

Universidade Estadual Paulista “Júlio de Mesquita Filho”

Instituto de Biociências de Botucatu

PPG – Farmacologia e Biotecnologia

**Células tronco embrionárias e
induzidas ao estado de pluripotência em
bovinos: características moleculares e
aplicação em quimeras embrionárias**

Ramon Cesar Botigelli

2021

UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

Células tronco embrionárias e induzidas ao estado de pluripotência em bovinos: características moleculares e aplicação em quimeras embrionárias

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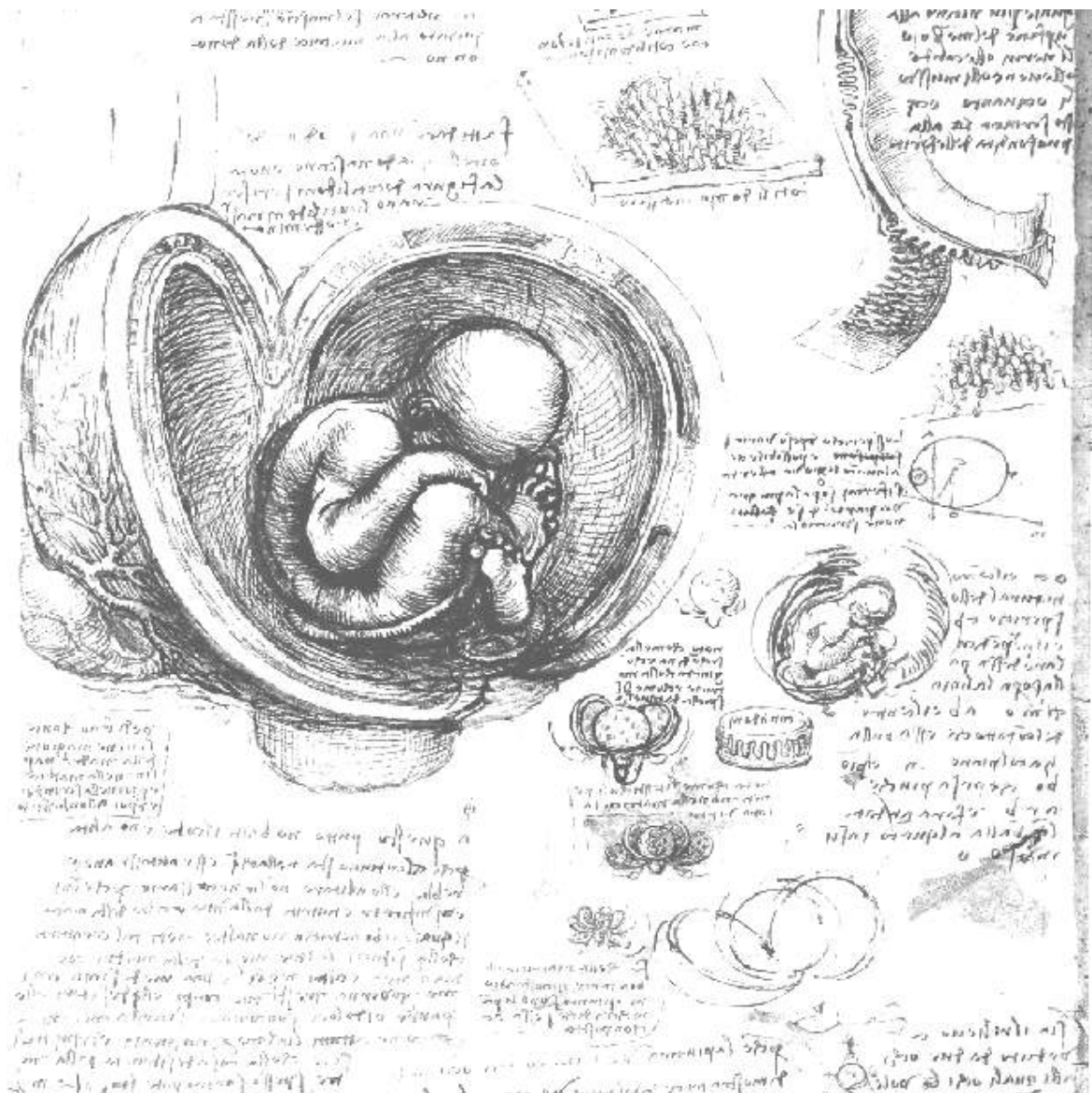
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"Learning Never Exhausts the Mind."

Leonardo da Vinci (1452-1519)

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Ethics statement



UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

CERTIFICADO

Certificamos que a proposta intitulada "Investigação de mecanismos celulares e moleculares da aquisição da toti- e pluripotência induzida in vitro - modelo translacional", protocolada sob o CEUA nº 3526250717, sob a responsabilidade de **Fabiana Fernandes Bressan** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprova**da pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 19/12/2017.

We certify that the proposal "Investigation of cellular and molecular mechanisms on in vitro induced toti- and pluripotency acquisition - a translational approach", utilizing 6 Heterogenic mice (males and females), 3 Swines (males and females), protocol number CEUA 3526250717, under the responsibility of **Fabiana Fernandes Bressan** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 12/19/2017.

Finalidade da Proposta: [Pesquisa \(Acadêmica\)](#)

Vigência da Proposta: de [03/2017](#) a [01/2020](#) Área: [Medicina Veterinária](#)

Origem:	Animais provenientes de estabelecimentos comerciais			
Espécie:	Camundongos heterogênicos	sexo:	Machos e Fêmeas	idade: 2 a 4 semanas N: 6
Linhagem:	Balb/c		Peso:	15 a 30 g
Origem:	Suinocultura VNP (Laboratório de pesquisa em Suínos)			
Espécie:	Suínos	sexo:	Machos e Fêmeas	idade: 3 a 12 meses N: 3
Linhagem:	comercial		Peso:	15 a 50 kg

Resumo: A manipulação da reprogramação celular é uma ferramenta importante para o entendimento da biologia celular de células pluripotentes e para o estudo de diferentes doenças teciduais ou genéticas, possibilitando a investigação dos eventos moleculares responsáveis pela competência celular à toti- e pluripotência. A presente proposta objetiva consolidar a tecnologia de produção de células reprogramadas pluripotentes e células germinativas in vitro em espécies de animais domésticos de interesse comercial ou biomédico (em especial bovinos e suínos), com ênfase tanto no aspecto técnico, com a disseminação de protocolos e material biológico para novos estudos e colaborações, como no aspecto científico, através da investigação dos mecanismos celulares e moleculares envolvidos na reprogramação celular. São propostas 3 metas: 1) A padronização do protocolos reprodutíveis e seguros de geração de iPS animais, 2) A geração de gametas, células germinativas e embriões a partir de células cultivadas in vitro, e 3) A investigação dos mecanismos transcricionais e epigenéticos de reprogramação celular. Cada uma das metas está composta por atividades descritas específicas. É esperada com a realização desta proposta a consolidação da pesquisa nacional na temática reprogramação nuclear e epigenética, assim como a inserção internacional a partir de colaborações e publicações científicas de alto impacto, resultando na formação de um grupo de pesquisa multidisciplinar, competitivo e colaborativo nacionalmente e internacionalmente.

Local do experimento: Manutenção do animal no Setor de Suinocultura da FMVZ/USP Análise do material coletado no Laboratório de Morfofisiologia Molecular e Desenvolvimento - FZEA/USP

Pirassununga, 24 de agosto de 2018



UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

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UNIVERSIDADE DE SÃO PAULO
Faculdade de Zootecnia e Engenharia de Alimentos
Comitê de Ética em Pesquisa da FZEA

Pirassununga, 22 de maio de 2019
CEUA N [3526250717](#)

Ilmo(a). Sr(a).
Responsável: Fabiana Fernandes Bressan
Área: Medicina Veterinária

Título da proposta: "Investigação de mecanismos celulares e moleculares da aquisição da toti- e pluripotência induzida in vitro - modelo translacional".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FZEA (ID 000893)

A Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP, no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 14/janeiro/2019) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Para a realização do ensaio de produção de embriões quiméricos bovinos neste experimento, foi associado ao Projeto de Jovem Pesquisador uma proposta de doutorado que incluirá a transferência dos embriões produzidos in vitro a receptoras, e posterior abate das mesmas. Os animais utilizados são provenientes da Prefeitura do Campus Fernando Costa. A solicitação à prefeitura encontra-se em anexo.".

Comentário da CEUA: "Esta CEUA FZEA aprova a emenda do projeto.".

Profa. Dra. Daniele dos Santos Martins
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Resumo

Células tronco pluripotentes são uma ferramenta fundamental para os estudos da embriologia, para o desenvolvimento de estudos clínicos in vitro, para a medicina regenerativa e mais recentemente, para a produção de gametas in vitro. Em 2006, Takahashi e colaboradores demonstraram ser possível a obtenção de células-tronco pluripotentes por indução gênica (induced pluripotent stem cells ou iPSCs). No entanto, condições ambientais e de cultura in vitro, como a utilização de diferentes suplementos para a manutenção da pluripotência podem diminuir e modular a eficiência da reprogramação à pluripotência. Dessa forma, a presente tese teve como objetivo estudar os efeitos do oxigênio e de diferentes suplementos para a manutenção da pluripotência no cultivo e reprogramação de células iPSCs de bovinos (biPSCs). No primeiro estudo, fibroblastos fetais de bovinos foram reprogramados em diferentes tensões de oxigênio (baixo ou alto oxigênio) e diferentes suplementos para a manutenção de pluripotência, bFGF ou bFGF+LIF+CHIR+PD (bFL2i). Em nossas condições experimentais, baixo oxigênio não aumentou a eficiência de reprogramação quando comparada a reprogramação em alto oxigênio. Por outro lado, a suplementação bFL2i aumentou a eficiência de reprogramação das biPSCs. As biPSCs derivadas com bFL2i em baixo oxigênio indicaram ausência de habilidade de auto renovação. As linhagens caracterizadas foram positivas para fosfatase alcalina, positivas para a expressão de genes marcadores de pluripotência (SOX2, OCT4 e STELLA), no entanto, negativas para NANOG. Ainda, baixo oxigênio aumentou a expressão de GLUT1 e GLUT3. As biPSCs também foram positivas na imunofluorescência para OCT4 e SOX2, mas negativas para NANOG. As biPSCs foram competentes em formar corpos embrióides e estes expressam marcadores de mesoderme e ectoderme, ainda, uma linhagem apresentou expressão de endoderme. No segundo estudo, fibroblastos fetais de bovinos foram reprogramados com diferentes suplementos para a manutenção da pluripotência (fatorial 2x2; bFGF, bFGF2i, LIF, LIF2i), ainda, linhagens de células tronco embrionárias foram isoladas para serem comparadas com as biPSCs geradas. A reprogramação de biPSCs foi mais eficiente com bFGF. As linhagens derivadas com LIF2i indicaram ausência de habilidade de auto renovação. As linhagens biPSCs e bESCs foram positivas para fosfatase alcalina. As biPSCs e bESCs foram positivas para a expressão de genes marcadores de pluripotência (SOX2, OCT4, STELLA, LIFr e OTX2) e somente as biPSCs expressaram NANOG e ESRRb. Ainda, biPSCs e bESCs foram positivas para SOX2, OCT4 e H3K27me3, negativas para NANOG na imunofluorescência. As biPSCs e bESCs foram competentes em formar corpos embrióides, estes expressaram marcadores de mesoderme, ectoderme e endoderme. Ainda, nossos resultados indicaram que em nossas condições, as biPSCs foram mais competentes comparadas as bESCs para a contribuição de blastocistos quiméricos quimeras. Por fim, nossos resultados demonstram que a reprogramação de biPSCs é possível em baixo e alto oxigênio. No entanto, a suplementação LIF2i e bFL2i (baixo oxigênio) não promoveram a auto renovação das biPSCs. Também, as biPSCs e bESCs geradas e isoladas apresentaram características de pluripotência, onde, biPSCs foram mais competentes em contribuir em quimeras embrionárias.

