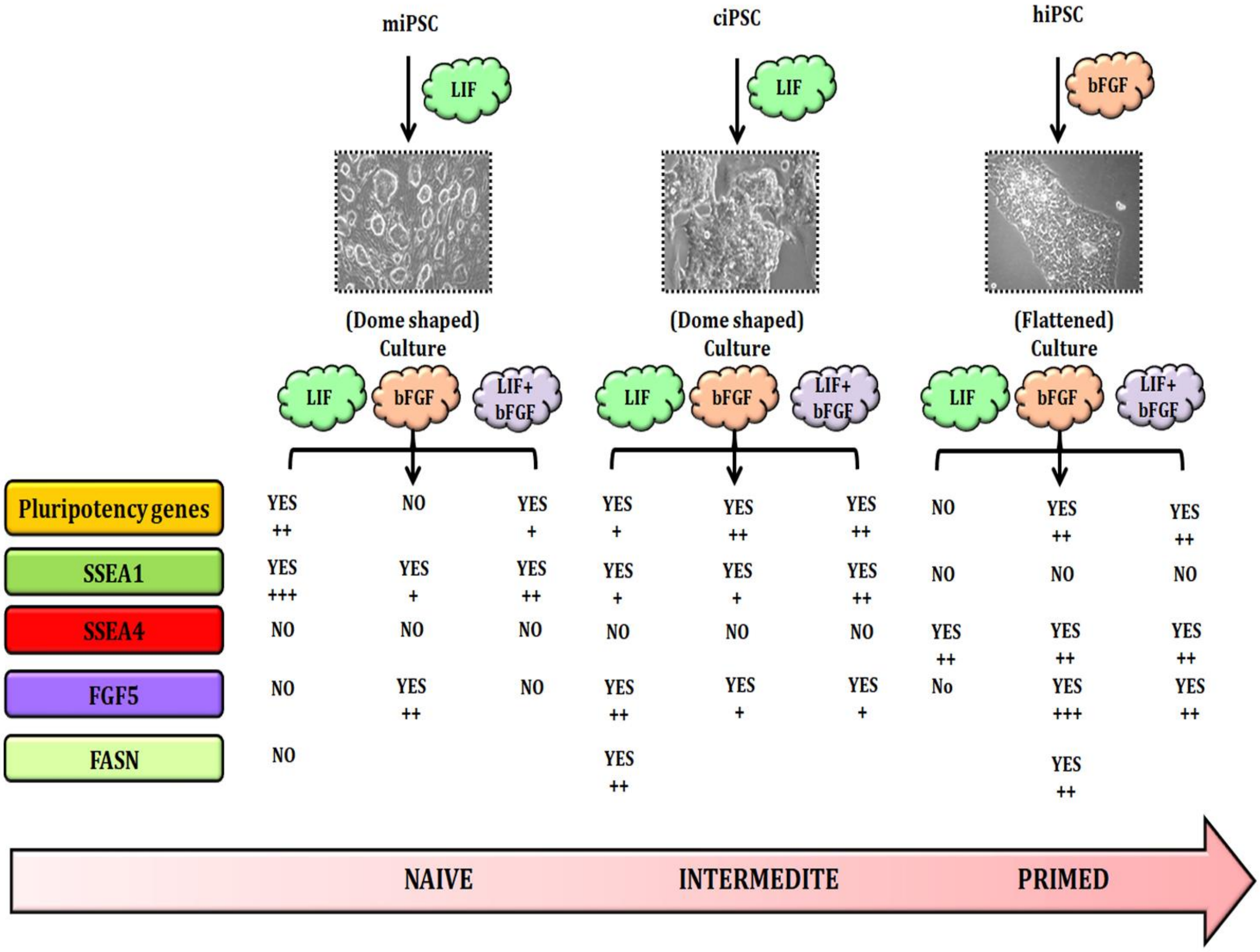
Keywords: Canine induced Pluripotent Stem Cells; Reprogramming; Naïve and Prime Pluripotency; Regenerative Medicine; Stem cell therapy models; lipid bodies.

Abbreviations

PSCs, Pluripotent stem cells; ciPSCs, canine induced pluripotent stem cells; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; miPSCs, mouse induced pluripotent stem cells; hiPSCS, human induced pluripotent stem cells; EpiESCs, Epiblast Embryonic Stem Cells; LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor; iMEF, inactivated mouse embryonic fibroblasts; EB, embryoid body; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; STAT3, Signal Transducer and Activator 3, JAK, Janus Kinase; SSEA; Surface Specific Embryonic antigen

GRAPHICAL ABSTRACT:

ciPSCs derived in presence of LIF showed characteristic properties resembling both naïve and primed pluripotent states. In contrast to miPSCs and similar to hiPSCs, ciPSCs demonstrated the expression of pluripotent genes, *FGF5* and expression of *FASN*, the gene involved lipid metabolism. However, similar to miPSCs, ciPSCs expressed SSEA1 in all the conditions and not the SSEA4, the characteristic property of naïve state. Considering these observations we propose ciPSCs to probably belong to the intermediary state of pluripotency.



	miPSC			ciPSC			hiPSC		
	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF
Pluripotency genes	YES ++	NO	YES +	YES +	YES ++	YES ++	NO	YES ++	YES ++
SSEA1	YES +++	YES +	YES ++	YES +	YES +	YES ++	NO	NO	NO
SSEA4	NO	NO	NO	NO	NO	NO	YES ++	YES ++	YES ++
FGF5	NO	YES ++	NO	YES ++	YES +	YES +	No	YES +++	YES ++
FASN	NO			YES ++				YES ++	

Introduction

Due to their quintessential properties of self-renewal and pluripotency, induced pluripotent stem cells (iPSCs) offer unprecedented opportunities in regenerative medicine [1,2]. iPSCs can be derived from any cell types of mammalian and non-mammalian origin. However, increased availability of these numerous iPSC model systems has often led to confusion regarding the appropriate model to be used [3].One of the criteria to select the appropriate PSC model is based on whether they exist in a naïve or primed state. Naïve PSCs which are exemplified by mouse embryonic stem cells (mESCs) and miPSCs correspond to ICM of blastocysts and exhibit distinctive properties such as compact and dome-shaped morphology and dependence of self-renewal and proliferation on the leukemia inhibitory factor (LIF)–Jak/Stat signalling pathway [4]. Other key features of naïve PSCs is the specific expression of genes like SSEA1, REX1, and STELLA, possession of two active X chromosome (XaXa) and reduced single-cell mortality [5]. On the other hand, primed PSCs exemplified by mouse epiblast stem cells (mEpiSCs), human ESCs (hESCs), and human iPSCs correspond to the epiblast cells of the post-implantation stage [4]. Primed PSCs have key features of flattened morphology and dependent on basic fibroblast growth factor (bFGF)-rather than LIF for self-renewal and proliferation [6]. In contrast to naïve PSCs, primed PSCs exhibit an inactivated X chromosome (X_aX_i), increased single cell mortality, and express epiblast markers FGF5 and OTX2, along with core pluripotent genes *Oct4* and *Sox2* [7]. Recent reports have shown the successful conversion of human PSCs to naïve state resembling mESCs using chemical compounds GSK3β inhibitor and a MEK/ERK inhibitor (2i) [5]. These reports confirm that the primed PSCs are not restricted to one way forward differentiation but have the ability to dedifferentiate to native PSCs also.

It has been of dilemma whether the naïve or prime pluripotent state is species-specific or is it a culture condition mediated effect. Previously, the generation of naïve pluripotent state was confined to mESCs and PSCs derived from rat embryos. However, recently, the derivation of PSCs in naïve states has been extended to other species such as porcine fibroblasts, rhesus monkey fibroblasts, and rabbit embryos by modifying culture conditions applied during the course of reprogramming [8–10]. Several reports have also claimed the successful derivation of naïve human PSCs by either modifying culture conditions or by over-expressing key pluripotent transcription factors [11–13]. Various pathways are involved in the effective maintenance of iPSCs in all species, the prominent ones are LIF/STAT3, FGF, MEK/ERK, and BMP/SMAD pathways [14]. Depending on the state of pluripotency, two cytokines, LIF or bFGF is added to the culture media for the maintenance of PSCs. While naïve mESC pluripotency is maintained by LIF, FGF- mediated activation of MEK signaling drives differentiation of mESCs. On the other hand, primed mEpiSCs or hiPSCs require basic FGF signaling for maintenance of pluripotency whereas LIF signaling has no effect on pluripotency [15].

Canines being genomically and physiologically more similar to humans offer a better model compared to rodents in unraveling many of the human diseases [16-18]. Many reports have documented the derivation of canine iPSCs (ciPSCs) from various cell sources using different reprogramming approaches. Depending on the source of reprogramming factors, whether human or mouse, derived iPSCs showed subtle differences in their characteristic properties. Goncalves et al. observed that ciPSCs generated by mouse *Oct4*, *Sox2*, *Klf4*, *c-Myc* (OSKM) showed decreased silencing of expression of the exogenous gene, while complete silencing was observed in the ciPSC lines derived from human OSKM factors [19]. However, whether the generated ciPSC lines belong to naïve or pluripotent state remains unrevealed.

In this study, we attempted to understand the positioning of the ciPSCs in the reprogramming landscape. This was done by analyzing the differences in the pluripotency in ciPSCs, in comparison to miPSCs and hiPSCs, with respect to crucial growth factors, LIF, bFGF, and a dual supply of LIF and bFGF in the culture medium. Though the colonies exhibited specific morphology differences, the expression of pluripotency markers was observed in ciPSCs cultured in all the three conditions. We then tried to understand the differences in lipid metabolism in ciPSCs and miPSCs. Taken together, we propose a distinct pluripotent state for ciPSCs which probably stands between naïve and primed states as revealed by their gene expression differences and lipid metabolism.

Materials and methods

Generation of iPSCs:

Canine dermal fibroblasts (CFBs) were derived from a skin punch biopsy from the ventral abdomen of a 9-month-old Mongrel from the Department of Surgery, College of Veterinary Sciences and Animal Husbandry, Anand Agricultural University, with appropriate approval from the institutional animal ethics committee. Dermal fibroblasts were expanded from skin explants in DMEM F-12 medium with 10% FBS and 1x Penicillin Streptomycin (Gibco) at 37˚C in 5% CO2. CFBs were up-scaled in suitable culture vessels for subsequent experiments and also cryo-preserved and kept in liquid nitrogen conditions.

Retrovirus plasmids expressing human OCT4, SOX2, KLF4, and C-MYC,(Plasmids are a kind gift from Prof. Catherine Verfaillie, KU, Leuven) were formed by individually transfecting each of these constructs in HEK 293T cells with the retroviral packaging vectors pSPAX2 and pMD2G. 293T cells ($8x10^6$) were transfected with lipofectamine (1:3 ratio) in HEK 293T medium consisting of DMEM high glucose (Gibco) with 10% FBS, 0.1mM NEAA, 6mM L-glutamine. After 48 hours of transfection, the supernatant was collected and added to $1.5x10^6$cells per well of a 6 well plate of CFBs. This medium containing retroviral particles was replaced with a second round of concentrated supernatant (72 hours) from the transfected HEK 293T cells on the following day. After 24 hours, the medium was replaced with fresh HEK 293T medium. After 5 days, transduced fibroblasts were passaged on inactivated MEFs and cultured in iPSC media (DMEM F12, 15% FBS, 2mM L-glutamine, 0.1mM NEAA, 0.075mM β-mercaptoethanol, 1mM sodium pyruvate, 1x Penicillin Streptomycin and LIF-8ng/ml). Media compositions can be found in Supplementary Table 3. iPSC colonies with compact ES-like cells were observed after 20-22 days. Colonies were manually picked, trypsinized, and transferred to new feeder plates and maintained in iPSC medium at 37°C, 5% CO2. The iPSC colonies were maintained in this condition for fifteen passages before transferring to different conditions. ciPSCs were also transferred to feeder-free vitronectin and maintained up to forty passages.

miPSCs derivation :

miPSCs were derived using a previously published protocol (20). In brief, the protocol includes the transduction of mouse embryonic fibroblasts (MEFs) seeded at the density of 1.5×10^6 cells /well in six-well plates with retroviral vectors containing supernatant for mouse Oct3/4, Sox2, and Klf4 (Addgene). To enhance the efficiency of transduction, MEFs were transduced twice with an interval of 24hrs. Cells were maintained in fibroblast medium for two days and were later changed to mESC medium. On day 4, post-viral transduction, transduced fibroblasts were trypsinized into single-cell cultures and reseeded on 6 well plates at a density of 0.5×10^6 cells per well on mitomycin inactivated MEF feeders. Colonies observed after 20-25 days were manually picked and further propagated.

hiPSCs culture:

NCL-1 hiPSCs (passage 23) were procured from EyeStem research Pvt. Ltd., Bangalore and cultured on feeder- free conditions on 1% matrigel (BD Corning) coating. Stem MACS iPS-brew XF (MACS media) medium was used for everyday medium change. The cells were split using Accutase (Gibco) and seeded at a ratio of 1:5. Further, they were cultured in different conditions of LIF, bFGF, and LIF+bFGF addition in hiPSC medium (composition mentioned in supplementary table 3).

Embryoid body formation:

ciPSC colonies cultured in presence of LIF were transferred into iPSC medium devoid of LIF. iPSC`s were induced to differentiation into EBs by plating on low adherent plates (Nunc) with mESC medium without LIF. Media change was done every alternate day for 10 days before proceeding to RNA isolation.

RNA extraction, cDNA synthesis and PCR:

Cells were lysed with trizol reagent and total RNA was extracted using RNeasy Mini kit (Qiagen) as per the manufacturer`s protocol. Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Fermentas) according to the manufacturer`s instructions. Canine specific primers (Supplementary Table 2) were designed for detecting endogenous expression of stemness genes. PCR was performed using Emerald PCR master mix with Taq DNA polymerase (Takara) with the cycle parameters as denaturation at 95°C for 5 minutes, amplification for 35 cycles, annealing for 20 seconds at 58°C and extension at 72°C for 30seconds and a final extension at 72° C for 10 minutes. The primers and their product sizes are given as a separate table. PCR products were resolved on 2% agarose gels with Ethidium Bromide. Gels were photographed using alpha imager.

Quantitative PCR was carried out using SYBR Green (Takara). The samples were analysed using the 7500 RT-PCR (ABI Biosystems) and were normalized with a house-keeping gene *Gapdh* to obtain the relative fold change among samples.

Immunocytochemistry:

iPSC colonies were washed in phosphate-buffered saline (1xPBS) twice and then fixed in 4% paraformaldehyde for 20 minutes at room temperature (RT). Colonies were washed twice with 1xPBS for 1 min each and permeabilized with Triton-X-100 in PBS for 15 minutes. Colonies were incubated overnight at 4°C with primary antibodies of appropriate dilutions (Supplementary Table 1). The next day, colonies were washed twice with 0.05% PBST and secondary antibody was added and incubated at RT for 1hr. After three washes in PBS for 1 minute each, cells were stained with 1:10,000 diluted DAPI for 3-5minutes. Cells were visualized and photomicrographed on an inverted fluorescence microscope. The images were processed with ImageJ software.

Blue Fluorescence imaging and Bodipy staining:

Confluent cultures of ciPSCs and miPSCs on vitronectin were analysed for blue fluorescence detection. PSC culture medium was replaced by DMEM high glucose basal medium without phenol red to avoid interference of phenol red during blue fluorescence imaging. The images were captured with a Nikon Eclipse TE2000 U attached to a Qicam Fast 1394 digital camera and Q Capture Pro software. The blue fluorescence was visualized using DAPI filter Cube (Nikon EPI-FL filter). The lipid body associated retinyl ester blue fluorescence images were acquired first followed by the acquisition of phase-contrast images. The images were merged to obtain the final images.

The ciPSCs and miPSCs were seeded onto culture dishes with suitable cell density and were subjected to bodipy staining, after 48 hours of culture. The cells were washed with PBS and incubated with bodipy solution (1:2000 dilution in basal medium) for 15 minutes at 37°C. The culture dishes were covered with aluminium foil to protect from light. After 15 minutes, the cells were washed with PBS and then fixed with 4% PFA for 30 minutes at room temperature. After removing the PFA, the cells were washed thrice with PBS and were immediately observed under the fluorescence microscope.

Statistical analysis:

Student's t-test was used to analyse the difference between the cells and their respective controls. The values with $p<0.05$ were considered statistically significant and those with $p<0.001$ were considered highly significant. Graph pad prism and Excel software tools were used for qPCR analysis and graph preparation. ANOVA was used for analysing the QPCR triplicate values.

RESULTS

Characterization of ciPSCs generated in presence of LIF:

Adult canine dermal fibroblasts were reprogrammed into ciPSCs by transduction with retroviruses expressing human transcription factors OCT4, SOX2, C-MYC, and KLF4. After 17 days, ES-like colonies with high nuclear to cytoplasmic ratio started emerging and were picked and re-plated onto inactivated MEFs and cultured in iPSC media with murine LIF. Colonies displayed a tightly packed morphology. Out of the total 5 clones isolated, three clones were enzymatically dissociated for further passaging and characterization. Various stages of reprogramming are depicted in figure 1a. For further characterization of ciPSCs, stemness marker expression was analysed by semi-quantitative PCR and immunofluorescence. Compared to CFBs, ciPSCs expressed endogenous pluripotency markers *OCT4*, *SOX2*, *KLF4,* and *NANOG* as analysed by semi-quantitative PCR (Figure 1b). Along with this, significant up-regulation of the epigenetic marker, *de novo* methyltransferase, *DNMT3A* was witnessed in ciPSCs (Figure 1b). In line with expectation, reprogramming resulted in the down-regulation of fibroblast marker *VIMENTIN* in ciPSCs as compared to canine fibroblasts (Figure 1c). Expression of pluripotent markers OCT4, SOX2, and SSEA1 at protein level further confirmed the reprogramming of canine fibroblasts (Figure 1d). Analysis of transgene silencing across different passages showed reduced expression of exogenous *OCT4*, *SOX2*, and *KLF4* with increase in the passage, however, the decrease in *OCT4* and *KLF4* transgenes was not to the extent of that in *SOX2* transgene. (Figure 1e). ciPSCs could be passaged as single cells enzymatically and could be cultured on inactivated MEF up to passage 15 and maintained under feeder-free conditions up to passage 40 with vitronectin.

To understand its differentiation ability, the spontaneous differentiation approach of forming EBs showed efficient skewing towards all the three lineages. Semi-quantitative PCR analysis of differentiated EBs demonstrated expression of representative ectoderm markers *PAX6* and *FOXG1*, mesoderm markers *VEGF* and *FLK1*,and endoderm markers *SOX17* and *CXCR4,* all of which were absent in control ciPSCs. Control ciPSCs showed expression of *DNMT3A*, a marker for *de novo* DNA methylation which was absent in EB confirming the differentiation of ciPSCs (Figure 1f & 1g). These results demonstrate the authenticity of ciPSCs derived from canine fibroblasts generated in the presence of mouse LIF.

ciPSCs derived in the presence of LIF exhibit mixed naïve and primed state properties:

To understand whether the generated ciPSCs resembles more of a naïve or primed state of pluripotency, we compared their morphology and gene expression profile with miPSCs representing the naive state and hiPSCs being primed state. ciPSCs cultured in presence of LIF possessed dome-shaped morphology similar to miPSCs and were unlike hiPSCs that were flattened (Figure 2a). All the iPSCs expressed higher levels of pluripotency markers compared to their fibroblast counterparts (Figure 2b i-iii). As SSEA1 expression in miPSCs and SSEA4 expression in hiPSCs represent the naïve and the primed state respectively, the identity of the pluripotent state of ciPSCs was tested by using these two markers. Similar to miPSCs, ciPSCs cultured in presence of LIF expressed SSEA1 but not SSEA4, thus advocating their naive state

(Figure 2c and d). Surprisingly, upon transcript analysis, ciPSCs belonging to distinct class of PSCs, exhibited characteristic features of naïve PSCs by expression of REX1 similar to miPSCs, and significantly lesser expression of OTX2 compared to hiPSCs. On the other hand, their expression levels of reduced KLF4 compared to hiPSCs and increased expression of FGF5 compared to miPSCs, resembled the signatures of primed PSCs (Figure 2E). These results strongly indicated ciPSCs to belong to a distinct state of pluripotency compared to that of naïve miPSCs and primed hiPSCs.

To test whether ciPSCs cultured in presence of LIF switched to that of bFGF have an altered pluripotent state, we cultured ciPSCs in presence of either LIF, bFGF or a combination of LIF+ bFGF conditions, along with controls miPSCs and hiPSCs. Similar to miPSCs, ciPSCs exhibited a dome-shaped morphology in the presence of LIF but exhibited a differentiated flattened morphology in bFGF and LIF+bFGF conditions (Figure 3a). However, ciPSCs could be maintained for up to fourteen passages in the bFGF and LIF+bFGF conditions, whereas miPSCs could be maintained only for two passages in similar culture conditions. hiPSCs maintained their stem cell-like compact morphology in presence of bFGF and failed to do so under LIF and LIF+bFGF supplementation. Evaluation of gene expression of ciPSCs in these culture conditions, similar to hiPSCs, showed enhanced expression of pluripotency genes *OCT4*, *NANOG*, and *SOX2* in bFGF and LIF+bFGF culture conditions compared to that miPSCs (Figure 3bi). As expected, bFGF deprivation and LIF supplementation in hiPSCs culture showed reduced *OCT4*, *NANOG*, and *SOX2* expression considerably. Interestingly, ciPSCs cultured in LIF, the cytokine used to maintain naïve pluripotency, expressed a higher amount of primed marker *FGF5* compared to miPSCs, which was not sustained upon bFGF or LIF+bFGF addition. hiPSCs expressed higher levels of *FGF5* than miPSCs in all the culture conditions, reaffirming their primed status (Figure 3b ii). The analysis of these results of *FGF5* expression revealed the characteristic features of primed stem cells in ciPSCs.

ciPSCs cultured in all three conditions were allowed to form EBs and RNA isolation was done on the 10th day. Transcripts of undifferentiated canine iPSCs were used as control. However, ciPSCs cultured in bFGF and miPSCs cultured in bFGF and LIF+bFGF conditions failed to form EBs (data not shown). The lineage marker expression of canine EBs cultured in LIF only and LIF+bFGF conditions were evaluated by q-PCR and the representative genes of all three lineages showed enhanced expression (Figure 3c). SSEA1 expression in miPSCs, ciPSCs and hiPSCs in three different culture conditions was evaluated by immunofluorescence, and percent positive cells in miPSCs and ciPSCs was quantified. Interestingly, similar to miPSCs, ciPSCs cultured in presence of bFGF showed two-fold lesser expression of SSEA1 compared to that of cells cultured in presence of LIF or LIF+bFGF (Figure 3d, e and f). SSEA1 expression was not detectable in hiPSCs cultured in all three conditions (figure 3d). While hiPSCs in all three conditions showed sustained expression of SSEA4, miPSCs and ciPSCs lacked SSEA4 expression (Figure 3g). The combined analysis of the results of *FGF5*, SSEA1, and SSEA4 expression encouraged us to categorize ciPSCs to belong intermediate state of pluripotency.

ciPSCs exhibit characteristic blue fluorescence and neutral lipid staining different from miPSCs

A previous report showed the use of characteristic blue fluorescence emitted by the primed pluripotent stem cells, but neither the differentiated cells nor the naïve mESCs, as an approach to identify and isolate the pure primed pluripotent population [21]. To understand the identity of the ciPSCs, we also looked into the emission of blue fluorescence from ciPSCs. Surprisingly, in contrast to naïve pluripotent miPSCs, ciPSCs exhibited characteristic blue fluorescence (Figure 4).

As the emission of blue fluorescence by the primed PSCs is due to the sequestration of retinyl esters in cytoplasmic lipid bodies, we analysed the lipid phenotypes by bodipy staining in ciPSCs and miPSCs cultured in presence of either LIF, bFGF or LIF+bFGF. We found enhanced bodipy staining in miPSCs cultured in presence of LIF, but not in bFGF and LIF+bFGF- the conditions which led miPSCs to differentiate. In contrast, the bodipy staining was observed in ciPSCs cultured in all three conditions and the highest staining was observed in ciPSCs cultured in LIF+bFGF conditions (Figure 5a). Bodipy staining in canine dermal fibroblasts and mouse fibroblasts showed minimal staining which is similar to that observed in miPSCs cultured in presence of bFGF and LIF+bFGF (Figure 5b). We looked at the expression of Fatty acid synthase (*FASN*), the gene responsible for long-chain fatty acid synthesis, in ciPSCs cultured under three conditions as shown in figure 5c. ciPSCs showed significant up-regulation in *FASN* expression in all three conditions compared to that of cells cultured in presence of LIF(Figure 5c). In contrast to miPSCs, ciPSCs showed enhanced expression of *FASN* cultured in presence of LIF, similar to that of hiPSCs cultured with bFGF (Figure 5d). These results reiterated the classification of ciPSCs under intermediate state of pluripotency.

DISCUSSION

There are many limitations in using human patients and also hESCs for stem cell research. Efficient animal models like canine models can accelerate the progress in stem cell therapy and the preclinical trials using iPS cells. Dogs share disease pathogenesis similar to that of humans which makes them an alternative model for understanding disease development from an early stage. Several studies have shown the generation of ciPSCs and their differentiation potential to different lineages. Lee et al. derived endothelial cells from the ciPSCs and studied their efficacy in immune-deficient mice models of hind limb ischemia and myocardial infarction [22]. It has also been reported that ciPSC- derived mesenchymal stem cells (iMSCs) displayed proficient differentiation into osteo, chondro and adipogenic cells and also suggested the use of iMSCs in cell therapy in osteoarthritis in canine patients and also as a model system for degenerative joint disease in humans [23]. In a similar study by Chow et al., canine iMSCs exhibited efficient proliferation and immune-modulatory features, similar to that of canine Ad-MSCs and BM-MSCs [24].