Abstract

Pluripotent stem cells are a crucial tool to embryology studies, to development *in vitro* clinical studies, to regenerative medicine and more recently, to gametes *in vitro* production. In 2006, Takahashi and collaborators demonstrated can be possible generate pluripotent stem cells by gene expression induction (induced pluripotent stem cells or iPSCs). However, *in vitro* culture and environmental conditions can modulate the reprogramming efficiency to pluripotency. Thus, the present thesis aimed to study the effects of oxygen and different sets of supplements for maintenance the pluripotency state during the bovine iPSC reprogramming window (biPSCs). In the first study, bovine fetal fibroblasts were reprogrammed in low or high oxygen and in different sets supplements for maintenance of pluripotency, bFGF or bFGF+LIF+CHIR+PD (bFL2i). In our conditions, low oxygen did not increase the reprogramming efficiency when compared to normoxia. On the other hand, bFL2i supplementation increased the reprogramming efficiency of biPSCs. The biPSCs derived in bFL2i low oxygen indicated the absence of self-renewal ability. The characterized biPSCs were positive for alkaline phosphatase, positive for expression of pluripotency marker genes (SOX2, OCT4 and STELLA), however, negative to NANOG. In addition, low oxygen increased the expression of GLUT1 and GLUT3. biPSCs were positive for OCT4 and SOX2, but negative for NANOG in immunofluorescence. The biPSCs were competent in forming embryoid bodies (EBs), also, EBs expressed markers of mesoderm and ectoderm, also, one biPSCs line expressed endoderm marker. In the second study, fetal bovine fibroblasts were reprogrammed with different supplements to maintain pluripotency (factorial 2x2; bFGF, bFGF2i, LIF, LIF2i), and embryonic stem cell lines were isolated to be compared with the generated biPSCs. The bFGF supplementation increased the reprogramming efficiency of biPSCs. The biPSCs derived with LIF2i indicated the absence of self-renewal ability. The biPSCs and bESCs were positive for alkaline phosphatase. The biPSCs and bESCs were positive for the expression of pluripotency marker genes (SOX2, OCT4, STELLA, LIFr and OTX2) and only the biPSCs expressed NANOG and ESRRb. In addition, biPSCs and bESCs were positive for SOX2, OCT4 and H3K27me3, negative for NANOG in immunofluorescence. The biPSCs and bESCs were competent to form embryoid bodies, which expressed markers of mesoderm, ectoderm and endoderm. Still, our results indicate biPSCs were more competent compared to bESCs for the chimeric blastocyst contribution. Finally, our results demonstrate the reprogramming of biPSCs is possible in low and high oxygen. However, supplementation LIF2i and bFL2i (low oxygen) did not promote self-renewal ability on biPSCs. Also, biPSCs and bESCs were generated and isolated and showed pluripotency characteristics, where biPSCs were more competent in contributing to embryonic chimeras.

Introduction

Pluripotent stem cells (PSCs) have two particular features, which are self-renewal and pluripotency. Self-renewal is the capacity to generate new cells with similar characteristics as the original cell, whereas pluripotency is the cell's ability to differentiate into all cell lines (somatic and germ line) from the adult organism (Ying *et al.* 2008). PSCs can be derived from blastocysts, by isolating embryonic stem cells (ESCs) lines or reprogramming differentiated cells, the called induced pluripotent stem cells (iPSCs). Both ESCs and iPSCs are pluripotent and have been receiving attention from the scientific community due to their potential applications such as: cellular agriculture, gamete generation, gene-edited animals (agricultural production or creating large animal models for human conditions), tissue generation and more. Associated with the numeric expansion, several studies investigate how to improve derivation of ESCs, the reprogramming efficiency of iPSCs, and the pluripotent state of ESCs and iPSCs. On this subject, several molecules, small molecules and inhibitors have been used to promote such characteristics.

A general compilation of these methods is presented in Chapter 1. This literature review addresses the first reports of PSCs, main strategies to derivate ESCs and reprogramming iPSCs on livestock species, their limitations and applications.

One of the challenges when working with iPSCs derived from differentiated cells of livestock species is the impossibility to compare results with other studies, especially because different transcription factors delivery methods, medium composition, growth factors and small molecules are often used. Still, different approaches were used to characterize cell lines and to test the differentiation capacity.

In the **Chapter 2**, bovine iPSCs were generated by lentiviral reprogramming using mouse OCT4, KLF4, SOX2 and C-MYC (mOKSM) transcription factors (TFs) into the fetal fibroblasts genome. The reprogramming was conducted in a 2x2 factorial experiment design, when we tested different oxygen tension (low x high oxygen, or 5% x 20%, respectively) and different sets of supplementations during culture (bFGF x bFGF+LIF+CHIR+PD, called bFL2i) to improve the reprogramming efficiency in biPSCs.

In the **Chapter 3**, bovine iPSCs were generated by lentiviral reprogramming on bovine fetal fibroblasts. The reprogramming was conducted in a 2x2 factorial experiment design, four sets of supplementations during culture (bFGF, bFGF+2i, LIF and LIF+2i) were tested to improve the reprogramming efficiency, and the pluripotent state in biPSCs. Additionally, bovine embryonic stem cells (bESCs) were derived to compare the pluripotent profile and characteristics to biPSCs. The pluripotent profile of biPSCs and bESCs were analyzed by RT-qPCR and immunostaining. Also, we evaluated biPSCs and bESCs on gold standard method to assess the differentiation ability of PSCs, producing and analyzing the contribution of biPSCs and bESCs on chimeric embryos. Finally, in **Chapter 4**, the final considerations of the findings of these studies are presented.

Hypothesis

Generation of bovine induced pluripotent stem cells is possible using mouse exogenous transcription factors and using the supplementation described on mouse iPSCs known molecules. Also, the low oxygen tension improves the reprogramming efficiency on biPSCs. Additionally, these biPSCs can contribute to bovine chimeric embryos.

Objectives

- To generate biPSCs in low and high O₂ tension
- To isolate and maintain *in vitro* bovine embryonic stem cells
- To characterize bESCs and biPSCs and the influence of different environments
- To investigate whether bESCs and biPSCs can integrate and contribute in bovine chimeric embryos

Experimental design

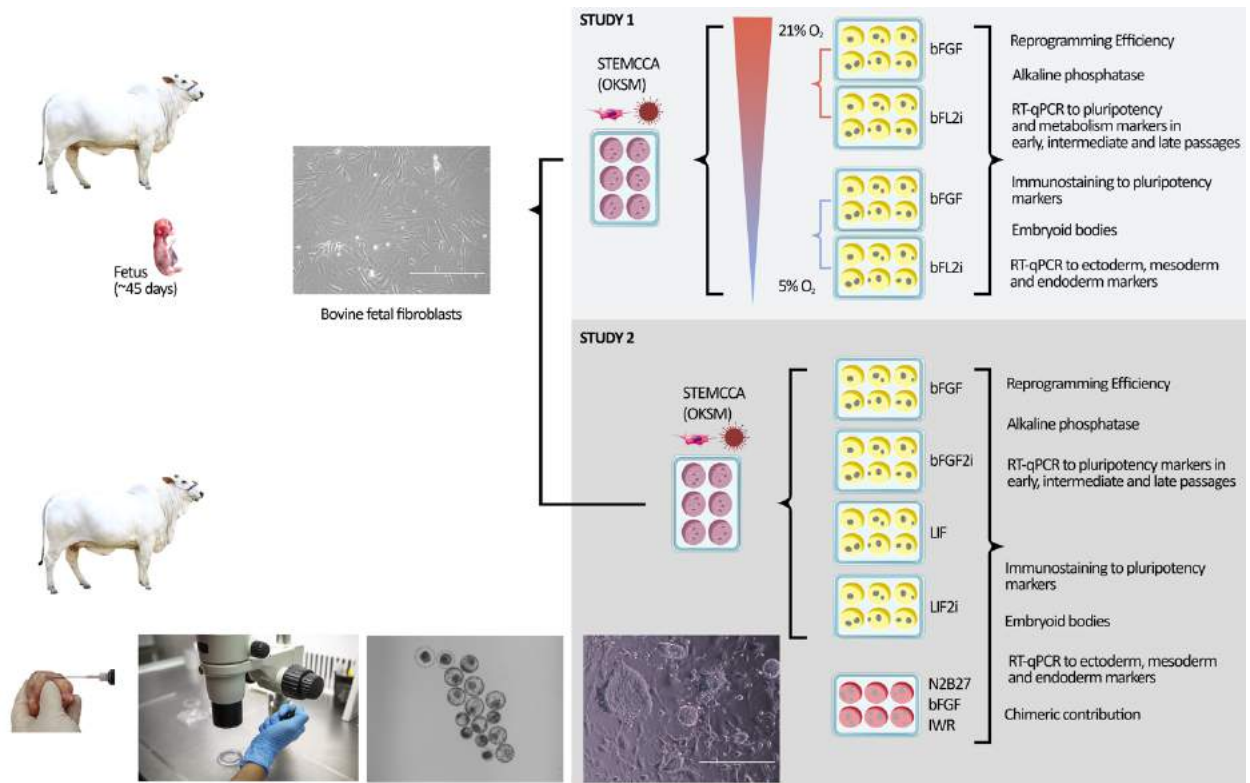


Figure 1. Experimental design overview over “bovine iPSCs and bovine ESCs: molecular profiles and chimeric contribution”

Chapter 1: Literature review

Background

In mammals, all cells present in the adult organism (somatic and germ cells) derive from the pluripotent cells present in the embryos prior of their compaction (Martin 1981). During the initial embryo development, the totipotent cells differentiate into inner cell mass (ICM) and trophectoderm, visibly distinct in the blastocyst stage. The trophectoderm originates the extraembryonic tissues, such as the amnion and chorion in the bovine species. While ICM cells, classified as pluripotent, differ in hypoblast and epiblast. The later, differentiates further in endoderm, mesoderm, ectoderm and primordial germ cells (PGCs)(Evans and Kaufman 1981; Rossant 2001; Blomberg *et al.* 2008; Nichols and Smith 2009; Hyttel *et al.* 2010; Sánchez and Smitz 2012).

In the early 1980s, Evans and Kaufman (1981) and Martin (1981) successfully described the isolation of ICM from mouse embryos in in vitro culture. Thus, mice embryonic stem cells (ESCs) could be cultured in vitro for undetermined time, due to their unlimited proliferation capacity. The success of human ESCs derivation only occurred 17 years later by Thomson *et al.* (1998). For domestic animals, the derivation process even required more time, such as: swine (Telugu *et al.* 2011), goats (Behboodi *et al.* 2011) and cattle (Bogliotti *et al.* 2018).

Another possibility for obtaining PSCs is through cell reprogramming. Initially, cell reprogramming was described by somatic cell nuclear transfer (SCNT)(Gurdon 1962a; b). In 1997, Dolly, the sheep, was born, and the authors proved the capacity of SCNT in mammals (Wilmut *et al.* 1997). Another approach of reprogramming differentiated cells was described by Takahashi and Yamanaka (2006). The authors reported a significant advance in the understanding of the acquisition of pluripotency when they induced differentiated somatic cells into a state of pluripotency similar to ESCs. The combination of four transcription factors (OCT4, KLF4, SOX2 and C-MYC), introduced by retroviruses into mouse embryonic fibroblasts, reprogrammed the cells to a pluripotent state. These cells were called induced pluripotent stem cells (iPSCs)(Takahashi and Yamanaka 2006).

The cell reprogramming technology that produced iPSCs has been successfully applied in many domestic species, such as: sheep (Li *et al.* 2011; Liu *et al.* 2012), equines (Nagy *et al.* 2011; Whitworth *et al.* 2014), swine (Cheng *et al.* 2012; Fujishiro *et al.* 2012; Zhang *et al.* 2015) and cattle (Huang *et al.* 2011; Heo *et al.* 2014; Lin *et al.* 2014). For the reprogramming of differentiated cells in iPSCs, different methodologies are often used, such as integrative (lentivirus, retrovirus, retroviral induction, transposons, PiggyBAC and CRISPR) and non-integrative systems (sendai virus, adenovirus, episomal plasmids and modified synthetic RNA)(Kumar 2015; Singh *et al.* 2015). Additionally, different culture media compositions, growth factors, supplements and inhibitors of cell differentiation have been described (Navarro *et al.* 2019).

PSCs (iPSCs or ESCs) characterization is usually analyzed by morphology, molecular markers (mRNA transcripts and proteins), epigenetic marks and cell differentiation characteristics. Still, PSCs can be classified into two categories, naïve and primed (Nichols and Smith 2009; Hanna *et al.* 2010). Naïve PSCs have similar characteristics to ICM cells, while cells classified as primed can be compared to cells from the post-implantation epiblast in mice (Nichols and Smith 2009). For ESCs and iPSCs of mice, it is highlighted the dependence of leukemia inhibitory factor (LIF) for the maintenance of the naïve pluripotent state. Also, when these murine cells are cultured with fibroblast growth factor (FGF), they are classified as in a primed state of pluripotency (Nichols and Smith 2009; Hassani *et al.* 2014).

Due to the different characteristic of PSCs (ESCs or iPSCs) in differentiating into embryonic tissues (ectoderm, mesoderm and endoderm) and germ cells, different methodologies are used to assess the differentiation potential, such as embryoid bodies formation, spontaneous in vitro cell differentiation, directed in vitro cell differentiation, formation of in vivo teratomas and the ability to contribute on chimeric embryos. In order to PSCs contribute on chimeric embryos and contribute on all tissues (somatic or germline) these cells need to have a naïve pluripotent profile (Weissman 2000). However, Yu *et al.*, (2020) recently described an intermediate pluripotent profile able to contribute with chimeric embryos generation all tissues and germ line cells.

Thus, the present study aims to generate bovine iPSCs and derivate bovine ESCs to compare both pluripotent profiles and evaluate their ability to contribute in chimeric embryos.

Definition and different types of pluripotent stem cells

Pluripotent means “many” potent. A pluripotent stem cell is defined as a cell that can undergo prolonged undifferentiated cell proliferation *in vitro* and has the capacity to differentiate into all embryonic tissues (endoderm, mesoderm and ectoderm) as well as germ line lineages. The classical example of PSCs is embryonic stem cells.