Considering different applications of ciPSCs, it is necessary to understand the state of ciPSCs for their efficient culture and maintenance. Optimization of culture conditions for their self-renewal and maintenance are key points in obtaining stable and reproducible ciPSC lines. We derived ciPSCs from canine dermal fibroblasts of mongrel breed by a retroviral approach using human reprogramming factors. Various reprogramming approaches have been performed for the generation of ciPSCs [25]: retroviral [3,26,27], lentiviral [19, 22, 28–31] and sendai virus [24,32] methods. Tsukamota et.al reprogrammed embryonic fibroblasts by an auto-erasable sendai virus vector but with lower efficiency [32]. Shimada et al derived ciPSCs by canine OSKM [26], but most groups reported ciPSC derivation by using either mouse [28, 33] or human [22, 27, 29, 30, 34] reprogramming factors. ciPSCs derived by Goncalves et al. reported the use of murine and human OSKM factors separately and in combination [19, 34], by the lentiviral method. Further in-depth studies have to be performed to elucidate whether species difference in reprogramming factors might influence canine iPSC derivation. Understanding ideal culture conditions for efficient passaging and maintenance of ciPSCs is necessary for maintaining their quality and also for further differentiation experiments. We derived ciPSCs on inactivated MEF and compared them with naïve pluripotent miPSCs and primed pluripotent hiPSCs. To authenticate the pluripotency of ciPSCs, we performed several pluripotent assays and found, except for the differential suppression of transgenes, ciPSCs fulfilled majority of the criteria required to be confirmed it as a bonafide iPSCs. However, we can’t negate the residual transgene expression having the possibility of potentially affecting the pluripotent state and differentiation ability of the cells. Derived ciPSCs were able to maintain on vitronectin for more than 40 passages. Most reports used inactivated mouse embryonic fibroblasts (MEFs) as the feeder layer for maintaining canine iPSC cultures except for Nishimura et al. who reported a feeder-free culture of ciPSCs in a doxycycline-inducible system [28].

The majority of the reports showed the pluripotency of ciPSCs to be maintained in culture conditions containing both LIF and bFGF [22, 26, 27, 29, 31, 33, 34]. Few reports also demonstrated the possibility of maintaining ciPSCs' pluripotency in the presence of either LIF or bFGF alone [30, 34, 35]. Vaags et al., derived the cESCs in presence of hLIF and bFGF and found the absence of LIF to result in spontaneous differentiation [36]. Similarly, Wilcox et al.,also reported the derivation of cESCs with the dual combination of LIF and bFGF [37]. Using LIF and inhibitors of glycogen synthase kinase 3β and mitogen-activated protein kinase 1/2 [called 2i and LIF (2iL)], Tobias et al., converted cESCs resembling primed PSCs toward a naïve pluripotent state [38]. LIF-dependent ciPSC colonies, derived by Whitworth et al. differentiated into fibroblast cells in the presence of LIF and bFGF, similar to cESCs derived by Wilcox et al [30, 37]. But ciPSCs derived in the presence of bFGF exhibited no change in pluripotency or proliferation when cultured with or without LIF [19]. Previous reports showed loss of pluripotency expression in ciPSCs when LIF or bFGF was removed [33]. Though AKT and ERK1/2 remained consistently activated, the loss of LIF resulted in STAT3 dephosphorylation and thereby differentiation [29]. In a subsequent report, the authors implied the role of bFGF in pluripotency similar to that of primed state cells. Removal of bFGF or inhibition of the SMAD2/3 pathway led to significant repression of NANOG[39].Comparison of ciPSCs with miPSCs and hiPSCs showed ciPSCs to harbour the characteristic properties of both naïve and primed pluripotent state. ciPSCs showed the characteristics of naïve PSCs by expression of SSEA1 and lacking the expression of SSEA4. On the other hand, ciPSCs also cultured in LIF showed the inherent expression of FGF5, similar to that of primed PSC hiPSCs cultured in presence of bFGF. Surprisingly, switching of culture conditions of ciPSCs from naïve to that of primed PSCs showed an enhanced expression of pluripotent genes in the presence of bFGF and LIF+bFGF compared to the cells cultured in presence of LIF alone, a phenotype contrast to that of miPSCs but similar to that of hiPSCs. Similar report of increased expression of *NANOG* was observed in bFGF cultured ciPSCs by Luo et al.,[39].In our experimental conditions, culturing miPSCs, hiPSCs and ciPSCs in different culture conditions probably does not facilitate them in switching from primed to naïve state or visa-versa, as naive miPSCs are not converted to a primed-like state by simple culture in bFGF alone, nor hiPSCs can be converted to naïve state by mere culturing them in presence of LIF [40,41]. When these PSCs are shifted from the culture that supports their native pluripotent state to non-permissible condition, they lose their pluripotent state and fails to differentiate as observed by their inability to form EBs by ciPSCs cultured in bFGF and miPSCs cultured in bFGF and LIF+bFGF conditions.

Morphological analysis of ciPSCs showed more of dome-shaped colonies, similar to that of miPSCs rather than flat-shaped hiPSC colonies. Different colony morphologies were reported in cESCs and ciPSCs by different groups. Dome-shaped cells, a characteristic feature of naïve states were reported by a few groups[28, 30, 40]. Flat colony morphology similar to primed state were observed in some reports[22, 26, 27,27,29,31, 33]. Interestingly, cESCs derived by two groups reported a heterogeneous colony morphology[36, 37]. Among these, Wilcox et al isolated canine embryos at morulae and blastocyst stages with 2 distinct cESC lines; one set by immunodissection of ICM (OVC.ID) and another set by embryo explants (OVC.EX). The cESC lines derived from the former set showed flat morphology and the latter set showed dome-shaped colonies[37].

Understanding the metabolic signatures is essential to discern the similarities and differences in different pluripotent stem cells. Previous studies have reported the difference in lipid content between the primed and naïve states [43, 44]. A significant abundance of FASN, the gene involved in lipid metabolism, and an enhanced accumulation of intracellular lipids were detected in primed LIF-FGF2 cultured cESCs compared to that of chemical inhibitor (2i)+ LIF cultured naive cESCs [44]. Further Muthuswamy et al., showed that the primed cells sequester retinol/ retinyl esters and maintain them in non-oxidized form to ensure prevention of differentiation of primed hiPSCs. Also, the primed cells possess the transcripts required to metabolize retinol and for its reuptake [21]. This intrigued us to question the lipid status of ciPSCs which will facilitate to place ciPSCs in the landscape of naïve and primed pluripotent state. The emission of blue fluorescence and bodipy staining reiterated the epiblast like characteristic feature of the ciPSCs generated in the presence of LIF. The control miPSCs which belongs to the naïve state also showed convincing bodipy staining but not the blue fluorescence. Similar to previous report, we also observed the enhanced expression of *FASN* in ciPSCs similar to that of hiPSCs[44]. The lack of the blue fluorescence of lipid bodies in miPSCs, despite enhanced lipogenesis, is probably due to the absence of retinyl ester sequestration. These observations confirm the high occurrence of lipogenesis in PSCs which is a distinct feature compared to that of somatic cell source.

A methodical analysis of various features is necessary for effective classification of iPSCs into specific pluripotency states [45]. SSEA marker expression suggests a naïve or prime state of pluripotency; mouse PSCs express SSEA-1 and human PSCs express SSEA-3 and SSEA-4 markers. In canine PSC reports, SSEA-4 expression was reported by more groups [22, 27, 29–31, 35] and some groups reported SSEA1 [32, 33, 42, 46] expression. Vaags et al reported the expression of both SSEA-3 and SSEA-4 and low levels of SSEA-1 expression in the derived cESCs [36]. Though many of the parameters analysed in this study showed the primed state of ciPSCs, the cell surface analysis of the expression of SSEA1 and not the SSEA4 in ciPSCs and the formation of EBs only in presence of LIF impedes us in categorically placing ciPSCs in the group of the primed pluripotent state. This is probably due to the derivation of ciPSCs in presence of LIF and not in the presence of bFGF, which is routinely used to generate the primed induced pluripotent stem cells. The time duration of iPS culture in particular conditions also can influence their characteristics [47]. Although, it is a formidable task to decisively position the pluripotent state of cells of different species, the in-depth characterization of ciPSCs through multiple approaches suggested ciPSCs to belong to its own distinct pluripotent state. However, to ascertain conclusively the pluripotent state of ciPSCs, further utilization of genomic assay such as RNA-sequencing and insilico comparisons between species, live-cell imaging and in-depth study of different parameters including lipid profile and functional assays such as chimera generation into pre- and post-implantation embryos and derivation of germ-like cells are imperious to decipher the actual pluripotent state of ciPSCs[48]. A previous report suggested that reprogramming pathways in higher animals like dogs and pigs are more similar to that of the human than to mice, validated by the similarity search and phylogenetic analysis[49]. Understanding the species-specific differences in reprogramming and state of pluripotency helps in drawing their evolutionary significance in development.

Conclusions

The dog is the best model to understand the complexities of inherited genetic diseases and also for precise modelling of neurodegenerative diseases unlike that of mice. We derived stable ciPSCs that exhibited a majority of features that resembled that of primed pluripotent stem cell state and a few of the qualities which mimicked naïve pluripotent stem cells. These data reflect the probability of ciPSCs to fall between prime and naïve states. Information obtained from our study, ciPSCs probably being in an intermediate state of pluripotency, makes us to think that ciPSCs will become a practical and promising tool to understand the animal evolution on a molecular basis. However, to conclusively annunciate the pluripotent state of ciPSCs, ATAC-Seq and epigenomic approach should be followed to have a better insight on the distinction between naïve and prime state. In a nutshell, unravelling the characteristic features of ciPSCs can be effectively harnessed for understanding the developmental aspects, disease pathology, biomarker and drug development which will benefit both human and veterinary medicine.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS:

Figure 1. Characterization of canine iPSCs generated in the presence of LIF.

a) Morphology of transduced canine dermal fibroblasts on day0, day 6 and day 17. **b)** RT-PCR analysis of pluripotency genes *OCT4, SOX2, KLF4,* and *NANOG* along with loading control *GAPDH*, **c)** RT-qPCR analysis of fibroblast gene *VIMENTIN* in ciPSCs and CFBs. Ct values were normalized to the value of *GAPDH,* **d)** Immunofluorescence images of pluripotency markers OCT4 (red), SOX2 (green), SSEA1 (red) in canine iPSCs. The nuclei were counterstained with DAPI, **e)** qRT-PCR analysis of *OCT4*, *SOX2* and *KLF4* transgenes across different ciPSC passages. Ct values were normalized to the value of *GAPDH*. **f)** RT-PCR analysis of lineage genes, ectoderm genes (*FOXG1* and *PAX6*), endoderm genes (*CXCR4* and *SOX17)* and mesoderm genes *FLK1* and *VEGF* along with *DNMT3A* in EBs of ciPSCs. *GAPDH* was used as a loading control. **g)** qRT-PCR analysis of relative expression of lineage markers in ciPSCs and EBs. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3), ***p<0.001. Scale bar represents 100 µm.

Figure 2. ciPSCs derived in the presence of LIF exhibit partial epiblastic characteristic properties. a) Phase contrast images of miPSCs, ciPSCs cultured in presence of LIF and hiPSCs grown in presence of bFGF, **b)** Expression analysis of pluripotency markers, *OCT4, SOX2* and *NANOG* in miPSCs (i), ciPSCs (ii) and hiPSCs (iii) with respect to their fibroblast controls, Ct values were normalized to the value of *GAPDH*, Protein expression analysis of SSEA1 **(c)** and SSEA4 **(d)** in naïve miPSCs, ciPSCs and primed hiPSCs, **e)** Comparative analysis of *REX1*, *KLF4*, *OTX2* and *FGF5* expression in miPSCs, ciPSCs cultured in presence of LIF and hiPSCs cultured in presence of bFGF. Data represented as mean ±S.E.M (n=3), *p<0.05, **p<0.01, ***p<0.001. Significance in figure 2e is calculated with respect to hiPSCs. Scale bar represents 100 µm.

Figure 3: ciPSCs cultured in presence of bFGF and LIF+ bFGF exhibit characteristic properties similar to that of primed pluripotent state. a) Comparison of morphological features of miPSCs, ciPSCs and hiPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **b)** Gene expression analysis of pluripotency markers *OCT4*, *NANOG*, *SOX2* (i) and primed marker *FGF5* (ii) in miPSCs, ciPSCs, and hiPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **c)** Relative expression of lineage markers in EBs cultured in LIF only and LIF+bFGF conditions. ciPSCs were taken as control. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3). **d)** Immunofluorescence images of SSEA1 expression by miPSCs, ciPSCs and hiPSCs cultured in the presence of either LIF or bFGF or LIF +bFGF conditions. Quantification of SSEA1 positive cells in miPSCs **(e)** and ciPSCs **(f)** cultures in LIF, bFGF and LIF+bFGF conditions. **g)** Comparative analysis of SSEA4 expression in miPSCS, ciPSCs and hiPSCs in three culture conditions; LIF, bFGF, and LIF+bFGF conditions. Data represented as mean ±S.E.M (n=3), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Scale bar represents 100 µm.

Figure 4. ciPSCS exhibit characteristic blue fluorescence distinct from miPSCs. Comparative analysis of blue fluorescence (excitation, 325–375 nm; emission, 460–500 nm) in ciPSCs and miPSCs. ciPSCs expressed characteristic blue fluorescence whereas miPSCs failed to show the blue fluorescence. Scale bar represents 100 µm.

Figure 5. ciPSCS exhibit neutral lipid staining distinct from miPSCs. a) Comparative analysis of bodipy expression in ciPSCs and miPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **b)** Comparative analysis of bodipy expression in MEF and CFB. **c)** Relative expression of Fatty acid synthase marker, *FASN* in ciPSCs cultured in LIF (L), bFGF and LIF+bFGF conditions was analysed. Ct values were normalized to the value of *GAPDH*. **d)** Relative expression of *FASN* in miPSCs, ciPSCs cultured in presence of LIF and hiPSCs

cultured in presence of bFGF. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3). Scale bar represents 100 µm.