Teratocarcinomas/ Embryonic carcinoma cells

The first cell report with the abilities of PSCs appeared in the early 1950. Stevens and Little (1954) described testicular teratomas in an inbred mouse strain. The mouse strain had a high frequency of about 1% of spontaneous testicular teratocarcinomas which was a great advantage as teratomas (benign) and teratocarcinomas (malignant) are rare in natural conditions. The higher frequency of teratocarcinomas allowed researchers to study them on macroscopically as well as a cellular level.

In 1960's, the characterization of the teratocarcinomas was described in these cells with capacity to replicate indefinitely, and also to differentiate into multiple adult cell types (Kleinsmith and Pierce 1964).

In 1970 two important experiments by Solter et al. (1970) and Stevens (1970) proved that teratocarcinomas could be generated by transferring mouse embryos to extrauterine sites. This confirmed that pluripotent cells from mouse embryos and cells from teratocarcinomas were highly similar and made it possible to produce embryonic carcinoma cells. The methods for culturing cells improved drastically in the beginning of the 1970's and this allowed the derivation and controlled propagation of cell types from teratocarcinomas (Martin and Evans 1975). The term embryoid body (EB) was defined from structures containing tissues representing the three germ layers in the teratocarcinomas (Martin and Evans 1975).

Interestingly, some of the cell lines derived from the teratomas proliferated fast and had a high capacity for differentiation when applied to teratocarcinomas and in vitro EBs. These cell lines had similar cell and colony morphology: they grew in small tight colonies and the cells had a large clear nucleus with visible nucleoli and sparse dark cytoplasm (Martin and Evans 1975). These cells were named embryonal carcinoma cells (ECCs) and considered as the tumor's stem cells (Martin 1975).

The most relevant pluripotency test was also established in the 1970's using ECCs. The test is conducted by injecting cells into the blastocoel cavity of a mouse embryo and transferring the embryo into a surrogate mother. If the cells are truly pluripotent, they will contribute to all three germ layers and germline of the offspring. The first report of production of ECCs chimeras was published in 1974 by Brinster (1974).

The research comprising the teratomas showed that it was possible to culture mouse and human cells that could be maintained infinitely in vitro and differentiate into all cell types of the adult mouse or human. The usual colony and cell morphology of pluripotent stem cells were described using ECCs and the stem cell markers as alkaline phosphatase, SSEA- and TRA- markers were identified. Furthermore, the first embryoid body, teratoma and chimeric contribution assay was conducted. All three assays are widely used today. Finally, the first experiments showing the possibility to direct differentiation of pluripotent stem cells were conducted on ECCs. However, most of the research using ECCs ceased shortly after the first ESC lines were derived in 1981.

Embryonic Stem Cells (ESCs)

In mammals, embryonic development begins with the fertilization. Following consecutive rounds of cleavage, blastomeres with totipotent ability can differentiate into any cell type as the transient extra-embryonic annexes, embryonic tissues and germline cells. The totipotent ability of single blastomeres from eight-cell stage embryos (uncompromised cells) of rabbits, pigs and sheep were reported competent to produce viable offspring (Moore *et al.* 1968; Willadsen 1980; Saito and Niemann 1991). Thus,

differentiation of totipotent cells is promoted by embryo genomic activation, transcripts, proteins, chromatin and histones modifications and its interactions (Christians 2019).

The first commitment during the development of totipotent cells consists of two major cell types: the pluripotent ICM cells and trophectoderm, and this segregation generates the blastocyst (~Embryonic day 3.5 (Ed3.5) in mice and ~Ed6 in humans). On later blastocyst (~Ed4.5 in mice and ~Ed7 in humans), ICM cells undergo a second round of cell commitment to generate the pluripotent epiblast cells and the hypoblast (Wang and Dey 2006).

Evans and Kaufman (1981) were the first researchers to successfully describe the isolation and establishment of mouse pluripotent ESCs from the inner cell mass (ICM) of expanded blastocysts. The authors had success with the in vitro culture using Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10% newborn calf serum, and feeder of inactivated Sandos inbred mice (SIM) 6-thioguanine-resistant, ouabain-resistant (STO) fibroblasts. In the same year, Martin (1981) also derived mouse ESCs, however, from ICM isolated by immune-surgery and cultured in DMEM supplemented with 10% Hyclone fetal calf serum.

After these mESCs reports, the first reports to derive ESCs from livestock sheep (Handyside *et al.* 1987), pig (Strojek *et al.* 1990), cattle (Saito *et al.* 1992) and goat (Meinecke-Tillmann and Meinecke 1996) were based on standard conditions for mouse ESC culture. When ICMs were isolated from in vivo derived embryos, they were cultured DMEM and a-minimum essential medium (aMEM), supplemented with FBS and cultured on inactivated mouse embryonic fibroblasts (MEFs) feeders. Also, these reports used growth factors to improve the success, such as insulin, epidermal growth factor (EGF) and LIF.

The successful of primate ESCs derivation was not reported until one decade later; hence, Thomson *et al.* (1995) firstly described the successful of derivation of rhesus macaque ESCs, and after 3 more years the same group published the isolation of human ESCs (Thomson *et al.* 1998). These cells were isolated from the ICM and it became clear that the culture conditions used for growing primate ESCs were distinct from that used for mouse ESCs; as these required Activin A and FGF2.

Subsequently to the primate reports, livestock ESCs incorporated FGF2 or bFGF into the culture conditions as described on cattle (Wang *et al.* 2005; Yadav *et al.* 2005; Roach *et al.* 2006), goat (Tian *et al.* 2006) and pigs (Miyoshi *et al.* 2000; Li *et al.* 2003, 2004). Mostly of the FGF-dependent livestock ESC lines reported show self-renewal ability and expressed markers of pluripotency, but those livestock ESCs lines demonstrated low efficiency to contribute to chimeric embryos.

Although ESCs from these different species exhibit differences in colony morphology, growth factor dependency and culture requirements, they were considered equivalent cell lines for many years because they are derived from the ICM. In 2007, another type of PSCs was described, the epiblast-derived stem cells (EpiSCs), this PSCs line was isolated from the post-implantation epiblast in mice (Tesar *et al.* 2007). The EpiSCs are FGF2 dependent, also, this cell line demonstrated the presence of pluripotency biomarkers and the capacity to produce teratomas and differentiation to ectoderm, mesoderm and endoderm tissues.

Since then, states of pluripotency were classified as naïve and primed (Nichols and Smith 2009). While distinct naïve and primed pluripotent states have been described, that correspond to the pre and post-implantation epiblast respectively (Nichols and Smith 2009).

More recently, Wu *et al.*, (Wu *et al.* 2015) isolated a novel primed pluripotent cell type, the region-selective epiblast stem cells (rsEpiSCs) are derived from pre- and post-implantation epiblasts under a FGF2 and IWR1 (Wnt canonical pathway inhibitor) based medium. Furthermore, similar supplementation (FGF2 and IWR) combined with CTFR medium (custom basal medium similar to mTeSR medium) were successful at isolate and stabilizing bovine ESCs from blastocysts produced by IVM-IVF, OPU-IVF, SCNT (Bogliotti *et al.* 2018).

Hypoxia in Early Embryonic Development

The *in vivo* embryogenesis occurs in the female reproductive tract and this environment is heavily influenced by oxygen gradients. Thus, before establishment of the circulatory system during the embryogenesis, delivery of oxygen (O₂) to the embryo

is limited by diffusion and, as a result, early mammalian development occurs in a relatively oxygen-poor (~1.5-8.7%) environment (Fischer and Bavister 1993).

This indicates that the early embryo has to be adapted to low oxygen availability. In agreement, several studies reported the benefits of hypoxia during the initial stages of mammalian blastocyst development produced on *in vitro* environment. The number of embryos, progressed into expanded and/or hatched blastocyst was higher when cultured in 7% oxygen in comparison to 20% oxygen (Batt *et al.* 1991). The same results were described in other mammalian species. Also, the reduction of oxygen tension to 5% has been associated with an improvement in embryo quality and pregnancy rates, especially when embryos were transferred at the blastocyst stage (Sciorio and Smith 2019).

The identification of hypoxia inducible factors (HIFs) helped explain many of hypoxia's direct effects on embryos and ESCs. Thus, on hypoxia conditions, HIFs activates the transcription of genes responsible to affect the energy metabolism, autophagy, translation inhibition, erythropoiesis and angiogenesis (Raquel DUNWOODIE, 2009).

The hypoxia conditions reduced the spontaneously differentiation when hESCs are cultured under 3% or 5% O₂, also, this condition promote full pluripotency and enhanced formation of embryoid bodies. Additionally, 3% or 5% O₂ did not compromise the cells proliferation rates, however, in ultra-hypoxia conditions (1% O₂) the cells maintain pluripotency state but reduce the proliferation, suggesting that different tension of oxygen can modulate proliferation and quiescence in stem cells (Ezashi *et al.* 2005).

In 2010 Forristal *et al.* (2010) showed the important function of HIF (HIF2 α and HIF3 α) on maintenance and self-renewal of human ESCs, using HIF knock down model, the authors described the reduction of OCT4, SOX2 and NANOG expression. Interestingly, C-MYC transcription factor is an important actor for self-renewal of pluripotent stem cells, in hypoxic conditions the expression remained high after prolonged culture, possibly sustaining the undifferentiated phenotype of hESCs (Närvä *et al.* 2013).

The hypoxia benefits to ESCs on maintenance, self-renewal and undifferentiation also are connected with metabolism regulation and all of these actors work on different

pluripotent state of stem cells. Additionally, Wu, Ocampo and Belmonte (2016) published a elegant review about the interactions between metabolism, transcriptional factors, supplements to maintenance the pluripotency on PSCs.

Naïve, intermediate and primed pluripotent states

In order to explain the characteristics of mouse ESCs and mouse EpiSCs, a two-phase model of embryonic development was proposed by Nichols and Smith (2009), consisting of a naïve state, which is closer to the ICM - early epiblast, and a primed state, which is closer to the late pre-gastrulating epiblast (Nichols and Smith 2009). Naïve and primed cells have a distinctive colonies morphology: primed cells are flat and more two-dimensional, while naïve cells have a domed shape and are more three-dimensional. However, common characteristics shared between naïve and primed PSCs includes characteristics of pluripotency, teratoma formation and embryoid body formation.

Mouse ESCs can also be maintained in 2iLIF medium, which consists of two small molecules, that inhibit GSK3 β (CHIR99021) and ERK/MAPK signaling (PD0325901) combine with LIF, promoting naïve pluripotency state (Ying *et al.* 2008). Mechanistically, LIF binds resulting in the phosphorylation of JAK kinases (pJAK), which activates several downstream signaling pathways, including transcription factor STAT3, PI3K/AKT and ERK/MAPK (Hirai *et al.* 2011; Hassani *et al.* 2014). LIF, which activates STAT3, induces mitochondrial respiration and the activity of several transcription factors (OCT4, NANOG, SOX2) that have a decisive role in the gene regulatory network of naïve pluripotency and induce important genes for self-renewal (Ying *et al.* 2008; Nichols and Smith 2009).

Also, the PI3K/Akt pathway upregulates mTOR and inhibits GSK3 β and is related to NANOG expression. The MAPK/ERK pathway induces differentiation but the effect is inhibited by BMP4 that activates the ID genes and DUSP9 through the SMAD1,5,8 signaling pathway (Hassani *et al.* 2014).

Interestingly, 2iLIF cells have an altered chromatin state and reduced levels of repressive marks; as H3K27me3 levels are reduced at developmental genes (Ross *et*

et al. 2008) and lower levels of other repressive histone modifications (Sim *et al.* 2017). The global DNA demethylation in naïve cells is similar to the DNA demethylation that occurs in early embryonic development between the two-cell (2C) and blastocyst stages (Guo *et al.* 2014).

The epigenetic status of the X chromosome in female PSCs, is another characteristic to classify naïve and primed cells. In mice, ESCs with naïve pluripotency state, are characteristics of the ICM and possess two activated X chromosomes. Additionally, *XIST*, a long non-coding RNA that silences one X chromosome is not expressed; in the other hand, in primed EpiSCs, *XIST* is expressed and one X chromosome is epigenetically inactivated (Bao *et al.* 2009). This epigenetic status matches with *in vivo* embryonic states: in the late inner cell mass, both X chromosomes are active, and during differentiation in the late epiblast, one random X chromosome is silenced (Okamoto *et al.* 2011).

In human cells, the epigenetic status of the X chromosome in female PSCs is more complex. Human naïve cells are affected by different protocols on epigenetic status, variances of X chromosome inactivation, including intermediate states of *XIST* expression and epigenetic silencing (Sahakyan *et al.* 2017). The *XIST* is expressed in the inner cell mass, but its expression is not correlated to the with epigenetic suppression of the X chromosome (Okamoto *et al.* 2011). One X chromosome is “dampened” by an uncertain mechanism, before later full X chromosome inactivation by chromatin silencing, possibly as late as post-implantation. This added complexity scenery in humans PSCs, may explain the differences in X chromosome status in naïve and primed hESCs or may reflect species-specific epigenetic regulatory differences between rodents x humans.

Naïve and primed stem cells are distinct also regarding metabolism. Naïve cells are associated to elevated mitochondrial function, increased oxidative phosphorylation (OXPHOS), and a lower level of glycolysis. The higher OXPHOS activity observed in naïve cells is, driven by STAT3, a downstream effector of the LIF-dependent self-renewal pathway. STAT3 also facilitated metabolic resetting during primed-to-naïve state transition (Carbognin *et al.* 2016). In the other way, the transcription factor HIF-1 α

promote switches mESC metabolism and impulses cells toward a primed state (Zhou *et al.* 2012).

Other player involved on OXPHOS differences on naïve and primed cells is LIN28, working on the suppression of OXPHOS and maintained low mitochondrial function in primed stem cells (Zhang *et al.* 2016). Additionally, Zhang *et al.* (2016) identified LIN28 is a key regulator during the metabolic transition from naïve-to-primed pluripotent state. Interestingly, primed stem cells show lower levels of mitochondrial respiration, however, have higher mitochondrial DNA (mtDNA) copy number and more mature mitochondria than naïve cells. Folmes *et al.*, (2012) described more developed mitochondria in primed pluripotent cells can serve as preparation for the elevated OXPHOS activities acquired upon differentiation.

Comparing naïve and primed hESC states revealed that naïve cells express nicotinamide N-methyltransferase (NNMT) at high levels, which act on S-adenosylmethionine (SAM) metabolizing, the major chemical donor for histone methyltransferases. Consequently, naïve cells have low levels of SAM and correspondingly low levels of histone methylation, while primed cells have the inverse, high SAM levels and high histone methylation (Sperber *et al.* 2015). Importantly, these metabolic modifications are crucial for naïve to primed transition, as *NNMT* knockout cells transition to a primed state (Sperber *et al.* 2015).

Hence, the states of pluripotency, naïve and primed, are interconvertible, based on the growth conditions/factors used and these transitions enriched our molecular understanding of pluripotency progression *in vivo* and mechanisms of cellular reprogramming (Weinberger *et al.* 2016). In 2iLIF conditions, which supports naïve pluripotency, when mESCs are continuously exposed to bFGF/ACTIVIN A, they develop to a primed state of pluripotency, which is similar to mEpiSCs derived from post-implantation embryo (Tesar *et al.* 2007). In the other direction, mouse primed cells can be converted into a naïve state by maintaining them in a 2iLIF condition, combined with over-expression of *Klf4* (Guo *et al.* 2009).

In 2015, a new type of PSCs was described, when Wu *et al.*, (Wu *et al.* 2015) showed the isolation of a stem-cell type with unique spatial characteristics and distinct molecular and functional features, designated as region-selective pluripotent stem cells

(rsPSCs), these cells can be efficiently obtained from mouse embryos and primate pluripotent stem cells, including humans. They used a culture condition with FGF2 and WNT/b-catenin signaling pathways inhibitor (IWR1). These rsPSCs proven efficient generation of intra- and interspecies chimeric embryos.

Recently, Yu et al., (2020) using the same approach, with N2B27 medium supplemented with FGF2 and IWR1, described the intermediate PSCs with transcriptomic connections with embryos day 5–6 epiblasts and retain high competency for direct primordial germ cells-like cells induction *in vitro* and germ-line chimera formation *in vivo*. Also, the authors described the successful to derivate ESCs from a large livestock species (horse) using the same protocol.

Before of these advances on the field, reports have indicated that only naive cells could generate chimeric offspring (Rossant 2008; Nichols and Smith 2009). The dynamics of pluripotency regulation, however, has been shown a lot more plastic, at least in the murine model, in which stem cells derived from rsPSCs and EpiSCs and intermediate PSCs are efficiently form chimeras in gastrulation-stage embryos, colonizing somatic and germ cell lineages (Huang *et al.* 2012; Wu *et al.* 2015; Yu *et al.* 2020)(Huang et al. 2012). The inability of some types of stem cells to form or not chimeras is, therefore, better explained by their compatibility between environment and developmental stage more than capacity of *in vivo* differentiation.

Induced pluripotent stem cells (iPSCs)

Pluripotency, the ability of a single cell to generate all lineages of an adult organism, cells with pluripotent ability exists as an orderly continuum during a brief window of early development. This pluripotency continuum state can be sustained *in vitro* as self-renewable PSCs through ESCs isolation from embryos.

In 2006, Shinya Yamanaka's group (2006; 2007) reported that somatic cells could be reprogrammed into pluripotent stem cells by retroviral overexpression of the four embryonic transcription factors: OCT4, KLF4, SOX2 and C-MYC (OKSM). These cells, referred to as induced pluripotent stem cells (iPSCs) exhibit morphology and growth properties of ESCs, express ESCs cell marker genes were proven to be self-

renewing and could be derived all tissues, demonstrated by formation of embryonic bodies, *in vitro* cell differentiation, formation of teratomas *in vivo* and the capacity to contribute on chimeric embryos (Takahashi and Yamanaka 2006; Okita *et al.* 2007; Takahashi *et al.* 2007). The generation of iPSCs promote a drastic change on the PSCs field. Consequently, after promote this advanced on the field professor Shinya Yamanaka was contemplated with Noble prize award (Laureates 2012).

To promote the overexpression of TFs many methodologies can be used as viral vectors (retrovirus, adenovirus, sendai virus and lentivirus), electroporation or liposome to delivery nonviral vectors (episomal vectors, minicircle vectors, piggyBac or mRNAs), sleeping beauty transposons and more recently CRISPR/cas9 system (Robinton and Daley 2012; Soto and Ross 2016; Navarro *et al.* 2019; Pieri *et al.* 2019). In contrast to ESCs, iPSCs have already been produced in several species, which iPSCs has been reported on livestock species as cattle (Huang *et al.* 2011; Sumer *et al.* 2011; Cao *et al.* 2012; Heo *et al.* 2014; Lin *et al.* 2014), sheep (Bao *et al.* 2011; Li *et al.* 2011; Liu *et al.* 2012), equine (Nagy *et al.* 2011; Whitworth *et al.* 2014) and pig (Cheng *et al.* 2012; Fujishiro *et al.* 2012; Zhang *et al.* 2015). In general, iPSC technology introduces numerous opportunities to study the effect of genetic engineering, stem cell-based therapies, and other aspects related to developmental biology.

To reprogramming somatic cells into iPSCs, many combinations of TFs can be used. The first combination of TFs was described by Takahashi and Yamanaka (2006), the combination of OKSM TFs were called as Yamanaka factors. Few years later, Nakagawa *et al.*, (2008) described the competence to reprogramming mouse fibroblasts into iPSCs using OCT4, KLF4 and SOX2, however, the efficiency was lower than OKSM TFs reported before.

In human iPSCs, other combination of TFs composed by OCT4, SOX2, NANOG and LIN28 was reported with efficiency on reprogramming fibroblasts into iPSCs (Yu *et al.* 2007). Interestingly, LIN28 is a negative regulator of miRNA biogenesis and suggest that Lin28 may play a central role in blocking miRNA-mediated differentiation in stem cells, also, these miRNAs act on C-MYC translation (Viswanathan *et al.* 2008), thus, thus establishing the connection between the two reprogramming cocktails (Oct4, Klf4, Sox2, and c-Myc, and Oct4, Sox2, Nanog, and Lin28).

In livestock, iPSCs were derived using different overexpression of TFs, with either classic “Yamanaka factors” OKSM or 6 reprogramming factors - OKSM plus NANOG and LIN28 (OKSMNL), and most importantly, most of them did not show robust evidence, if any, of exogenous vector expression silencing, even when considered naïve-like and were able to contribute to embryonic and fetal tissues (Cao *et al.* 2012; Fujishiro *et al.* 2012; Whitworth *et al.* 2014; Canizo *et al.* 2018).

Recently, episomal iPSC reprogramming vectors system was highlighted, especially because the system is competent to produce iPSCs, is a non-integrating system, provide the optimal system for generating transgene-free and virus-free iPSCs in a feeder-free environment (Yu *et al.* 2009). Thus, Yu *et al.*, (2009) combined OCT4, SOX2, NANOG, LIN28, KLF4 and L-MYC, also, the vector had SV40LT.

In livestock species, episomal vectors are not widespread. In swine, few reports described the iPSCs derived using episomal vectors (Telugu *et al.* 2010). Unfortunately, porcine iPSCs derived from episomal vectors transfection showed the lack of exogenous DNA lost due to either integration of plasmid DNA into the host genome or retention of plasmid DNA as an episomal fraction over extended passages, suggesting these porcine iPSCs were not truly pluripotent and remained dependent on the exogenous expression of pluripotency genes (Telugu *et al.* 2010). Years later, Li *et al.*, (2018) described the efficiency on generation of transgene-free porcine intermediate type iPSCs, using the combination of three episomal plasmids (pCXLE-hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL) porcine iPSCs were isolated without episomal vectors detection after 20 passages.

Different environments can be used to promote the reprogramming of differentiate cells into iPSCs, as well, the oxygen tension. Considering the important role of low oxygen tension for maintaining the undifferentiated phenotype of hESCs and on the function of other stem cell populations, Yoshida *et al.* demonstrated in that low oxygen significantly increases the efficiency of both miPSCs and hiPSCs generation and the NANOG expression (Yoshida *et al.* 2009a; b).

Also, metabolic changes are other crucial event during the reprogramming process. Folmes *et al.*, (2011) modulated the energy metabolism using glycolyze inhibitors and the OXPHOS stimulators, these pharmacology approach reduced the

reprogramming efficiency. However, the glucose supplementation during the reprogramming process promote and increase the iPSCs generation (Folmes *et al.* 2011).

This suggests that different routes may lead to a common pluripotent ground state, or, alternatively, that different transcription factors activate the same program by reinforcing each other's synthesis.

Chimerism

The term “chimera” stems from Greek mythology, describing a creature composed of three distinct animals. Modern techniques of chimera production combine cells of more than one embryonic origin to make one genetically mixed embryo and animal. Thus, the chimeric embryo is, by definition, a structure whose cells are derived from more than one embryo, that is, from more than one fertilization. Consequently, each cell has a genetic/phenotypic identity and the distribution is aleatory in the offspring (Nagy A. ; Vintersten K. ; Behringer R. 2003).

There are some types of chimerism, which are:

i) embryonic chimerism - occurs when an embryo is formed from two or more original embryos, containing complete sets of chromosomes from at least two fathers and two mothers.

ii) tissue chimerism - exists when an adult organism have tissues from two or more embryonic origins, for example, from individuals undergoing organ transplants.

iii) cell chimerism - characterized when there is the transfer of the nucleus from one cell to another enucleated one, which will then have a nuclear DNA of a different origin than mitochondrial DNA, for example, cloning by the somatic cell nuclear transfer technique).

iv) genetic chimerism - established when a DNA molecule receives the insertion of a genetic sequence from other origin (another individual), for example, of transgenic individuals.

The embryonic chimerism techniques were first standardized and established in murine species (rats and mice). Among the chimerism techniques, the most widespread are the aggregation of embryos by proximity; co-cultivation of embryos with PSCs; and microinjection of PSCs on embryos in different stages (Lee *et al.* 2007).

The first mouse embryonic chimeras was generated independently by Tarkowski (Tarkowski 1961) through aggregating two eight-cell embryos. The aggregation methodology presents benefits to produce chimeric embryos, a good example, is the capacity to use embryos with different stages of development, with different ploidies (diploid or tetraploid) and with PSCs (Nagy A. ; Vintersten K. ; Behringer R. 2003). For increase the adhesion during the aggregation, a simple methodology was developed by Vajta *et al.*, (2000) to culture zona-free embryos, the Well of the Well (WOW) system. Small wells (WOWs) were formed in four-well dishes by melting the bottom with heated steel rods. The WOWs were then rinsed, the wells were filled with medium, and the embryos were placed into the WOWs. Other approach to produce chimeric embryos with PSCs is basically the co-cultivation of 8-cell embryos (zona-free) on PSCs.

Microinjection is the most used method to produce chimeric embryos with PSCs, although other cell types can also be injected, such as teratocarcinogenic cells, and embryonic stem cells and iPSCs. The first report using microinjection to produce chimeric embryos was described by Gardner (1968), when PSCs were injected into the blastocoel cavity of mouse blastocysts . Since then, chimerism has been performed by micromanipulation of ESCs in many species of mammals including mice (Evans and Kaufman 1981), rats (Iannaccone *et al.* 1994), rabbits (Schoonjans *et al.* 1996), sheep (Butler *et al.* 1987), goats (Jia *et al.* 2008), pigs (Onishi *et al.* 1994) and cattle (Iwasaki *et al.* 2000).

Interspecies chimeras also are able to produce by microinjection. These reports started with Rossant and Frels (1980) combining *Mus musculus* and *Mus caroli*, were produced by blastocyst injection. More recently, Wu *et al.*, (2017) and Yu *et al.*, (2020) described interspecies (rodents and livestock species combined with human PSCs) chimeras success for medical applications in the future.

Livestock pluripotent stem cells applications

The majority of studies concerning the use of stem cells in reproductive medicine have been carried out in animals; also, livestock PSCs have a high potential to facilitate the conception of gene-edited animals, to be used in the development of regenerative medicine applications, to constitute a source of cellular agriculture and, more recently, through differentiation to gametes, could enable in vitro breeding to accelerated genetic improvement.