Positioning Canine Induced Pluripotent Stem Cells (iPSCs) in the reprogramming landscape of naïve or primed state in comparison to Mouse and Human iPSCs

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ABSTRACT

Aims

Deriving canine-induced pluripotent stem cells (ciPSCs) have paved the way for developing novel cell-based disease models and transplantation therapies in the dog. Though ciPSCs have been derived in the presence of Leukemia inhibitory factor (LIF) as well in the presence of basic fibroblast growth factor (bFGF), the positioning of ciPSCs in the naïve or the primed state of pluripotency remains elusive. This study aims to understand whether canine iPSCs belong to naïve or prime state in comparison to mouse (m) iPSCs and human (h) iPSCs.

Main Methods

In the present study, we derived ciPSCs in presence of LIF and compared their state of pluripotency with that of miPSCs and hiPSCs by culturing them in the presence of LIF, bFGF, and LIF+bFGF. Gene expression level at transcript level was performed by RT-PCR and qRT-PCR and at the protein level was analysed by immunofluorescence. We also attempted to understand the pluripotency state using lipid body analysis by bodipy staining and blue fluorescence emission.

Key findings

In contrast to miPSCs, the naïve pluripotent stem cells, ciPSCs showed the expression of FGF5 similar to that of primed pluripotent stem cell, hiPSCs. Compared to miPSCs, ciPSCs cultured in presence of LIF showed enhanced expression of primed pluripotent marker FGF5, similar to hiPSCs cultured in presence of bFGF. Upon culturing in hiPSC culture condition, ciPSCs showed enhanced expression of core pluripotency genes compared to miPSCs cultured in similar condition. However, ciPSCs expressed naïve pluripotent marker SSEA1 similar to miPSCs and lacked the expression of primed state marker SSEA4 unlike hiPSCs. Interestingly, for the first time, we demonstrate the ciPSC pluripotency using lipid body analysis wherein ciPSCs showed enhanced bodipy staining and blue fluorescence emission, reflecting the primed state of pluripotency. ciPSCs expressed higher levels of fatty acid synthase (*FASN),* the enzyme involved in the synthesis of palmitate,similar to that of hiPSCs and higher than thatof miPSCs. As ciPSCs exhibit characteristic properties of both naïve and primed pluripotent state, it probably represents a unique intermediary state of pluripotency that is distinct from that of mice and human pluripotent stem cells.

Significance

Elucidating the pluripotent state of ciPSCs assists in better understanding of the reprogramming events and development in different species. The study would provide a footprint of species-specific differences involved in reprogramming and the potential implication of iPSCs as a tool to analyse evolution.

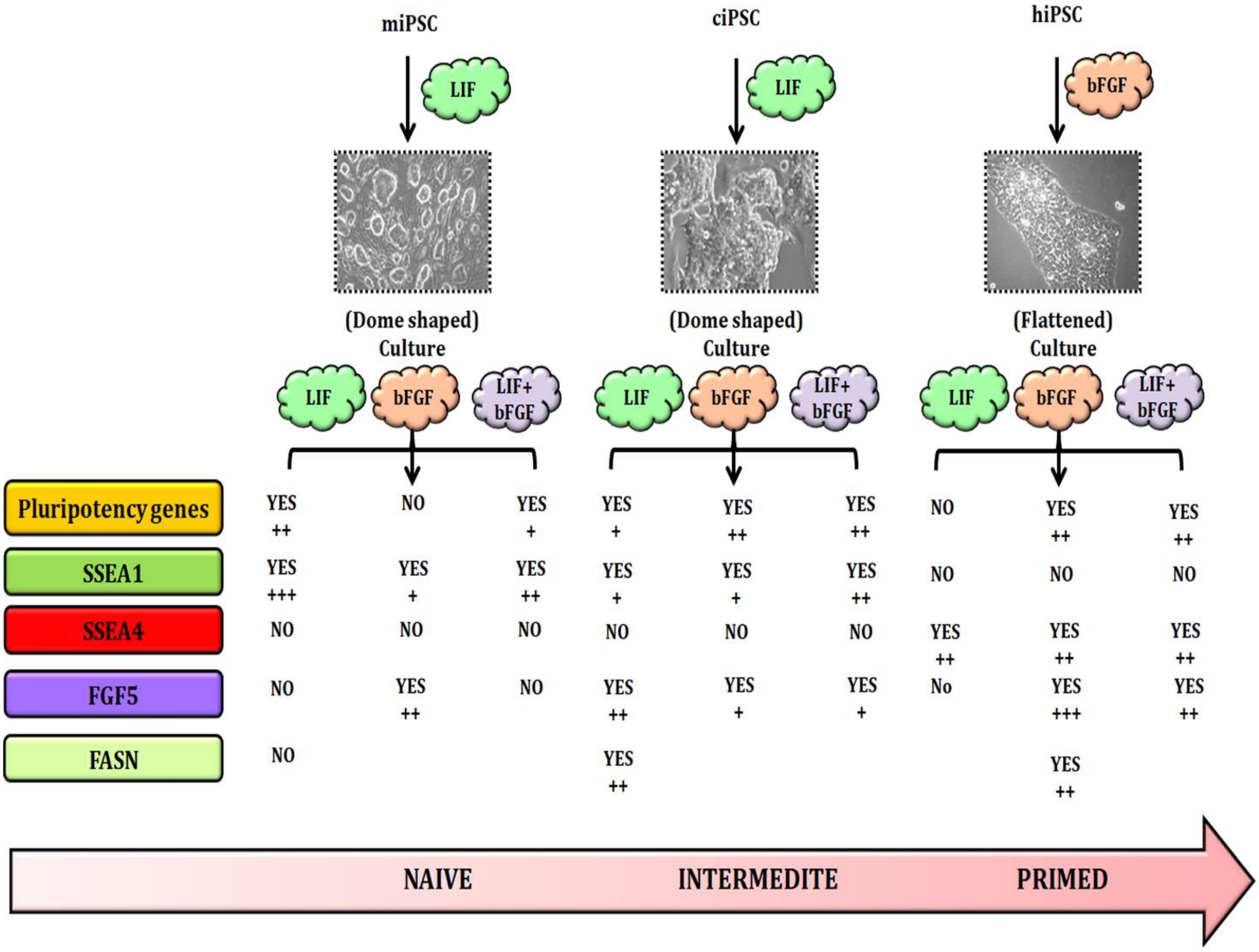
Keywords: Canine induced Pluripotent Stem Cells; Reprogramming; Naïve and Prime Pluripotency; Regenerative Medicine; Stem cell therapy models; lipid bodies.

Abbreviations

PSCs, Pluripotent stem cells; ciPSCs, canine induced pluripotent stem cells; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; miPSCs, mouse induced pluripotent stem cells; hiPSCS, human induced pluripotent stem cells; EpiESCs, Epiblast Embryonic Stem Cells; LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor; iMEF, inactivated mouse embryonic fibroblasts; EB, embryoid body; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; STAT3, Signal Transducer and Activator 3, JAK, Janus Kinase; SSEA; Surface Specific Embryonic antigen

GRAPHICAL ABSTRACT:

ciPSCs derived in presence of LIF showed characteristic properties resembling both naïve and primed pluripotent states. In contrast to miPSCs and similar to hiPSCs, ciPSCs demonstrated the expression of pluripotent genes, *FGF5* and expression of *FASN*, the gene involved lipid metabolism. However, similar to miPSCs, ciPSCs expressed SSEA1 in all the conditions and not the SSEA4, the characteristic property of naïve state. Considering these observations we propose ciPSCs to probably belong to the intermediary state of pluripotency.



	miPSC			ciPSC			hiPSC		
	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF	LIF	bFGF	LIF+ bFGF
Pluripotency genes	YES ++	NO	YES +	YES +	YES ++	YES ++	NO	YES ++	YES ++
SSEA1	YES +++	YES +	YES ++	YES +	YES +	YES ++	NO	NO	NO
SSEA4	NO	NO	NO	NO	NO	NO	YES ++	YES ++	YES ++
FGF5	NO	YES ++	NO	YES ++	YES +	YES +	No	YES +++	YES ++
FASN	NO			YES ++				YES ++	

Introduction

Due to their quintessential properties of self-renewal and pluripotency, induced pluripotent stem cells (iPSCs) offer unprecedented opportunities in regenerative medicine [1,2]. iPSCs can be derived from any cell types of mammalian and non-mammalian origin. However, increased availability of these numerous iPSC model systems has often led to confusion regarding the appropriate model to be used [3].One of the criteria to select the appropriate PSC model is based on whether they exist in a naïve or primed state. Naïve PSCs which are exemplified by mouse embryonic stem cells (mESCs) and miPSCs correspond to ICM of blastocysts and exhibit distinctive properties such as compact and dome-shaped morphology and dependence of self-renewal and proliferation on the leukemia inhibitory factor (LIF)–Jak/Stat signalling pathway [4]. Other key features of naïve PSCs is the specific expression of genes like SSEA1, REX1, and STELLA, possession of two active X chromosome (XaXa) and reduced single-cell mortality [5]. On the other hand, primed PSCs exemplified by mouse epiblast stem cells (mEpiSCs), human ESCs (hESCs), and human iPSCs correspond to the epiblast cells of the post-implantation stage [4]. Primed PSCs have key features of flattened morphology and dependent on basic fibroblast growth factor (bFGF)-rather than LIF for self-renewal and proliferation [6]. In contrast to naïve PSCs, primed PSCs exhibit an inactivated X chromosome (X_aX_i), increased single cell mortality, and express epiblast markers FGF5 and OTX2, along with core pluripotent genes *Oct4* and *Sox2* [7]. Recent reports have shown the successful conversion of human PSCs to naïve state resembling mESCs using chemical compounds GSK3β inhibitor and a MEK/ERK inhibitor (2i) [5]. These reports confirm that the primed PSCs are not restricted to one way forward differentiation but have the ability to dedifferentiate to native PSCs also.

It has been of dilemma whether the naïve or prime pluripotent state is species-specific or is it a culture condition mediated effect. Previously, the generation of naïve pluripotent state was confined to mESCs and PSCs derived from rat embryos. However, recently, the derivation of PSCs in naïve states has been extended to other species such as porcine fibroblasts, rhesus monkey fibroblasts, and rabbit embryos by modifying culture conditions applied during the course of reprogramming [8–10]. Several reports have also claimed the successful derivation of naïve human PSCs by either modifying culture conditions or by over-expressing key pluripotent transcription factors [11–13]. Various pathways are involved in the effective maintenance of iPSCs in all species, the prominent ones are LIF/STAT3, FGF, MEK/ERK, and BMP/SMAD pathways [14]. Depending on the state of pluripotency, two cytokines, LIF or bFGF is added to the culture media for the maintenance of PSCs. While naïve mESC pluripotency is maintained by LIF, FGF- mediated activation of MEK signaling drives differentiation of mESCs. On the other hand, primed mEpiSCs or hiPSCs require basic FGF signaling for maintenance of pluripotency whereas LIF signaling has no effect on pluripotency [15].

Canines being genomically and physiologically more similar to humans offer a better model compared to rodents in unraveling many of the human diseases [16-18]. Many reports have documented the derivation of canine iPSCs (ciPSCs) from various cell sources using different reprogramming approaches. Depending on the source of reprogramming factors, whether human or mouse, derived iPSCs showed subtle differences in their characteristic properties. Goncalves et al. observed that ciPSCs generated by mouse *Oct4*, *Sox2*, *Klf4*, *c-Myc* (OSKM) showed decreased silencing of expression of the exogenous gene, while complete silencing was observed in the ciPSC lines derived from human OSKM factors [19]. However, whether the generated ciPSC lines belong to naïve or pluripotent state remains unrevealed.

In this study, we attempted to understand the positioning of the ciPSCs in the reprogramming landscape. This was done by analyzing the differences in the pluripotency in ciPSCs, in comparison to miPSCs and hiPSCs, with respect to crucial growth factors, LIF, bFGF, and a dual supply of LIF and bFGF in the culture medium. Though the colonies exhibited specific morphology differences, the expression of pluripotency markers was observed in ciPSCs cultured in all the three conditions. We then tried to understand the differences in lipid metabolism in ciPSCs and miPSCs. Taken together, we propose a distinct pluripotent state for ciPSCs which probably stands between naïve and primed states as revealed by their gene expression differences and lipid metabolism.

Materials and methods

Generation of iPSCs:

Canine dermal fibroblasts (CFBs) were derived from a skin punch biopsy from the ventral abdomen of a 9-month-old Mongrel from the Department of Surgery, College of Veterinary Sciences and Animal Husbandry, Anand Agricultural University, with appropriate approval from the institutional animal ethics committee. Dermal fibroblasts were expanded from skin explants in DMEM F-12 medium with 10% FBS and 1x Penicillin Streptomycin (Gibco) at 37˚C in 5% CO2. CFBs were up-scaled in suitable culture vessels for subsequent experiments and also cryo-preserved and kept in liquid nitrogen conditions.