Also, stem cell research and the generation of induced pluripotent stem cells in livestock species presents a new route of possibilities and hopes for the veterinary reproductive and regenerative medicine field, including, for example, the conservation of rare species and breeds.

However, protocols that work well with human and mouse PSCs may not be directly applied to livestock species, and additional adjusts and optimization will likely be necessary.

Overall, more than 30 years from the initial attempts for derivation of livestock ESCs and more recently iPSCs, we can now look to the future and see the applications of PSC technology will have a significant effect on advancing animal agriculture and the the use of large animal models in basic and applied research.

Chapter 2: Oxygen levels during in vitro induced reprogramming alter pluripotency acquisition and maintenance in cattle

Submit to: **Cells**

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Abstract

The event of cellular reprogramming into pluripotency is influenced by several factors, such as *in vitro* culture conditions (e.g., culture medium and oxygen concentration). Herein, bovine iPSCs (biPSCs) were generated in different levels of oxygen tension (5% or 20% of oxygen) and additional supplementation sets (bFGF or bFGF+LIF+2i - bFL2i), to evaluate the efficiency of pluripotency induction and maintenance *in vitro*. Reprogramming was observed in all groups. bFL2i supplementation increased the initial reprogramming efficiency. However, bFL2i supplementation in low oxygen led to the loss of self-renewal and pluripotency maintenance. All clonal lines were positive for alkaline phosphatase; they were positive for endogenous pluripotency-related genes *SOX2*, *OCT4*, and *STELLA*; however, expression was decreased throughout passages without the oxygen influence. *GLUT1* and *GLUT3* were upregulated by low oxygen. The biPSCs were immunofluorescence-positive stained for OCT4 and SOX2 (all groups) and negative to NANOG. They formed embryoid bodies, which were spontaneously differentiated in ectoderm and mesoderm (all groups) and endoderm (one line from bFL2i in high oxygen) embryonic germ layers. Our study is the first to compare high and low oxygen environment during and after induced reprogramming in cattle, and, in our conditions, the low oxygen did not favor the pluripotency of biPSCs.

Keywords: oxygen environmental, acquisition of pluripotency, bovine iPSCs, pluripotent state

Introduction

The success of reprogramming somatic cells into cells with pluripotent characteristics (induced pluripotent cells, iPSCs) has been long reported and well-studied, in special, in humans and mice, providing researchers a valuable source of different cell types [1,2] to use in regenerative medicine, and also for biotechnology and reproductive purposes in domestic and wild animals [3].

iPSCs can be genetically manipulated by different genome editing tools, providing a powerful platform for the production of transgenic animals, especially by chimeric animals or somatic cell nuclear transfer (SCNT). Indeed, multiple genetic alterations in livestock could be used to improve traits connected with animal production and create large animal models for *in vitro* and *in vivo* disease modeling [4,5].

The generation of livestock iPSCs has been reported, including sheep [6,7], equines [8,9], swine [10–12] and cattle [13–15]. species. These livestock iPSCs showed pluripotent characteristics such as higher proliferation capacity, expression of pluripotency marks, and the ability to generate three germ layers tissues on *in vitro* and *in vivo* tests; however, most of the characteristics and protocols are still divergent and often describes a “pluripotency-like” state.

Importantly, pluripotent stem cells are mainly classified into two different states, naïve or primed. Naïve ESCs and iPSCs, mostly observed in murine models, rely on

leukemia inhibitory factor (LIF) supplementation to retain pluripotency, whereas primed mouse EpiSC and human ESCs and iPSCs, relies on fibroblast growth factor 2 (FGF2) stimulation [16]. Also, two small molecules are widely used to improve the pluripotent state by blocking the mitogen-activated protein kinase (MEK), and Glycogen synthase kinase 3-beta (GSKB3) activity pathways and are called 2i (2 inhibitors) [17].

Different methodologies have been explored to improve the efficiency during the reprogramming process, and one promising field the in vitro culture environment, which enables the use of different molecules as inhibitors and supplements and enables the control of the oxygen tension. In mouse ESCs, hypoxia conditions (5% oxygen) increased colonies with alkaline phosphatase activity, enhanced cell proliferation, and prevented differentiation [18]. Additionally, for mouse and human iPSCs, low oxygen tension increased reprogramming efficiency on promoting colony numbers [19]. However, livestock iPSCs have not yet been described on low oxygen.

Therefore, this study aims to reprogram cattle fibroblasts under different culture conditions and oxygen tension to generate biPSCs, and these were evaluated regarding pluripotency related transcripts, immunostaining, and in vitro differentiation capacity, and therefore, to obtain a better understanding of the failures in the establishment of the pluripotency state in biPSCs that are mainly linked to the mechanisms of self-renewal and related to the in vitro culture condition.

Material and Methods

All experiments were approved by the Ethics Committee on Animal Experimentation at the Faculty of Animal Science and Food Engineering of the University of São Paulo (CEUA 2192250918 and 3526250717).

Cells and Media

Mouse embryonic fibroblasts (MEFs), bovine fetal fibroblasts (bFFs), and 293FT cells were cultured in complete Iscove's Modified Dulbecco's Medium (IMDM, Gibco Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS)(GE Healthcare Life Sciences), 1% GlutaMAX (Gibco Thermo Fisher Scientific), 1% MEM non-essential amino acids solution (Gibco Thermo Fisher Scientific) and 1%

penicillin/streptomycin (Gibco Thermo Fisher Scientific). biPSCs were cultured in KnockOut DMEM-F12 (Gibco Thermo Fisher Scientific) supplemented with 20% KnockOut Replacement Serum (Gibco Thermo Fisher Scientific), 1% GlutaMAX (Gibco Thermo Fisher Scientific), 1% MEM non-essential amino acids solution (Gibco Thermo Fisher Scientific), 1% penicillin/streptomycin (Gibco Thermo Fisher Scientific) and 1:1000 β -mercaptoethanol (Gibco Thermo Fisher Scientific) supplemented with 10ng/mL recombinant human FGF-basic (Peprotech), 1000UI/mL recombinant mouse LIF Protein (ESGRO, Millipore) and 2i (1mM PD0325901 and 3mM CHIR99021, Sigma).

Feeder cells

MEFs were isolated from 13.5d mouse fetuses and cultured on complete IMDM. Cells were treated with mitomycin and used as a feeder monolayer at 1×10^5 cells in 6 well dishes. The dishes were treated with 0,1% gelatin previously to seeding.

Lentivirus production

Lentiviral production was performed as described before [20]. Briefly, 5×10^6 HEK-293FT cells were seeded in t75 cm² flasks and maintained in IMDM with 10% FBS overnight. The STEMCCA vector (12 μ g) containing the murine transcription factors OCT4, KLF4, SOX2, and c-MYC [21] and the auxiliary plasmids (1.2 μ g TAT, 1.2 μ g REV, 1.2 μ g Hgpm2 and 2.4 μ g VSVG) were transfected using Lipofectamine 3000 reagent (Invitrogen) following manufacturer's suggestions. The media containing the lentivirus particles were recovered at 24-72 h after transfection. The supernatant was filtered (0.45 μ m membrane, Millipore SCHVU01RE), distributed media evenly into ultracentrifuge tubes. The ultracentrifugation was performed at 48960 G, 1h 30 min at 4°C. The supernatant was removed, and approximately 100uL per tube was used to resuspend the lentivirus stock. The lentivirus stock was aliquoted and stored at - 150°C.

Bovine induced pluripotent stem cells generation

To reprogram fetal fibroblasts, the cells were seeded 24h before lentivirus transduction 2×10^4 per well on a 6-wells plate. A fresh medium with lentivirus stock was

added to the culture medium. After overnight transduction, the culture medium was replaced. On day 5 after transduction, the cells were harvested with TrypLE and split into plates with feeder layers and iPSC medium. Also, the culture was performed in two different oxygen levels (5% - low O₂ tension and 20% - high O₂), at 38.5° C, 5% CO₂ in a humidified atmosphere. Three clonal lineages were analyzed for each experimental group.

In vitro embryo production

Bovine ovaries were collected from a local abattoir, and cumulus–oocytes complexes (COCs) were aspirated from selected follicles. Follicles 2-6 mm in diameter were aspirated using a 21-gauge needle. Cumulus-oocyte complexes (COCs) cultured in Tissue Culture Medium 199 (TCM-199) supplemented with 10% FBS, 0.2 mM pyruvate, 50 µg/mL gentamicin, 0.5 mg/mL FSH, and hCG, 5 U/mL (Vetecor, Hertape Calier) for 22 h. The *in vitro* fertilization was performed with frozen-thawed semen from a single Holstein bull. Capacitated sperm were obtained after Percoll gradient (45% and 90%) separation. The IVF was performed in Tyrode/albumin/sodium lactate/sodium pyruvate (IVF-TALP) medium supplemented with 0.6% BSA. Presumptive zygotes were partially denuded after 18 h and cultured in SOF medium in an incubator with a humidified atmosphere of 5% CO₂ and 5% O₂ in air at 38.5 °C for 7 days (fertilization is considered day 0).

Histochemistry and Immunocytochemistry

Alkaline phosphatase activity was detected with an alkaline phosphatase kit (Leukocyte Alkaline Phosphatase kit, cat. # 86R, SIGMA) according to the manufacturer's instructions. For immunofluorescence, the cells were fixed with 4% paraformaldehyde (PFA) in Dulbecco's Phosphate Buffered Saline (DPBS) for 10 min, permeabilized with 1% Triton X-100 in DPBS at 20 min, blocked for 1 h with 1% BSA and 0.3% Triton X-100 in DPBS, and incubated with primary antibody overnight at 4 °C in blocking solution, anti-SOX2 (S9072-100UG, 1:500; sigma), anti-OCT4 (sc-8628, 1:100; Santa Cruz Biotechnology) and anti-NANOG (Ab80892, 1:100, Abcam). The next day, cells were washed with DPBS and 0.05% tween 20, incubated with secondary

antibodies (Alexa Fluor 568-conjugated goat anti-rabbit IgG, Life Tech A-11036, and Alexa Fluor 488-conjugate goat anti-mouse IgG, Life Tech, A-11029) for 1 h at room temperature. Then, cells were washed 3x for 10 min each; on the second wash, 10 ug/ml of Hoescht 33342 were included for 10 min. Samples were mounted on microscope slides with Prolong Gold Antifade Mountant (Life Tech, P36935). Images were captured using EVOS™ digital inverted microscope (Life Technologies).

RNA extraction and Reverse Transcription

All samples were submitted to total RNA extraction using Trizol® protocol (Invitrogen). After extraction, total RNA samples were quantified with a NanoDrop® (Thermo Scientific). For DNA digestion and reverse transcription, we adjusted the concentration to 1.000ng RNA per sample. All samples were submitted to DNA digestion, using a DNase I - Amplification Grade® (Invitrogen). For reverse transcription (RT) of samples, the High Capacity cDNA Reverse Transcription kit was utilized (Applied Biosystems Carlsbad, CA-USA).

Real time qPCR

Relative quantification of transcribed genes was analyzed by real-time qPCR using the PowerUP SybrGreen® PCR Master Mix reagent (Applied Biosystems TM) in the Applied Biosystems QuantStudio 6 equipment. qPCR reactions were run in a volume of 10 µL containing 100 nM of each primer, 1X Power UP SybrGreen (Applied Biosystems), 2.5 µL H₂O, and 1 µL template (four-fold diluted cDNA; 25ng). The primers were designed using the software Primer-BLAST (NCBI) based upon sequences available in GenBank (SM, Table 1). Cycling conditions for amplification were: 95° C for 10 min followed by 45 cycles at 95° C for 15 sec, 57° C for 20 sec, and 60° C for 40 sec. Each sample was analyzed in duplicate for each of the genes. Also, ultrapure DNase and RNase free water were used as a negative control of the reaction. For reference genes, ACTB and PPIA were selected. The relative gene expression was performed by $2^{-\Delta CT}$ [22].

Spontaneous differentiation in embryoid bodies

Spontaneous differentiation of bovine iPSCs through embryoid bodies (EBs) formation was performed in plates treated with agarose 0.1% and iPSCs media without bFGF, bFGF2i, or LIF. After 5 days in suspension culture, EBs were collected for RT-PCR analysis. EBs were placed on gelatin-coated culture plates and cultured in IMDM medium; EBs were documented 7 days after plating.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Data were tested for normality of residuals and homogeneity of variances using the Shapiro–Wilk test and analyzed. Gene expression on biPSCs lines data was analyzed by two-way ANOVA followed by Tukey's post hoc test. Gene expression on EBs data was analyzed by one-way ANOVA followed by Tukey's post hoc test. Differences with probabilities of $P < 0.05$ were considered significant. In the text, values are presented as the means $\pm$ the mean's standard error (S.E.M.). All experiments were repeated at least three times unless otherwise stated.

Results

Influence of low or high oxygen tension and different pluripotent supplements during the reprogramming process of bovine fibroblasts

Colonies were observed on day 14 after transduction, and the colony number was evaluated to analyze the reprogramming efficiency in each experimental group. A higher number of colonies was observed in the culture supplemented with bFL2i; this effect was observed in both oxygen tensions. (Table 2). Additionally, in our experimental conditions, the difference of colonies number in different oxygen tension was not observed.