Retrovirus plasmids expressing human OCT4, SOX2, KLF4, and C-MYC,(Plasmids are a kind gift from Prof. Catherine Verfaillie, KU, Leuven) were formed by individually transfecting each of these constructs in HEK 293T cells with the retroviral packaging vectors pSPAX2 and pMD2G. 293T cells (8x10^6) were transfected with lipofectamine (1:3 ratio) in HEK 293T medium consisting of DMEM high glucose (Gibco) with 10% FBS, 0.1mM NEAA, 6mM L-glutamine. After 48 hours of transfection, the supernatant was collected and added to 1.5x10^6cells per well of a 6 well plate of CFBs. This medium containing retroviral particles was replaced with a second round of concentrated supernatant (72 hours) from the transfected HEK 293T cells on the following day. After 24 hours, the medium was replaced with fresh HEK 293T medium. After 5 days, transduced fibroblasts were passaged on inactivated MEFs and cultured in iPSC media (DMEM F12, 15% FBS, 2mM L-glutamine, 0.1mM NEAA, 0.075mM β-mercaptoethanol, 1mM sodium pyruvate, 1x Penicillin Streptomycin and LIF-8ng/ml). Media compositions can be found in Supplementary Table 3. iPSC colonies with compact ES-like cells were observed after 20-22 days. Colonies were manually picked, trypsinized, and transferred to new feeder plates and maintained in iPSC medium at 37°C, 5% CO2. The iPSC colonies were maintained in this condition for fifteen passages before transferring to different conditions. ciPSCs were also transferred to feeder-free vitronectin and maintained up to forty passages.

miPSCs derivation :

miPSCs were derived using a previously published protocol (20). In brief, the protocol includes the transduction of mouse embryonic fibroblasts (MEFs) seeded at the density of 1.5×10^6 cells /well in six-well plates with retroviral vectors containing supernatant for mouse Oct3/4, Sox2, and Klf4 (Addgene). To enhance the efficiency of transduction, MEFs were transduced twice with an interval of 24hrs. Cells were maintained in fibroblast medium for two days and were later changed to mESC medium. On day 4, post-viral transduction, transduced fibroblasts were trypsinized into single-cell cultures and reseeded on 6 well plates at a density of 0.5×10^6 cells per well on mitomycin inactivated MEF feeders. Colonies observed after 20-25 days were manually picked and further propagated.

hiPSCs culture:

NCL-1 hiPSCs (passage 23) were procured from EyeStem research Pvt. Ltd., Bangalore and cultured on feeder- free conditions on 1% matrigel (BD Corning) coating. Stem MACS iPS-brew XF (MACS media) medium was used for everyday medium change. The cells were split using Accutase (Gibco) and seeded at a ratio of 1:5. Further, they were cultured in different conditions of LIF, bFGF, and LIF+bFGF addition in hiPSC medium (composition mentioned in supplementary table 3).

Embryoid body formation:

ciPSC colonies cultured in presence of LIF were transferred into iPSC medium devoid of LIF. iPSC`s were induced to differentiation into EBs by plating on low adherent plates (Nunc) with mESC medium without LIF. Media change was done every alternate day for 10 days before proceeding to RNA isolation.

RNA extraction, cDNA synthesis and PCR:

Cells were lysed with trizol reagent and total RNA was extracted using RNeasy Mini kit (Qiagen) as per the manufacturer`s protocol. Complementary DNA (cDNA) was synthesized using the cDNA synthesis kit (Fermentas) according to the manufacturer`s instructions. Canine specific primers (Supplementary Table 2) were designed for detecting endogenous expression of stemness genes. PCR was performed using Emerald PCR master mix with Taq DNA polymerase (Takara) with the cycle parameters as denaturation at 95˚C for 5 minutes, amplification for 35 cycles, annealing for 20 seconds at 58˚C and extension at 72˚C for 30seconds and a final extension at 72˚ C for 10 minutes. The primers and their product sizes are given as a separate table. PCR products were resolved on 2% agarose gels with Ethidium Bromide. Gels were photographed using alpha imager.

Quantitative PCR was carried out using SYBR Green (Takara). The samples were analysed using the 7500 RT-PCR (ABI Biosystems) and were normalized with a house-keeping gene *Gapdh* to obtain the relative fold change among samples.

Immunocytochemistry:

iPSC colonies were washed in phosphate-buffered saline (1xPBS) twice and then fixed in 4% paraformaldehyde for 20 minutes at room temperature (RT). Colonies were washed twice with 1xPBS for 1 min each and permeabilized with Triton-X-100 in PBS for 15 minutes. Colonies were incubated overnight at 4°C with primary antibodies of appropriate dilutions (Supplementary Table 1). The next day, colonies were washed twice with 0.05% PBST and secondary antibody was added and incubated at RT for 1hr. After three washes in PBS for 1 minute each, cells were stained with 1:10,000 diluted DAPI for 3-5minutes. Cells were visualized and photomicrographed on an inverted fluorescence microscope. The images were processed with ImageJ software.

Blue Fluorescence imaging and Bodipy staining:

Confluent cultures of ciPSCs and miPSCs on vitronectin were analysed for blue fluorescence detection. PSC culture medium was replaced by DMEM high glucose basal medium without phenol red to avoid interference of phenol red during blue fluorescence imaging. The images were captured with a Nikon Eclipse TE2000 U attached to a Qicam Fast 1394 digital camera and Q Capture Pro software. The blue fluorescence was visualized using DAPI filter Cube (Nikon EPI-FL filter). The lipid body associated retinyl ester blue fluorescence images were acquired first followed by the acquisition of phase-contrast images. The images were merged to obtain the final images.

The ciPSCs and miPSCs were seeded onto culture dishes with suitable cell density and were subjected to bodipy staining, after 48 hours of culture. The cells were washed with PBS and incubated with bodipy solution (1:2000 dilution in basal medium) for 15 minutes at 37°C. The culture dishes were covered with aluminium foil to protect from light. After 15 minutes, the cells were washed with PBS and then fixed with 4% PFA for 30 minutes at room temperature. After removing the PFA, the cells were washed thrice with PBS and were immediately observed under the fluorescence microscope.

Statistical analysis:

Student’s t-test was used to analyse the difference between the cells and their respective controls. The values with $p<0.05$ were considered statistically significant and those with $p<0.001$ were considered highly significant. Graph pad prism and Excel software tools were used for qPCR analysis and graph preparation. ANOVA was used for analysing the QPCR triplicate values.

RESULTS

Characterization of ciPSCs generated in presence of LIF:

Adult canine dermal fibroblasts were reprogrammed into ciPSCs by transduction with retroviruses expressing human transcription factors OCT4, SOX2, C-MYC, and KLF4. After 17 days, ES-like colonies with high nuclear to cytoplasmic ratio started emerging and were picked and re-plated onto inactivated MEFs and cultured in iPSC media with murine LIF. Colonies displayed a tightly packed morphology. Out of the total 5 clones isolated, three clones were enzymatically dissociated for further passaging and characterization. Various stages of reprogramming are depicted in figure 1a. For further characterization of ciPSCs, stemness marker expression was analysed by semi-quantitative PCR and immunofluorescence. Compared to CFBs, ciPSCs expressed endogenous pluripotency markers *OCT4*, *SOX2*, *KLF4,* and *NANOG* as analysed by semi-quantitative PCR (Figure 1b). Along with this, significant up-regulation of the epigenetic marker, *de novo* methyltransferase, *DNMT3A* was witnessed in ciPSCs (Figure 1b). In line with expectation, reprogramming resulted in the down-regulation of fibroblast marker *VIMENTIN* in ciPSCs as compared to canine fibroblasts (Figure 1c). Expression of pluripotent markers OCT4, SOX2, and SSEA1 at protein level further confirmed the reprogramming of canine fibroblasts (Figure 1d). Analysis of transgene silencing across different passages showed reduced expression of exogenous *OCT4*, *SOX2*, and *KLF4* with increase in the passage, however, the decrease in *OCT4* and *KLF4* transgenes was not to the extent of that in *SOX2* transgene. (Figure 1e). ciPSCs could be passaged as single cells enzymatically and could be cultured on inactivated MEF up to passage 15 and maintained under feeder-free conditions up to passage 40 with vitronectin.

To understand its differentiation ability, the spontaneous differentiation approach of forming EBs showed efficient skewing towards all the three lineages. Semi-quantitative PCR analysis of differentiated EBs demonstrated expression of representative ectoderm markers *PAX6* and *FOXG1*, mesoderm markers *VEGF* and *FLK1*,and endoderm markers *SOX17* and *CXCR4,* all of which were absent in control ciPSCs. Control ciPSCs showed expression of *DNMT3A*, a marker for *de novo* DNA methylation which was absent in EB confirming the differentiation of ciPSCs (Figure 1f & 1g). These results demonstrate the authenticity of ciPSCs derived from canine fibroblasts generated in the presence of mouse LIF.

ciPSCs derived in the presence of LIF exhibit mixed naïve and primed state properties:

To understand whether the generated ciPSCs resembles more of a naïve or primed state of pluripotency, we compared their morphology and gene expression profile with miPSCs representing the naive state and hiPSCs being primed state. ciPSCs cultured in presence of LIF possessed dome-shaped morphology similar to miPSCs and were unlike hiPSCs that were flattened (Figure 2a). All the iPSCs expressed higher levels of pluripotency markers compared to their fibroblast counterparts (Figure 2b i-iii). As SSEA1 expression in miPSCs and SSEA4 expression in hiPSCs represent the naïve and the primed state respectively, the identity of the pluripotent state of ciPSCs was tested by using these two markers. Similar to miPSCs, ciPSCs cultured in presence of LIF expressed SSEA1 but not SSEA4, thus advocating their naive state

(Figure 2c and d). Surprisingly, upon transcript analysis, ciPSCs belonging to distinct class of PSCs, exhibited characteristic features of naïve PSCs by expression of REX1 similar to miPSCs, and significantly lesser expression of OTX2 compared to hiPSCs. On the other hand, their expression levels of reduced KLF4 compared to hiPSCs and increased expression of FGF5 compared to miPSCs, resembled the signatures of primed PSCs (Figure 2E). These results strongly indicated ciPSCs to belong to a distinct state of pluripotency compared to that of naïve miPSCs and primed hiPSCs.

To test whether ciPSCs cultured in presence of LIF switched to that of bFGF have an altered pluripotent state, we cultured ciPSCs in presence of either LIF, bFGF or a combination of LIF+ bFGF conditions, along with controls miPSCs and hiPSCs. Similar to miPSCs, ciPSCs exhibited a dome-shaped morphology in the presence of LIF but exhibited a differentiated flattened morphology in bFGF and LIF+bFGF conditions (Figure 3a). However, ciPSCs could be maintained for up to fourteen passages in the bFGF and LIF+bFGF conditions, whereas miPSCs could be maintained only for two passages in similar culture conditions. hiPSCs maintained their stem cell-like compact morphology in presence of bFGF and failed to do so under LIF and LIF+bFGF supplementation. Evaluation of gene expression of ciPSCs in these culture conditions, similar to hiPSCs, showed enhanced expression of pluripotency genes *OCT4*, *NANOG*, and *SOX2* in bFGF and LIF+bFGF culture conditions compared to that miPSCs (Figure 3bi). As expected, bFGF deprivation and LIF supplementation in hiPSCs culture showed reduced *OCT4, NANOG,* and *SOX2* expression considerably. Interestingly, ciPSCs cultured in LIF, the cytokine used to maintain naïve pluripotency, expressed a higher amount of primed marker *FGF5* compared to miPSCs, which was not sustained upon bFGF or LIF+bFGF addition. hiPSCs expressed higher levels of *FGF5* than miPSCs in all the culture conditions, reaffirming their primed status (Figure 3b ii). The analysis of these results of *FGF5* expression revealed the characteristic features of primed stem cells in ciPSCs.

ciPSCs cultured in all three conditions were allowed to form EBs and RNA isolation was done on the 10th day. Transcripts of undifferentiated canine iPSCs were used as control. However, ciPSCs cultured in bFGF and miPSCs cultured in bFGF and LIF+bFGF conditions failed to form EBs (data not shown). The lineage marker expression of canine EBs cultured in LIF only and LIF+bFGF conditions were evaluated by q-PCR and the representative genes of all three lineages showed enhanced expression (Figure 3c). SSEA1 expression in miPSCs, ciPSCs and hiPSCs in three different culture conditions was evaluated by immunofluorescence, and percent positive cells in miPSCs and ciPSCs was quantified. Interestingly, similar to miPSCs, ciPSCs cultured in presence of bFGF showed two-fold lesser expression of SSEA1 compared to that of cells cultured in presence of LIF or LIF+bFGF (Figure 3d, e and f). SSEA1 expression was not detectable in hiPSCs cultured in all three conditions (figure 3d). While hiPSCs in all three conditions showed sustained expression of SSEA4, miPSCs and ciPSCs lacked SSEA4 expression (Figure 3g). The combined analysis of the results of *FGF5*, SSEA1, and SSEA4 expression encouraged us to categorize ciPSCs to belong intermediate state of pluripotency.

ciPSCs exhibit characteristic blue fluorescence and neutral lipid staining different from miPSCs

A previous report showed the use of characteristic blue fluorescence emitted by the primed pluripotent stem cells, but neither the differentiated cells nor the naïve mESCs, as an approach to identify and isolate the pure primed pluripotent population [21]. To understand the identity of the ciPSCs, we also looked into the emission of blue fluorescence from ciPSCs. Surprisingly, in contrast to naïve pluripotent miPSCs, ciPSCs exhibited characteristic blue fluorescence (Figure 4).

As the emission of blue fluorescence by the primed PSCs is due to the sequestration of retinyl esters in cytoplasmic lipid bodies, we analysed the lipid phenotypes by bodipy staining in ciPSCs and miPSCs cultured in presence of either LIF, bFGF or LIF+bFGF. We found enhanced bodipy staining in miPSCs cultured in presence of LIF, but not in bFGF and LIF+bFGF- the conditions which led miPSCs to differentiate. In contrast, the bodipy staining was observed in ciPSCs cultured in all three conditions and the highest staining was observed in ciPSCs cultured in LIF+bFGF conditions (Figure 5a). Bodipy staining in canine dermal fibroblasts and mouse fibroblasts showed minimal staining which is similar to that observed in miPSCs cultured in presence of bFGF and LIF+bFGF (Figure 5b). We looked at the expression of Fatty acid synthase (*FASN*), the gene responsible for long-chain fatty acid synthesis, in ciPSCs cultured under three conditions as shown in figure 5c. ciPSCs showed significant up-regulation in *FASN* expression in all three conditions compared to that of cells cultured in presence of LIF(Figure 5c). In contrast to miPSCs, ciPSCs showed enhanced expression of *FASN* cultured in presence of LIF, similar to that of hiPSCs cultured with bFGF (Figure 5d). These results reiterated the classification of ciPSCs under intermediate state of pluripotency.

DISCUSSION

There are many limitations in using human patients and also hESCs for stem cell research. Efficient animal models like canine models can accelerate the progress in stem cell therapy and the preclinical trials using iPS cells. Dogs share disease pathogenesis similar to that of humans which makes them an alternative model for understanding disease development from an early stage. Several studies have shown the generation of ciPSCs and their differentiation potential to different lineages. Lee et al. derived endothelial cells from the ciPSCs and studied their efficacy in immune-deficient mice models of hind limb ischemia and myocardial infarction [22]. It has also been reported that ciPSC- derived mesenchymal stem cells (iMSCs) displayed proficient differentiation into osteo, chondro and adipogenic cells and also suggested the use of iMSCs in cell therapy in osteoarthritis in canine patients and also as a model system for degenerative joint disease in humans [23]. In a similar study by Chow et al., canine iMSCs exhibited efficient proliferation and immune-modulatory features, similar to that of canine Ad-MSCs and BM-MSCs [24].

Considering different applications of ciPSCs, it is necessary to understand the state of ciPSCs for their efficient culture and maintenance. Optimization of culture conditions for their self-renewal and maintenance are key points in obtaining stable and reproducible ciPSC lines. We derived ciPSCs from canine dermal fibroblasts of mongrel breed by a retroviral approach using human reprogramming factors. Various reprogramming approaches have been performed for the generation of ciPSCs [25]: retroviral [3,26,27], lentiviral [19, 22, 28–31] and sendai virus [24,32] methods. Tsukamota et.al reprogrammed embryonic fibroblasts by an auto-erasable sendai virus vector but with lower efficiency [32]. Shimada et al derived ciPSCs by canine OSKM [26], but most groups reported ciPSC derivation by using either mouse [28, 33] or human [22, 27, 29, 30, 34] reprogramming factors. ciPSCs derived by Goncalves et al. reported the use of murine and human OSKM factors separately and in combination [19, 34], by the lentiviral method. Further in-depth studies have to be performed to elucidate whether species difference in reprogramming factors might influence canine iPSC derivation. Understanding ideal culture conditions for efficient passaging and maintenance of ciPSCs is necessary for maintaining their quality and also for further differentiation experiments. We derived ciPSCs on inactivated MEF and compared them with naïve pluripotent miPSCs and primed pluripotent hiPSCs. To authenticate the pluripotency of ciPSCs, we performed several pluripotent assays and found, except for the differential suppression of transgenes, ciPSCs fulfilled majority of the criteria required to be confirmed it as a bonafide iPSCs. However, we can’t negate the residual transgene expression having the possibility of potentially affecting the pluripotent state and differentiation ability of the cells. Derived ciPSCs were able to maintain on vitronectin for more than 40 passages. Most reports used inactivated mouse embryonic fibroblasts (MEFs) as the feeder layer for maintaining canine iPSC cultures except for Nishimura et al. who reported a feeder-free culture of ciPSCs in a doxycycline-inducible system [28].

The majority of the reports showed the pluripotency of ciPSCs to be maintained in culture conditions containing both LIF and bFGF [22, 26, 27, 29, 31, 33, 34]. Few reports also demonstrated the possibility of maintaining ciPSCs' pluripotency in the presence of either LIF or bFGF alone [30, 34, 35]. Vaags et al., derived the cESCs in presence of hLIF and bFGF and found the absence of LIF to result in spontaneous differentiation [36]. Similarly, Wilcox et al.,also reported the derivation of cESCs with the dual combination of LIF and bFGF [37]. Using LIF and inhibitors of glycogen synthase kinase 3β and mitogen-activated protein kinase 1/2 [called 2i and LIF (2iL)], Tobias et al., converted cESCs resembling primed PSCs toward a naïve pluripotent state [38]. LIF-dependent ciPSC colonies, derived by Whitworth et al. differentiated into fibroblast cells in the presence of LIF and bFGF, similar to cESCs derived by Wilcox et al [30, 37]. But ciPSCs derived in the presence of bFGF exhibited no change in pluripotency or proliferation when cultured with or without LIF [19]. Previous reports showed loss of pluripotency expression in ciPSCs when LIF or bFGF was removed [33]. Though AKT and ERK1/2 remained consistently activated, the loss of LIF resulted in STAT3 dephosphorylation and thereby differentiation [29]. In a subsequent report, the authors implied the role of bFGF in pluripotency similar to that of primed state cells. Removal of bFGF or inhibition of the SMAD2/3 pathway led to significant repression of NANOG[39].Comparison of ciPSCs with miPSCs and hiPSCs showed ciPSCs to harbour the characteristic properties of both naïve and primed pluripotent state. ciPSCs showed the characteristics of naïve PSCs by expression of SSEA1 and lacking the expression of SSEA4. On the other hand, ciPSCs also cultured in LIF showed the inherent expression of FGF5, similar to that of primed PSC hiPSCs cultured in presence of bFGF. Surprisingly, switching of culture conditions of ciPSCs from naïve to that of primed PSCs showed an enhanced expression of pluripotent genes in the presence of bFGF and LIF+bFGF compared to the cells cultured in presence of LIF alone, a phenotype contrast to that of miPSCs but similar to that of hiPSCs. Similar report of increased expression of *NANOG* was observed in bFGF cultured ciPSCs by Luo et al.,[39].In our experimental conditions, culturing miPSCs, hiPSCs and ciPSCs in different culture conditions probably does not facilitate them in switching from primed to naïve state or visa-versa, as naive miPSCs are not converted to a primed-like state by simple culture in bFGF alone, nor hiPSCs can be converted to naïve state by mere culturing them in presence of LIF [40,41]. When these PSCs are shifted from the culture that supports their native pluripotent state to non-permissible condition, they lose their pluripotent state and fails to differentiate as observed by their inability to form EBs by ciPSCs cultured in bFGF and miPSCs cultured in bFGF and LIF+bFGF conditions.

Morphological analysis of ciPSCs showed more of dome-shaped colonies, similar to that of miPSCs rather than flat-shaped hiPSC colonies. Different colony morphologies were reported in cESCs and ciPSCs by different groups. Dome-shaped cells, a characteristic feature of naïve states were reported by a few groups[28, 30, 40]. Flat colony morphology similar to primed state were observed in some reports[22, 26, 27,27,29,31, 33]. Interestingly, cESCs derived by two groups reported a heterogeneous colony morphology[36, 37]. Among these, Wilcox et al isolated canine embryos at morulae and blastocyst stages with 2 distinct cESC lines; one set by immunodissection of ICM(OVC.ID) and another set by embryo explants(OVC.EX). The cESC lines derived from the former set showed flat morphology and the latter set showed dome-shaped colonies[37].

Understanding the metabolic signatures is essential to discern the similarities and differences in different pluripotent stem cells. Previous studies have reported the difference in lipid content between the primed and naïve states [43, 44]. A significant abundance of FASN, the gene involved in lipid metabolism, and an enhanced accumulation of intracellular lipids were detected in primed LIF-FGF2 cultured cESCs compared to that of chemical inhibitor (2i)+ LIF cultured naive cESCs [44]. Further Muthuswamy et al., showed that the primed cells sequester retinol/ retinyl esters and maintain them in non-oxidized form to ensure prevention of differentiation of primed hiPSCs. Also, the primed cells possess the transcripts required to metabolize retinol and for its reuptake [21]. This intrigued us to question the lipid status of ciPSCs which will facilitate to place ciPSCs in the landscape of naïve and primed pluripotent state. The emission of blue fluorescence and bodipy staining reiterated the epiblast like characteristic feature of the ciPSCs generated in the presence of LIF. The control miPSCs which belongs to the naïve state also showed convincing bodipy staining but not the blue fluorescence. Similar to previous report, we also observed the enhanced expression of *FASN* in ciPSCs similar to that of hiPSCs[44]. The lack of the blue fluorescence of lipid bodies in miPSCs, despite enhanced lipogenesis, is probably due to the absence of retinyl ester sequestration. These observations confirm the high occurrence of lipogenesis in PSCs which is a distinct feature compared to that of somatic cell source.

A methodical analysis of various features is necessary for effective classification of iPSCs into specific pluripotency states [45]. SSEA marker expression suggests a naïve or prime state of pluripotency; mouse PSCs express SSEA-1 and human PSCs express SSEA-3 and SSEA-4 markers. In canine PSC reports, SSEA-4 expression was reported by more groups [22, 27, 29–31, 35] and some groups reported SSEA1 [32, 33, 42, 46] expression. Vaags et al reported the expression of both SSEA-3 and SSEA-4 and low levels of SSEA-1 expression in the derived cESCs [36]. Though many of the parameters analysed in this study showed the primed state of ciPSCs, the cell surface analysis of the expression of SSEA1 and not the SSEA4 in ciPSCs and the formation of EBs only in presence of LIF impedes us in categorically placing ciPSCs in the group of the primed pluripotent state. This is probably due to the derivation of ciPSCs in presence of LIF and not in the presence of bFGF, which is routinely used to generate the primed induced pluripotent stem cells. The time duration of iPS culture in particular conditions also can influence their characteristics [47]. Although, it is a formidable task to decisively position the pluripotent state of cells of different species, the in-depth characterization of ciPSCs through multiple approaches suggested ciPSCs to belong to its own distinct pluripotent state. However, to ascertain conclusively the pluripotent state of ciPSCs, further utilization of genomic assay such as RNA-sequencing and insilico comparisons between species, live-cell imaging and in-depth study of different parameters including lipid profile and functional assays such as chimera generation into pre- and post-implantation embryos and derivation of germ-like cells are imperious to decipher the actual pluripotent state of ciPSCs[48]. A previous report suggested that reprogramming pathways in higher animals like dogs and pigs are more similar to that of the human than to mice, validated by the similarity search and phylogenetic analysis[49]. Understanding the species-specific differences in reprogramming and state of pluripotency helps in drawing their evolutionary significance in development.

Conclusions

The dog is the best model to understand the complexities of inherited genetic diseases and also for precise modelling of neurodegenerative diseases unlike that of mice. We derived stable ciPSCs that exhibited a majority of features that resembled that of primed pluripotent stem cell state and a few of the qualities which mimicked naïve pluripotent stem cells. These data reflect the probability of ciPSCs to fall between prime and naïve states. Information obtained from our study, ciPSCs probably being in an intermediate state of pluripotency, makes us to think that ciPSCs will become a practical and promising tool to understand the animal evolution on a molecular basis. However, to conclusively annunciate the pluripotent state of ciPSCs, ATAC-Seq and epigenomic approach should be followed to have a better insight on the distinction between naïve and prime state. In a nutshell, unravelling the characteristic features of ciPSCs can be effectively harnessed for understanding the developmental aspects, disease pathology, biomarker and drug development which will benefit both human and veterinary medicine.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS:

Figure 1. Characterization of canine iPSCs generated in the presence of LIF.

a) Morphology of transduced canine dermal fibroblasts on day0, day 6 and day 17. **b)** RT-PCR analysis of pluripotency genes *OCT4, SOX2, KLF4,* and *NANOG* along with loading control *GAPDH*, **c)** RT-qPCR analysis of fibroblast gene *VIMENTIN* in ciPSCs and CFBs. Ct values were normalized to the value of *GAPDH,* **d)** Immunofluorescence images of pluripotency markers OCT4 (red), SOX2 (green), SSEA1 (red) in canine iPSCs. The nuclei were counterstained with DAPI, **e)** qRT-PCR analysis of *OCT4*, *SOX2* and *KLF4* transgenes across different ciPSC passages. Ct values were normalized to the value of *GAPDH*. **f)** RT-PCR analysis of lineage genes, ectoderm genes (*FOXG1* and *PAX6*), endoderm genes (*CXCR4* and *SOX17)* and mesoderm genes *FLK1* and *VEGF* along with *DNMT3A* in EBs of ciPSCs. *GAPDH* was used as a loading control. **g)** qRT-PCR analysis of relative expression of lineage markers in ciPSCs and EBs. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3), ***p<0.001. Scale bar represents 100 µm.