Six colonies from each experimental group were selected at day 21 post-transduction to expansion and clonal culture. All lines were cultured for at 25 passages (± 200 days), exceptionally the clonal lines derived on bFL2i group in low oxygen

conditions, which lost iPSCs characteristics after 8 passages, suggesting a loss of the self-renewal ability and the maintenance of pluripotency.

Firstly, the morphology and the presence of alkaline phosphatase were evaluated. All biPSCs lines generated in low oxygen or high oxygen tension were small and compact colonies, dome-shaped with clearly defined borders (Figure 1A) and were positive to alkaline phosphatase (Figure 1B). Additionally, we did not observe morphological differences between biPSCs groups (supplement and O₂ tension).

Molecular profile of biPSCs generated in low or high oxygen tension and different pluripotent supplements

For gene expression analysis on biPSCs generated in low oxygen and high oxygen conditions and different supplements, samples were collected in different moments during the reprogramming process as initials passages (5 and 10), intermediate passages (15 and 20), and late passage (25). Targets were pluripotent and metabolism genes, besides exogenous OSKM expression.

The SOX2 relative gene expression varied throughout passages ($p=0.0074$); however, experimental groups did not differ. Relative expression of OCT4 and STELLA was increased on the bFGF group cultured in high oxygen compared to low oxygen tension; in high oxygen, the bFGF supplementation showed higher expression than bFL2i for these genes. However, both genes were decreased after passaging ($p=0.0287$ and $p=0.0367$ for OCT4 and STELLA, respectively) (Figure 2).

SLC2A1 and SLC2A3 (also called GLUT1 and GLUT3, respectively) presented higher relative expression on low oxygen conditions ($p=0.0002$ and $p=0.0014$, respectively). In high oxygen tension, no differences in bFGF x bFL2i supplementation were observed (Figure 2). exogenous expression of mOKSM was reduced throughout passaging ($p=0.0002$); however, groups were not different ($p=0.6745$, Figure 2).

Immunofluorescence revealed that all lineages were positive to Sox2 and Oct4; however, Nanog was not observed in our conditions (Figure 3).

Spontaneous differentiation of biPSCs generated in low and high oxygen conditions and different pluripotent supplements

In vitro differentiation of cells was performed via spontaneous differentiation of embryoid body (EB) formation. All biPSCs lines generated in low and high oxygen conditions and different pluripotent supplements were cultured in suspension for 5 days without supplementation. All lineages were able to produce EBs (Figure 4). Also, these EBs were evaluated regarding ectoderm, mesoderm, and endoderm markers by RT-qPCR.

The relative expression of ectoderm marker (Vimentin - VIM), mesoderm markers (BMP4 and PECAM1) was observed for all lines. However, we did not observe the relative expression of TUBB3 and FOXA2, ectoderm, and endoderm markers, respectively. Interestingly, we observed the relative gene expression of AFP, an endoderm marker, in EBs derived from biPSCs culture in bFL2i on normoxia conditions only. The oxygen tension did not seem to affect the cell differentiation in our experimental conditions; still, we observed higher expression of PECAM1 and VIM on EBs produced from the bFL2i high oxygen tension group (Figure 5).

Additionally, we transferred EBs to gelatin-coated dishes and cultured them for another 7 days with IMDM supplemented with FBS. During the culture, it was possible to identify several different types of cells presenting varying morphology (Figure 6).

Discussion

The oxygen tension can modulate different routes, including pathways connected with maintenance of pluripotency. During the culture of human embryonic stem cells, Ezashi et al. [23] described the 5% O₂ tension described the same effects of hypoxia, acting on the maintenance of two active X chromosomes, an important naïve stem cells characteristic in human ESCs. In iPSCs, the initials studies with low oxygen tension described the enrichment of reprogramming efficiency [19,24,25]. However, reprogramming iPSC cells in a low oxygen atmosphere has not yet been studied in many species, including cattle.

Here, we describe for the first time the comparison between bovine iPSCs generated in low and high oxygen tension. We performed a factorial experiment design of different oxygen tensions (5% x 20%) with different pluripotent supplements as bFGF vs. bFL2i to maintain in vitro bovine reprogrammed cells. In our conditions, we observed 19 (0.95%) colonies in low oxygen and 15 colonies (0.75%) in high oxygen conditions after 14 days to promote the transduction with the mOKSM exogenous vector.

In human and mice, reprogramming efficiency to generate iPSCs was improved in low oxygen conditions [19]. However, De Castro et al. [26] demonstrated, in equines, results similar to our results using bovine cells. In both livestock species, the low oxygen tension did not improve the reprogramming efficiency like were described in humans and mice.

Additionally, our results demonstrated higher initial efficiency using bFL2i supplementation independent of oxygen tension. Pluripotency maintenance using different supplementation can work in many pluripotency pathways, increasing the transcriptional changes during the reprogramming process. In particular, we emphasize and discuss the action of CHIR99021 in the GSK3 pathway, and the consequence is the increment of C-MYC expression, a key gene to increase the proliferation and work in the reprogramming process. Also, LIF works on STAT3 and Pi3K pathways; both pathways promote the pluripotency core (OCT4, SOX2, and NANOG) regulation, working on pluripotency maintenance and self-renewal, two crucial characteristics of PSCs.

Nichols and Smith [16] described, in an elegant review, two different states of pluripotency, naïve and primed, when each state was described with typical colonies morphology. Pluripotent stem cells presenting the naïve profile grow as dome-shaped and compact colonies with well-defined borders; whereas primed profile grow as flat colonies. The first reports of bovine iPSCs in 2011-12 were different for the colonies morphology, when biPSCs showed dome-shaped [27,28] and flat colonies [14,29], however, those authors do not classified that lines for naïve and primed profile. More recently, Kawaguchi et al. [30] demonstrated differences in morphology of the colonies with different supplementations, and bFGF+LIF lineages presented flattened shape, whereas lineages cultured with PD0325901 (MEK pathway inhibitor) changed to dome-

shaped morphology. Also, higher efficiency of colony generation was reported using the combination of bFGF+LIF+2i+forskolin. De Castro et al. [26] also described different profiles on eiPSCs generated in low x high oxygen tension. In our experimental conditions, differences in the biPSCs colonies morphology derived in low or high oxygen, or when derived in different supplementations, were not observed.

The relative gene expression of pluripotency and metabolism targets was evaluated in our conditions. Sumer et al. [29] reported the dependence of endogenous NANOG expression on bovine iPSCs for generation and maintenance of iPSCs from bovine fetal fibroblasts. Controversially, our results generated biPSCs from fibroblasts, and Kawaguchi et al. [30] generated biPSCs from bovine amnion-derived cells, and both reports the endogenous expression of NANOG was not observed. Therefore, endogenous expression of NANOG may not be necessary for the production and maintenance of biPSCs. Also, we observed the endogenous expression of SOX2, OCT4, and STELLA (also known as Dppa3); for these genes, we observed the relative expression decreasing on early to late passages.

Lengner et al. [31] described the inefficiency of different oxygen tension on promote significant changes in the expression of the core pluripotency genes OCT4, SOX2, and NANOG in human ESCs. Herein, we did not observe changes in SOX2 and OCT4 expression when cultured in low or high oxygen tension. Another possibility to explain this inefficiency on the pluripotency core transcription are the oxygen levels, probable lower oxygen levels (2% of oxygen) can promote the pluripotency core transcription regulation.

The detailed description of time-lapse gene expression analyzes during the reprogramming process is still an empty space in recent literature. Several species-specific differences could influence the response of oxygen tension and the reprogramming process. In our conditions, we maintained pluripotent-like cells for at least 25 passages in both oxygen conditions.

In low O₂, conditions GLUT1 and GLUT3 expression were upregulated in our biPSCs. GLUT3 demonstrated a higher affinity for glucose than GLUT1 and has a high turnover, making it an efficient transporter [32]. Also, in mouse embryos, GLUT3 is the functional transporter for maternal glucose, and the GLUT3 expression is required for

blastocyst formation [33]. Similar our result, Harvey et al. [34] described the same upregulation in GLUT1 expression in bovine blastocysts cultured under 2% oxygen than blastocysts cultured under 7% and 20% oxygen. Also, in *in vitro* hypoxia condition (2% oxygen), the GLUT1 expression was similar to *in vivo*-derived embryos. Controversially, the low oxygen tension (5% oxygen) decreased the GLUT3 expression in equine iPSCs and human embryonic stem cells [26,35]. These controversial results demonstrated the differences in species-specific effects of low oxygen tension on metabolism pathways.

It is important to stress that most of the iPSC lines established in non-rodent species, especially in livestock species, are dependent on continuous transgene expression to maintain their pluripotency [4,36]. In bovine iPSCs, Kawaguchi et al. [30] described the same characteristic on naïve-type biPSCs under the control of transgenes; and after Dox removal, biPSCs readily changed their morphology and no longer expressed both exogenous and endogenous pluripotent genes. More studies are necessary to optimize the culture conditions for the establishment and maintenance of ESCs, and naïve iPSCs vary among species to resolve these challenges on livestock species.

In our conditions, one biPSCs line was positive for differentiation in all three embryonic tissues. This lineage was cultured in high O₂ tension and with bFL2i. These spontaneous differentiation results may present an incomplete ability of our EBs to express ectoderm, mesoderm, and endoderm markers after the reprogramming process in our conditions, probably due to insufficient period of differentiation *in vitro* or transgene expression. Indeed, we underline that however the low oxygen condition was described in mice cells as an enhancer to promote differentiation in the endoderm lineage [37], the same effects were not found in bovine EBs derived from biPSCs produced and cultured in low O₂ condition.

Finally, the acquisition and maintenance of pluripotency *in vitro* offers an important tool to improve early development knowledge. Our findings showed for the first time the biPSCs generated in low oxygen tension conditions. Also, the oxygen tension may lead to less pronounced differences in *in vitro* pluripotency maintenance in biPSCs when compared to previous reports on humans and mice. Importantly, in our conditions, it is evident that bFGF combined with LIF and 2i did not favor pluripotency

maintenance at 5% O₂. In addition, our findings are relevant for demonstrated the combination of oxygen and different supplementations sets impact the bovine iPSCs.

Declarations

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Authors' contributions

BWB and RCB carried out the design of the study, its execution, and writing. NCGP, LSM, JBC, PM, AFS, and KR participated in its execution and helped to draft the manuscript. FFB and MFGN participated in its design and coordination. The authors read and approved the final manuscript.

Conflicts of Interest

All other authors of this manuscript have nothing to declare and no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data Availability Statement

All data presented in this study is present within the manuscript.

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Tables

Table 1. The general list of primers used to characterize pluripotency and EBs differentiation.

Primer	Transcript id	Foward (5' → 3')	Reverse (5' → 3')	Product size
ACTB	NM_173979.3	CAGCAGATGTGGATCAGCAAGC	AACGCAGCTAACAGTCCGCC	89
PPIA	NM_178320.2	CATACAGGTCTGGCATC	CACGTGCTTGCCATCCAA	107
NANOG	NM_001025344.1	CTCAGCTACAAGCAGGTGAAGA	ACACCCCTGGTGGTAGGAAT	153
OCT4	NM_174580.2	GCAAACGATCAAGCAGTGACTAC	GGCGCCAGAGGAGAGGATACG	93
SOX2	NM_001105463.2	ATGGGCTCGGTGGTGAAGT	TGGTAGTGCTGGGACATGTGA	178
STELLA	NM_001111109.2	AGTGAGCGGAGGTACAGGAT	TCGCACTCTTGATCGAATCTCA	132
GLUT1	NM_174602.2	ATCCTCATTGCCGTGGTGCT	ACGATGCCAGAGCCGATGGT	133
GLUT3	NM_174603.3	CGCCTTTGGCACTCTCAAC	GCACTGGATGATGGCTGGTAA	88
TUBB3	NM_001077127.1	GGATAGACCCCACTGGCAAT	TTGTGTGAAGAAGCCTCGTTG	88
VIM	NM_173969.3	CTCCTACCGCAGGATGTTCG	TGGATGTGGTCACGTAGCTC	140
PECAM1	NM_174571.3	AATCAGAGCGTGGGCTCAAA	ATCCACTGGGGCTATCACCT	147
BMP4	NM_001045877.1	AGCTTCCACCACGAAGAACAT	CACCTCGTTCTCTGGGATGC	102
AFP	NM_001034262.2	CGGACCTTCCGAGCCATAAC	CTCTTTCCCCATCCTGCAGAC	154
FOXA2	XM_025001047.1	CGAGCCCGAGGGCTACTC	GTACGTGTTTCATGCCGTTCA	92

Table 2. Number of bovine induced pluripotent stem cells colonies on day 14 after the transduction derived in low and high oxygen tension and cultured with bFGF or bFL2i supplementation.

D14	Low oxygen	High oxygen	total
bFGF	4	1	5
bFL2i	15	14	29
total	19	15	

Figures

Figure 1.