Figure 2. ciPSCs derived in the presence of LIF exhibit partial epiblastic characteristic properties. a) Phase contrast images of miPSCs, ciPSCs cultured in presence of LIF and hiPSCs grown in presence of bFGF, **b)** Expression analysis of pluripotency markers, *OCT4, SOX2* and *NANOG* in miPSCs (i), ciPSCs (ii) and hiPSCs (iii) with respect to their fibroblast controls, Ct values were normalized to the value of *GAPDH*, Protein expression analysis of SSEA1 **(c)** and SSEA4 **(d)** in naïve miPSCs, ciPSCs and primed hiPSCs, **e)** Comparative analysis of *REX1*, *KLF4*, *OTX2* and *FGF5* expression in miPSCs, ciPSCs cultured in presence of LIF and hiPSCs cultured in presence of bFGF. Data represented as mean ±S.E.M (n=3), *p<0.05, **p<0.01, ***p<0.001. Significance in figure 2e is calculated with respect to hiPSCs. Scale bar represents 100 µm.

Figure 3: ciPSCs cultured in presence of bFGF and LIF+ bFGF exhibit characteristic properties similar to that of primed pluripotent state. a) Comparison of morphological features of miPSCs, ciPSCs and hiPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **b)** Gene expression analysis of pluripotency markers *OCT4*, *NANOG*, *SOX2* (i) and primed marker *FGF5* (ii) in miPSCs, ciPSCs, and hiPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **c)** Relative expression of lineage markers in EBs cultured in LIF only and LIF+bFGF conditions. ciPSCs were taken as control. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3). **d)** Immunofluorescence images of SSEA1 expression by miPSCs, ciPSCs and hiPSCs cultured in the presence of either LIF or bFGF or LIF +bFGF conditions. Quantification of SSEA1 positive cells in miPSCs **(e)** and ciPSCs **(f)** cultures in LIF, bFGF and LIF+bFGF conditions. **g)** Comparative analysis of SSEA4 expression in miPSCS, ciPSCs and hiPSCs in three culture conditions; LIF, bFGF, and LIF+bFGF conditions. Data represented as mean ±S.E.M (n=3), *p<0.05, **p<0.01, ***p<0.001. Scale bar represents 100 µm.

Figure 4. ciPSCS exhibit characteristic blue fluorescence distinct from miPSCs. Comparative analysis of blue fluorescence (excitation, 325–375 nm; emission, 460–500 nm) in ciPSCs and miPSCs. ciPSCs expressed characteristic blue fluorescence whereas miPSCs failed to show the blue fluorescence. Scale bar represents 100 µm.

Figure 5. ciPSCS exhibit neutral lipid staining distinct from miPSCs. a) Comparative analysis of bodipy expression in ciPSCs and miPSCs cultured in LIF, bFGF and LIF+bFGF conditions. **b)** Comparative analysis of bodipy expression in MEF and CFB. **c)** Relative expression of Fatty acid synthase marker, *FASN* in ciPSCs cultured in LIF (L), bFGF and LIF+bFGF conditions was analysed. Ct values were normalized to the value of *GAPDH*. **d)** Relative expression of *FASN* in miPSCs, ciPSCs cultured in presence of LIF and hiPSCs

cultured in presence of bFGF. Ct values were normalized to the value of *GAPDH*. Data represented as mean ±S.E.M (n=3). Scale bar represents 100 μm.

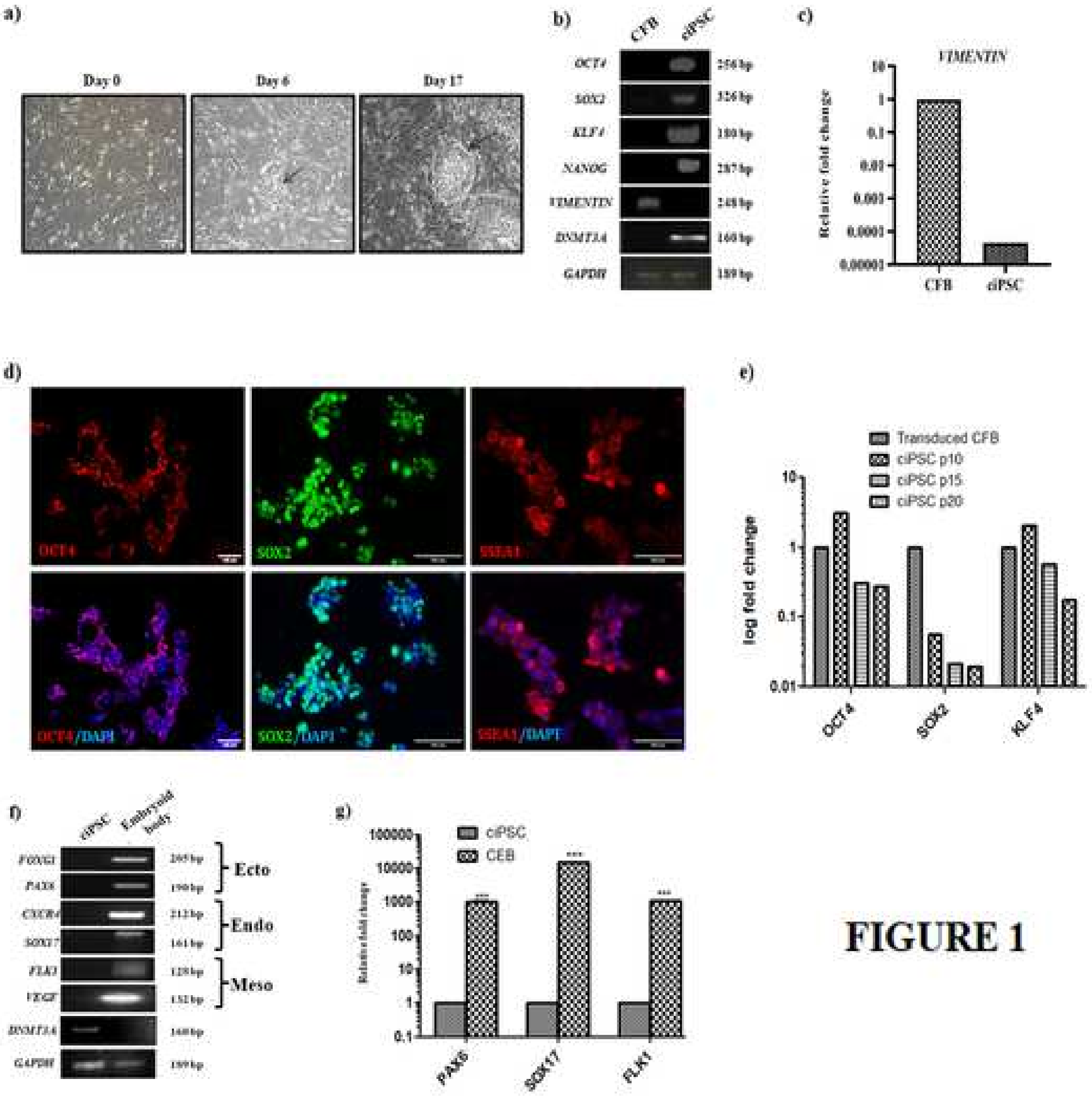
a)
Day 0
Day 6
Day 17
b)
CFB
ciPSC
OCT4
SOX2
KLF4
NANOG
VIMENTIN
DNMT3A
GAPDH
250 bp
326 bp
180 bp
287 bp
248 bp
160 bp
189 bp
c)
VIMENTIN
Relative fold change
10
1
0.1
0.01
0.001
0.0001
0.00001
CFB
ciPSC
d)
OCT4
SOX2
SSEA1
OCT4/DAPI
SOX2/DAPI
SSEA1/DAPI
e)
Transduced CFB
ciPSC p10
ciPSC p15
ciPSC p20
log fold change
10
1
0.1
0.01
OCT4
SOX2
KLF4
f)
ciPSC
Embryoid body
FOXG1
PAX6
CXCR4
SOX17
FLK1
VEGF
DNMT3A
GAPDH
205 bp
190 bp
212 bp
161 bp
128 bp
152 bp
160 bp
189 bp
Ecto
Endo
Meso
g)
ciPSC
CEB
Relative fold change
100000
10000
1000
100
10
1
0.1
***
***
***
PAX6
SOX17
FLK1

FIGURE 1

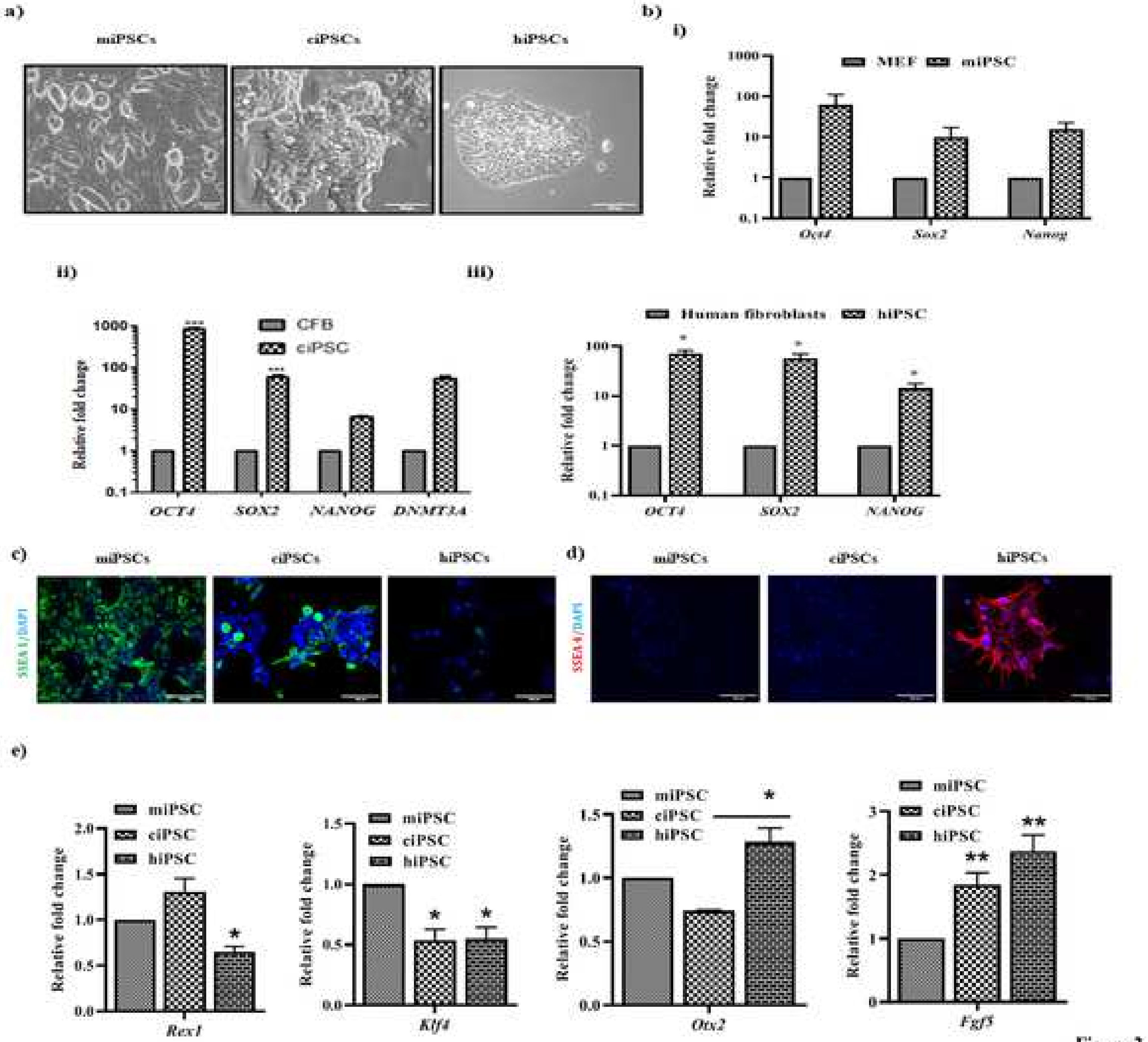
a)
miPSCs
ciPSCs
hiPSCs
b)
i)
MEF
miPSC
Relative fold change
1000
100
10
1
0.1
Oct4
Sox2
Nanog
ii)
CFB
ciPSC
Relative fold change
1000
100
10
1
0.1
***
***
OCT4
SOX2
NANOG
DNMT3A
iii)
Human fibroblasts
hiPSC
Relative fold change
100
10
1
0.1
*
*
*
OCT4
SOX2
NANOG
c)
miPSCs
ciPSCs
hiPSCs
SSEA1/DAPI
d)
miPSCs
ciPSCs
hiPSCs
SSEA4/DAPI
e)
miPSC
ciPSC
hiPSC
Relative fold change
2.0
1.5
1.0
0.5
0.0
*
Rex1
miPSC
ciPSC
hiPSC
Relative fold change
1.5
1.0
0.5
0.0
*
*
Klf4
miPSC
ciPSC
hiPSC
*
Relative fold change
1.5
1.0
0.5
0.0
Otx2
miPSC
ciPSC
hiPSC
Relative fold change
3
2
1
0
**
**
Fgf5