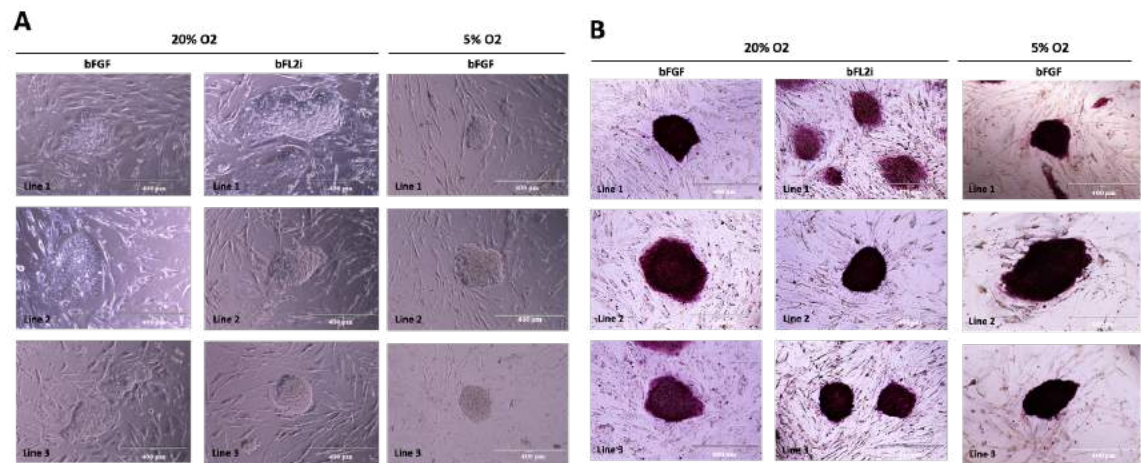


Figure 2.

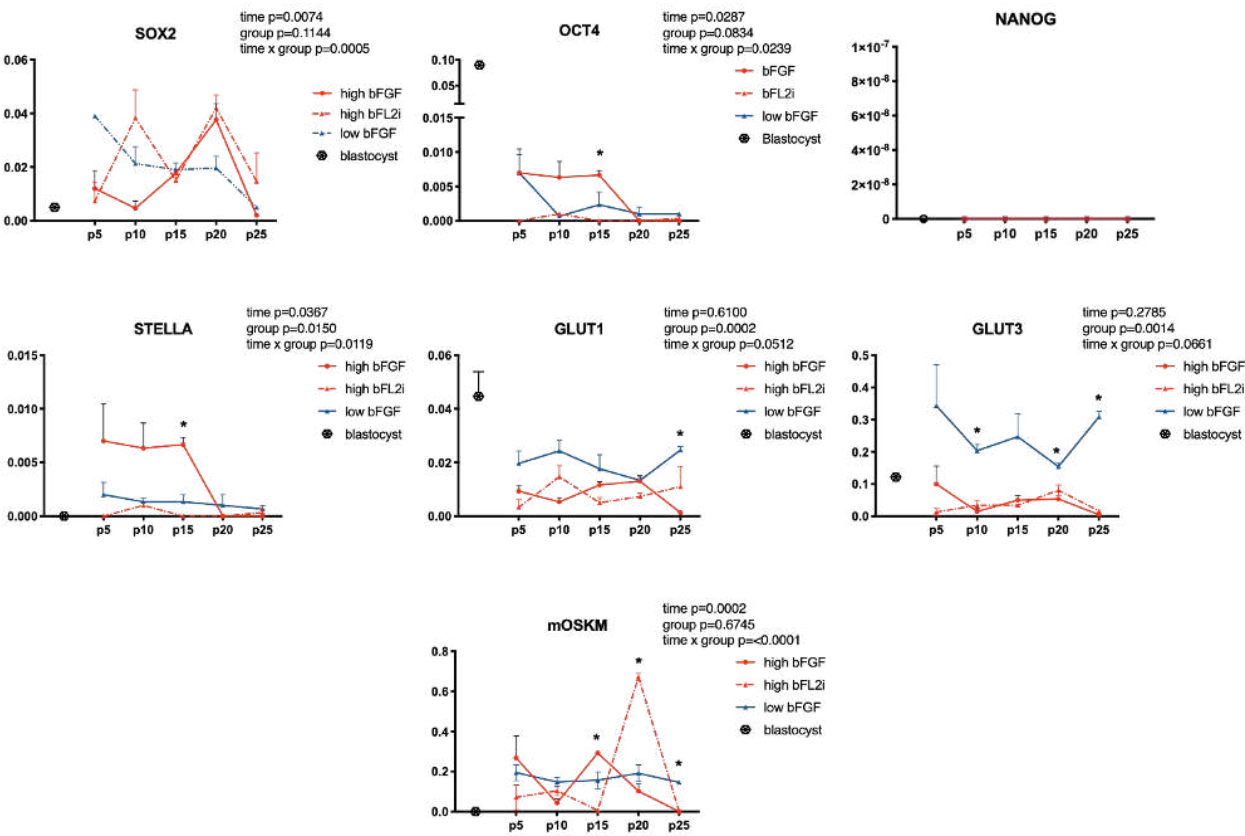


Figure 3.

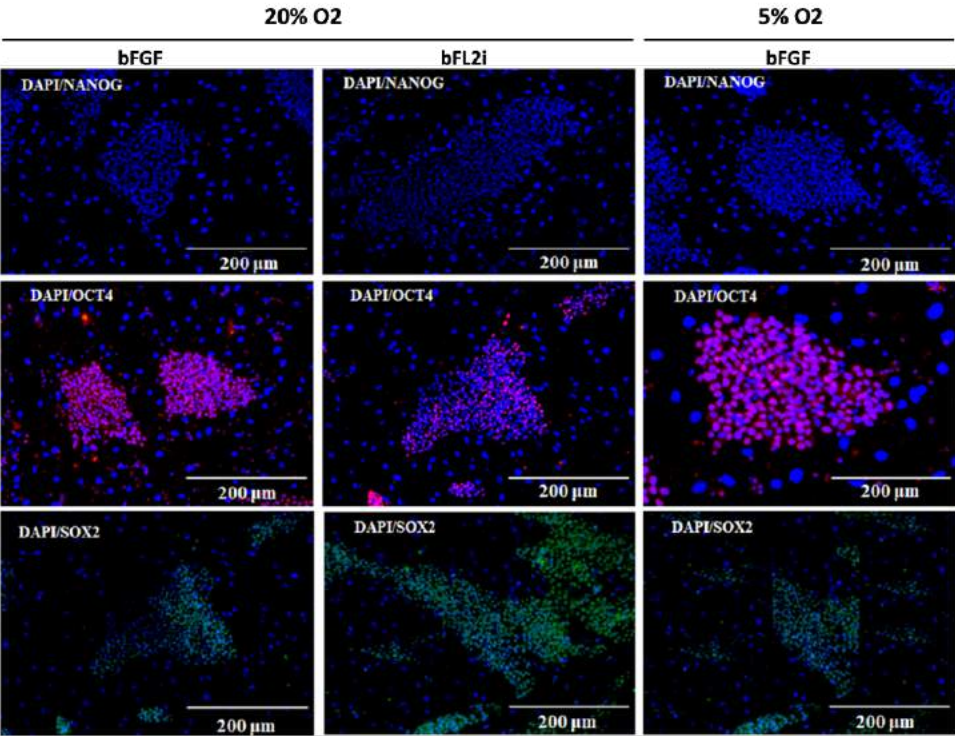


Figure 4.

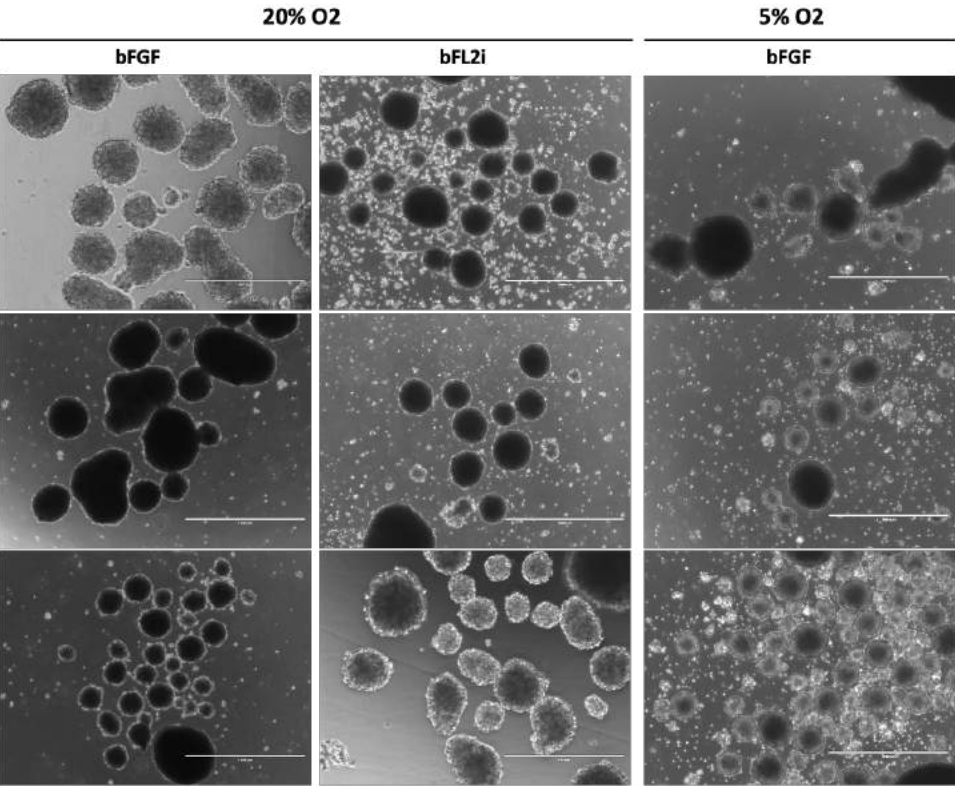


Figure 5.

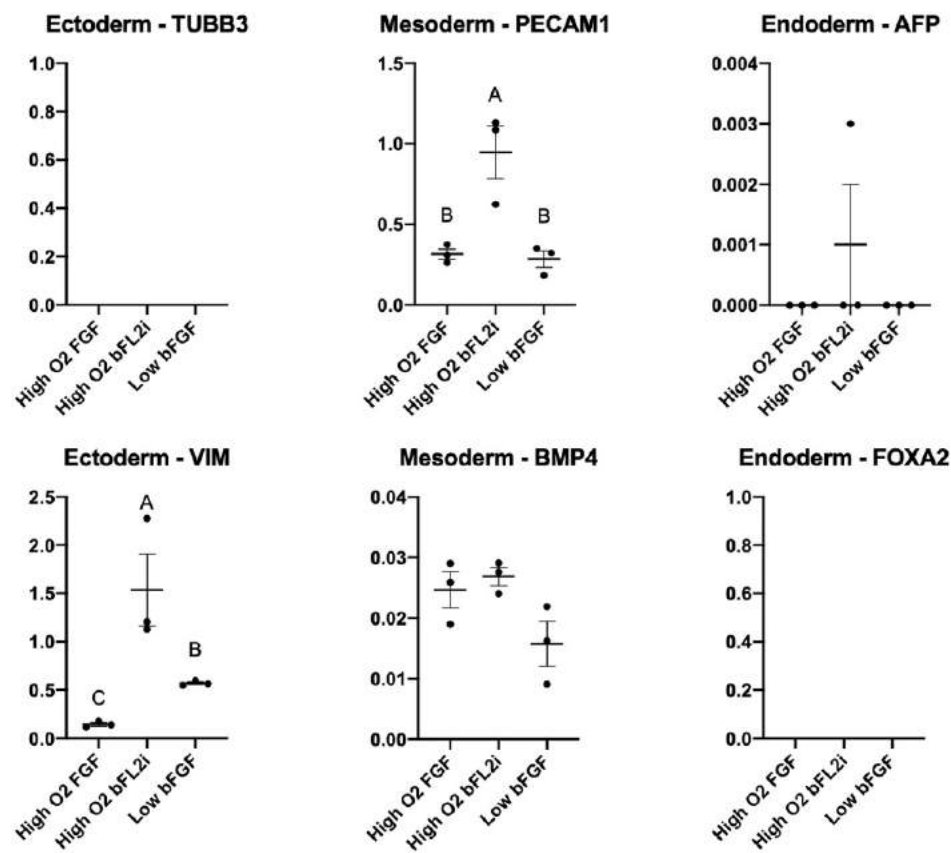
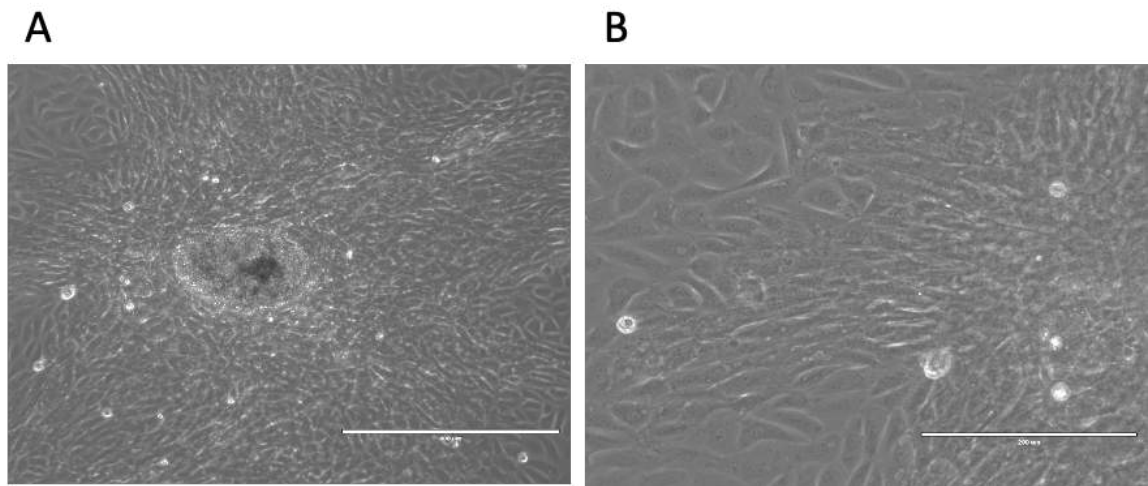


Figure 6.