Figure 2

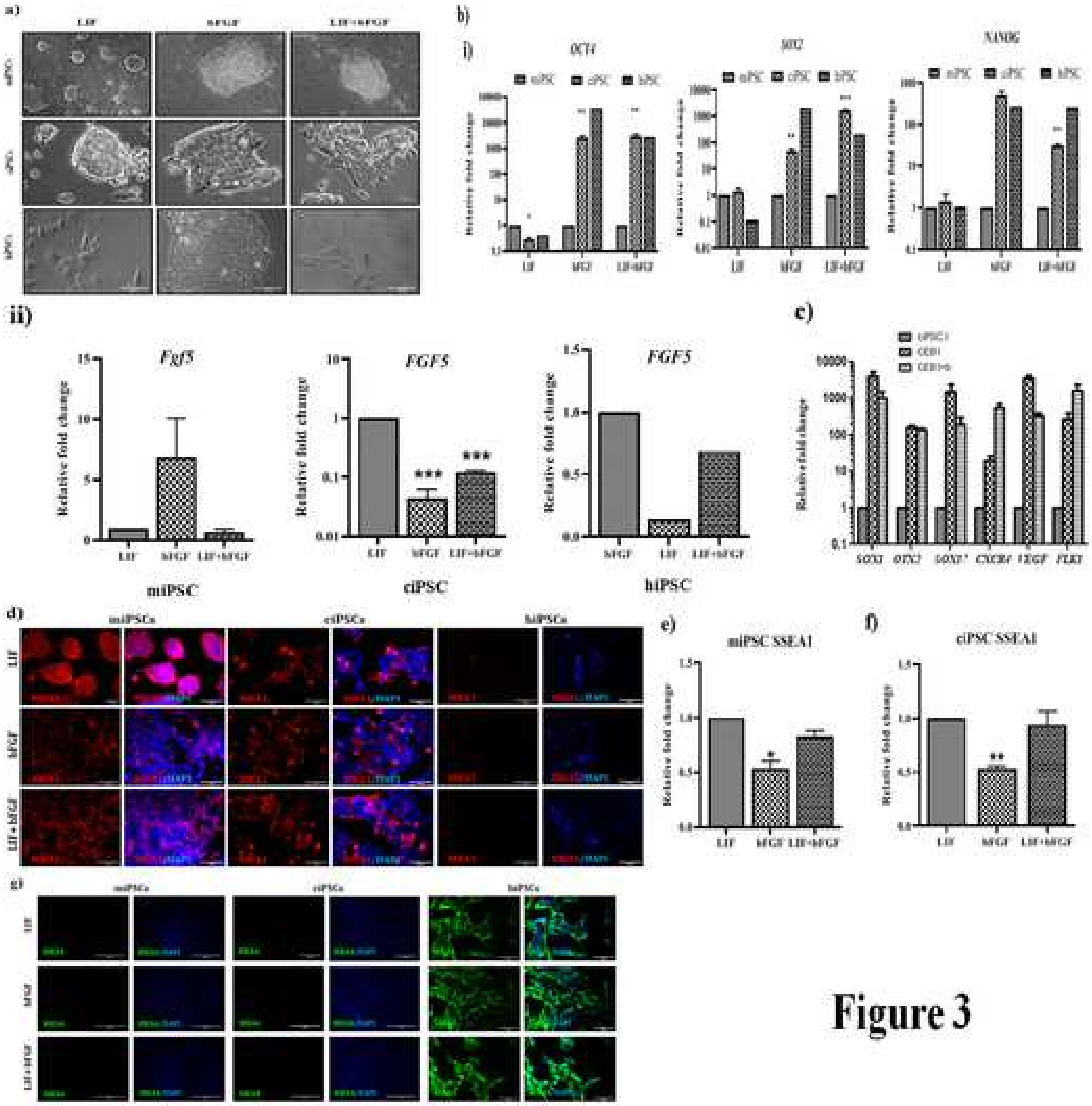
a)
LIF
bFGF
LIF+bFGF
miPSC
ciPSC
hiPSC
b)
i)
OCT4
SOX2
NANOG
miPSC
ciPSC
hiPSC
Relative fold change
LIF
bFGF
LIF+bFGF
ii)
Fgf5
FGF5
FGF5
Relative fold change
LIF
bFGF
LIF+bFGF
miPSC
ciPSC
hiPSC
***
***
c)
ciPSC
CEB
CEB-Hb
Relative fold change
SOX1
OTX2
SOX17
CXCR4
VEGF
FLK1
d)
miPSCs
ciPSCs
hiPSCs
LIF
bFGF
LIF+bFGF
SSEA1
SSEA1/DAPI
e)
miPSC SSEA1
Relative fold change
LIF
bFGF
LIF+bFGF
*
f)
ciPSC SSEA1
Relative fold change
LIF
bFGF
LIF+bFGF
**
g)
miPSCs
ciPSCs
hiPSCs
LIF
bFGF
LIF+bFGF
SSEA4
SSEA4/DAPI

Figure 3

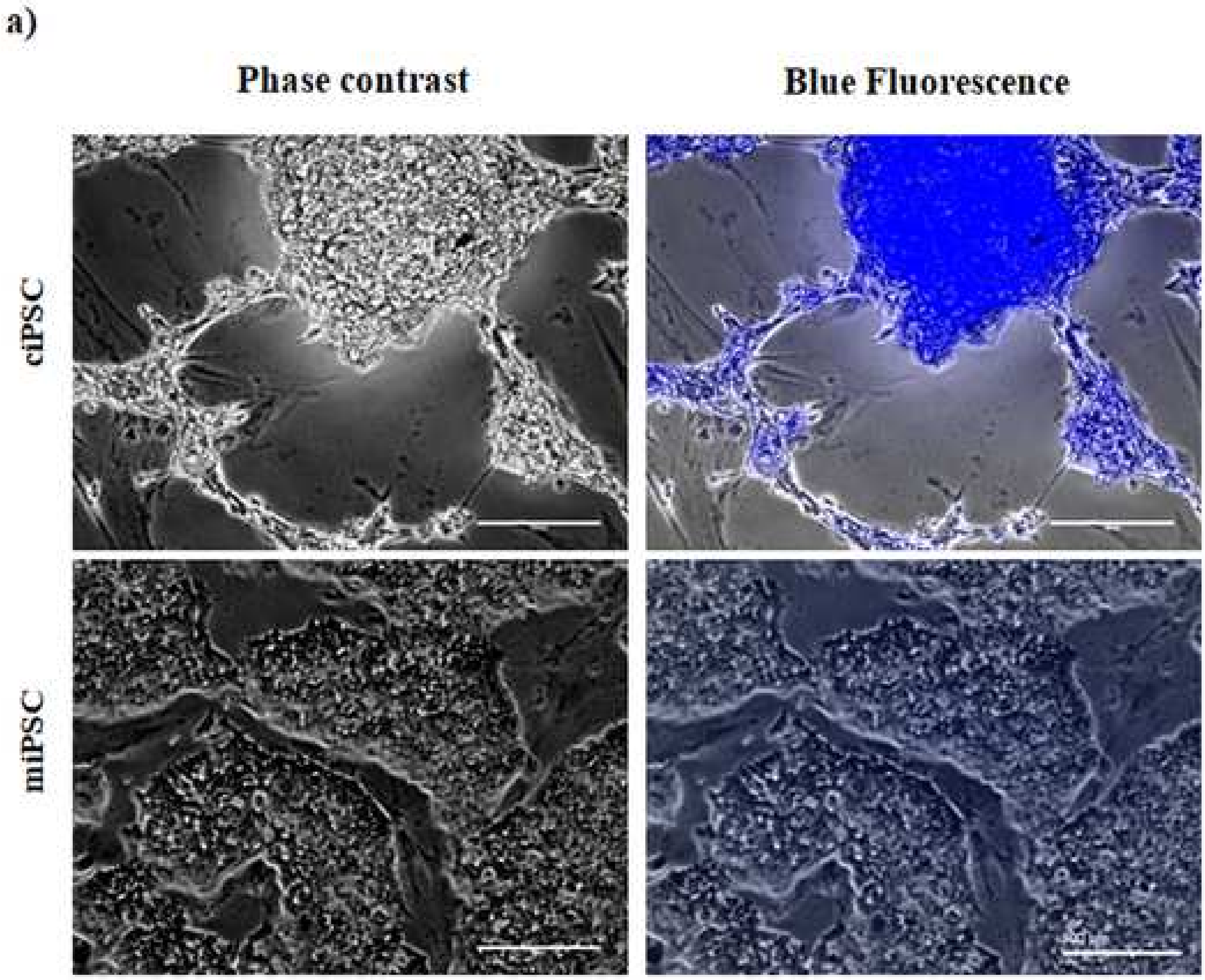

Figure 4

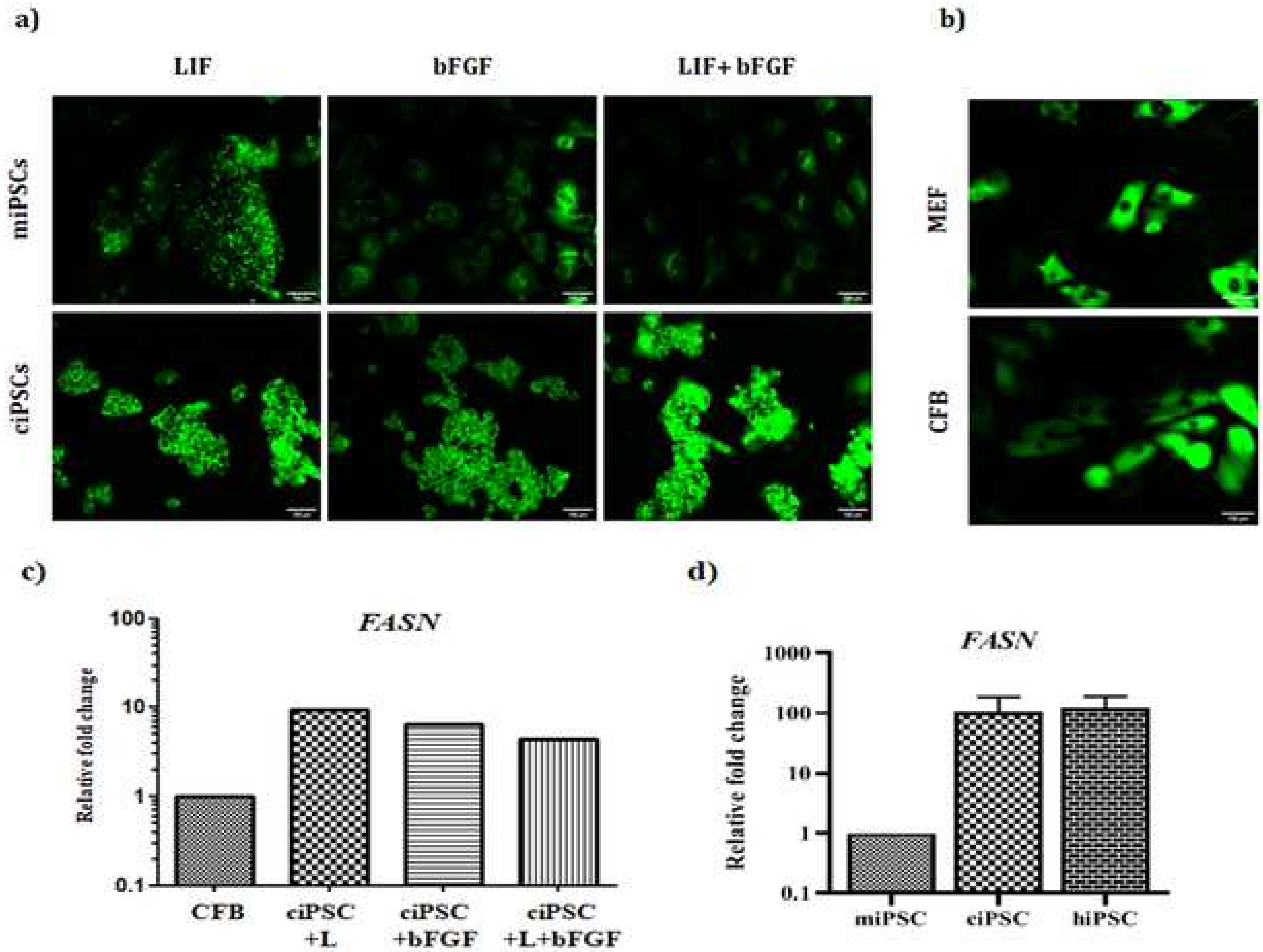
a)
LIF
bFGF
LIF+ bFGF
miPSCs
ciPSCs
b)
MEF
CFB
c)
FASN
Relative fold change
100
10
1
0.1
CFB
ciPSC +L
ciPSC +bFGF
ciPSC +L+bFGF
d)
FASN
Relative fold change
1000
100
10
1
0.1
miPSC
ciPSC
hiPSC

Figure 5

HIGHLIGHTS

- Canine iPSCs (ciPSCs) were derived in the presence of Leukemia Inhibiting Factor
- ciPSCs expressed SSEA1 and lacked the expression of SSEA4, characteristic of naïve PSCs
- ciPSCs showed enhanced expression of pluripotent genes in bFGF and LIF+bFGF culture conditions
- ciPSCs exhibit enhanced blue fluorescence and bodipy staining, characteristic of prime PSCs
- ciPSCs showed distinct properties compared to mouse and human iPSCs and probably belonged to intermediary state of PSCs