Legends of figures

Figure 1. Bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i). A. Colonies morphology. B. Alkaline phosphatase presence. Magnification 10x, Scale bars: 400 μ m.

Figure 2. Gene expression to SOX2, OCT4, NANOG, STELLA (pluripotent markers), GLUT1 and GLUT3 (metabolism pathway) and mouse OKSM (exogenous vector) in bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i). Statistical differences in time, group effect and the interaction between time*group were showed in each gene. Statistical differences on groups in the same passage were present with *.

Figure 3. Immunostaining to Nanog (red), Oct4 (red) and SOX2 (green) in bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i). The nucleous were stained with DAPI (blue). Magnification 10x, scale bars: 200 μ m.

Figure 4. Embryoid bodies produced from bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i).

Figure 5. Gene expression to TUBB3 and VIM (ectoderm markers), PECAM1 and BMP4 (mesoderm markers) and AFP and FOXA2 (endoderm markers) in embryoid bodies produced from bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i). Statistical differences were presented by different letters.

Figure 6. Embryoid bodies produced from bovine induced pluripotent stem cells derived in different oxygen tensions (low x high oxygen tension) and pluripotent supplements (bFGF x bFL2i) cultured during 5 days on gelatin coated plate. A, Magnification 4x, scale bar: 400 μ m. B, magnification 10x, scale bar: 200 μ m.

Chapter 3: Acquisition and maintenance of pluripotency are influenced by fibroblast growth factor, leukemia inhibitory factor and 2i on bovine-induced pluripotent stem cells

Submit to: **Stem Cell Research & Therapy**

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Abstract

Background: Several insights on embryo development, stem cells, cell fate and cell differentiation have emerged using induced stem cells (iPSCs). However, in the literature the difficulty to compare iPSCs with genuine ESCs keep hard. Here, we present a systematic molecular profile comparison of bovine induced pluripotent stem cells (biPSCs) lines derived from fetal fibroblasts and bovine embryonic stem cells (bESCs).

Methods: biPSCs lines were generated by expression of exogenous by the lentiviral transduction of the mouse OCT4, SOX2, KLF4, and c-MYC vector (mOSKM). The lines were derived using either bFGF and LIF in combination/or not with PD0325901 and CHIR9902. Additionally, bESC were also isolated and maintained and analyzed for comparison to the generated iPSCs. All the lines were tested for known pluripotent colonies' characteristics as alkaline phosphatase (AP) and gene expression to SOX2, OCT4, NANOG, ESRRb, STELLA, LIFr, OTX2, FGF5. The presence of Oct4, Sox2, Nanog, Gata6, and H3k27me3 were also evaluated by immunostaining. bPSCs were tested for embryonic bodies (EBs) formation and differentiation. Additionally, biPSCs and bESCs were injected on early embryos to assess the potential to contribute on chimeric blastocysts.

Results: It was possible to generate biPSCs in all groups. However, LIF2i treatment did not survive after the passage 8. All biPSCs and bESCs were to AP+ and expressed OCT4, SOX2, STELLA, ESRRb and LIFr (pluripotent and naïve markers). However, the relative expression detected by RT-qPCR decreased during the culture development. The same profile was observed on mOSKM expression. biPSCs and bESCs were positively stained for Oct4 and Sox2 proteins in each line, but Nanog was not observed. Additionally, one line of biPSCs cultured with bFGF2i supplement showed the presence of Gata6. The H3k27me3 presence was observed in all biPSCs lines, however, bFGF treatment reduced the H3k27me3 fluorescence intensity. All biPSCs lines were able to differentiate into EBs. The EBs showed ectoderm, mesoderm and endoderm gene expression. biPSCs and bESCs were able to contribute to chimeric blastocysts, also, the contribution rates of biPSCs was higher than bESCs.

Conclusion: Bovine iPSCs were generated by mOSKM on bFGF and mLIF combined with/ or not 2i. Also, the bFGF was more efficient in promoting the acquisition and maintenance of pluripotency. Additionally, the biPSCs are more efficient than bESCs.

Keywords: cattle, pluripotent cells, embryonic stem cells, cellular reprogramming, growth factors.

Background

In 2006, Shinya Yamanaka's group [1,2] reported that somatic cells could be reprogrammed into pluripotent stem cells (PSCs; e.g., induced pluripotent stem cells, iPSCs) by retroviral overexpression of four embryonic transcription factors: OCT4, SOX2, KLF4, and C-MYC (OSKM). These cells were proven to be self-renewing and could be derived from almost any type of differentiated cell, providing researchers with a valuable source of pluripotent stem cells. These iPSCs made new approaches to stem cell research, including the generation of patient-specific stem cells for regenerative disease modeling and autologous transplants possible. More recently, the generation of iPSCs from a wide range of species was achieved, including cow [3–5], sheep [6,7], equine [8,9] and pig [10–12].

In bovine, iPSCs were described using different delivery systems, base media and growth factors [4,13–20]. Also, the potential of cells differentiation is assessed by in vitro and in vivo approaches. This diversity of strategies coupled with the complexity of iPSCs make it challenging to compare the results obtained by different researchers. Additionally, the acquisition and maintenance of pluripotency on iPSCs derived from livestock species has been challenging, especially because comparisons with genuine embryonic stem cells are still unpublished [21,22].

The pluripotent stem cells (iPSCs or embryonic stem cells, ESCs) can be classified into two classic categories, namely naïve and primed. Cells classified as naïve correspond to cells in the inner cell mass (ICM) of blastocysts, while primed are similar to epiblast in the post-implantation stage [24–26]. Among the ESCs, it is well known that the murine ESCs since these cells are dependent on the leukemia inhibitory factor (LIF), 2i (MEK/ERK (PD0325901) and GSK-3 β (CHIR99021) inhibitors) for growth in culture and correspond to the naive state of pluripotency. However, human ESCs

resemble murine epiblast cells and are dependent on fibroblast growth factor 2 (FGF 2 or bFGF), being classified as primed in pluripotency [24].

The first report of bovine ESCs was published by Saito et al. [27]. In that moment, the protocol to isolate the bESCs was performed with MEM-Alpha supplemented with 10% FCS, 2-mercaptoethanol, high glucose and HEPES-buffer combine with MEM previously incubated on cell line 5637 containing LIF. The absence of define medium and specific growth factors increased the difficult to establish and maintenance of bESCs in in vitro culture and this difficult persisted during many years. Recently, bESCs establishment were described with efficient by Bogliotti et al. [28] using bFGF and IWR-1 inhibitor on customized mTsER-1 medium. Those cells were classified as primed by molecular characteristics.

The “gold standard” assay to classify PSCs on naïve state is the capacity of the cells contribute on chimeric embryos, contrarily, primed cells do not have this capacity [25]. Newly, Yu et al [23] demonstrated a “new” state of pluripotency with capacity to contribute on chimeric embryos, called intermediate state. This described state shows the opportunity to use those cells on direct primordial germ cells-like cells induction in vitro and can contribute to chimeras.

Here, we present a systematic comparison of biPSC lines derived from fetal fibroblasts and bESCs. The lines were derived using either bFGF and LIF in combination/or not with 2i. Characterization of the resultant bovine PSCs lines included assessment of pluripotency marker expression by alkaline phosphatase, transcripts abundance by qPCR and immunocytochemistry, as well the potential of differentiation was observed by embryoid bodies formation and chimeric blastocysts production. The availability of bovine PSCs, open new opportunities to genome editing, production of transgenic animals, genomic selection and other opportunities for reproductive biotechnology.

Material and Methods

Protocols were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (protocol number 2913/2013), and by the Ethics Committee on the Use of Animals of the Faculty

of Animal Science and Food Engineering, University of São Paulo, Brazil (protocols number 3526250717 and 2192250918).

Cells and Media

Mouse embryonic fibroblasts (MEFs), bovine fetal fibroblasts (bFFs) and 293FT cells were cultured in complete Iscove's Modified Dulbecco's Medium (IMDM) [12200036; Gibco Thermo Fisher Scientific] supplemented with 10% fetal bovine serum (FBS) [SH30071.03, GE Healthcare Life Sciences], 1% GlutaMAX [35050061, Gibco Thermo Fisher Scientific], 1% MEM non-essential amino acids solution [11140076, Gibco Thermo Fisher Scientific] and 1% penicillin/streptomycin [15140122, Gibco Thermo Fisher Scientific]. Derived biPSCs were cultured in KnockOut DMEM-F12 [12660012, Gibco Thermo Fisher Scientific] supplemented with 20% KnockOut Serum Replacement [10828028, Gibco Thermo Fisher Scientific], 1% GlutaMAX [35050061, Gibco Thermo Fisher Scientific], 1% MEM non-essential amino acids solution [11140076, Gibco Thermo Fisher Scientific], 1% penicillin/streptomycin [15140122, Gibco Thermo Fisher Scientific] and 1:1000 β -mercaptoethanol [21985023, Gibco Thermo Fisher Scientific].

Feeder cells

EmbryoMax® primary mouse embryonic fibroblasts (PMEF-CF, Sigma-Aldrich) were cultured on complete Iscove's Modified Dulbecco's Medium (IMDM) [12200036; Gibco Thermo Fisher Scientific] supplemented with 10% fetal bovine serum (FBS) [SH30071.03, GE Healthcare Life Sciences], 1% GlutaMAX [35050061, Gibco Thermo Fisher Scientific], 1% MEM non-essential amino acids solution [11140076, Gibco Thermo Fisher Scientific] and 1% penicillin/streptomycin [15140122, Gibco Thermo Fisher Scientific]. Cells were treated with mitomycin C (47589, Millipore) and used as a feeder monolayer at a concentration of 1×10^5 cells in 6 well dishes. The dishes were treated with 0,1% gelatin previously to seeding.

Lentivirus production

Lentiviral production was performed as described before [14]. Briefly, 5x10⁶ HEK-293FT cells were seeded in t75 cm² flasks and maintained in IMDM with 10% FBS overnight. The STEMCCA vector (12ug) containing the murine transcription factors OCT4, SOX2, KLF4 and c-MYC [29] and the auxiliary plasmids (1.2 µg TAT, 1.2 µg REV, 1.2 µg Hgpm2 and 2.4 µg VSVG), additionally, FUGW vector (5 µg) [30] and the auxiliary plasmids (1.2 µg pLP1, 1.2 µg pLP2 and 2.4 µg VSVG. Virapower lentiviral expression system, Thermo Fisher) were transfected using Lipofectamine 3000 reagent (Invitrogen) following manufacturer's suggestions. The media containing the lentivirus particles were recovered. The supernatant was filtered (0.45 µm membrane, Millipore SCHVU01RE). The ultracentrifuge was performed by 48,960 G, by 90 minutes at 4° C. The supernatant was removed and leave ~100 µL per tube was used to resuspend the lentivirus stock. The lentivirus stock was split in aliquots and store at - 150° C.

Bovine fibroblasts and induced pluripotent stem cells production (biPSC)

Bovine fetal fibroblasts were obtained from 40-45 days old male fetus and cultured in IMDM supplemented with 10% of FBS, 1% non-essential amino acid solution, and 1% pen/strep. For the reprogramming protocol, the isolated fibroblasts were seeded 24h before lentivirus transduction on 0.1% gelatin-coated wells of a 6-well plate at a density of 2x10⁴ cells/well in IMDM medium. A fresh medium with lentivirus stock was added to the culture medium, incubated overnight, and replaced by a lentivirus-free medium the following day. On day 5 after transduction, the cells were harvested with TrypLE and transferred into mitotic inactivated, by mitomycin-C, mouse embryonic fibroblasts (MEFs) feeder layers with iPSC medium, supplemented with 10ng/mL recombinant human FGF-basic [100-18B, Peprotech] or ESGRO® Recombinant Mouse LIF Protein [ESG1106 - Millipore] associated or not with 2i (1mM PD0325901 [444968, Sigma] and 3mM CHIR99021 [361571, Sigma]) at 38.5° C, 5% CO₂ and 5% O₂ in a humidified atmosphere. Additionally, biPSCs were frozen once they are ~70% confluent using cryopreservation medium, which contains 90% iPSCs medium and 10% (vol/vol) DMSO.

Bovine *in vitro* embryo production

Bovine ovaries were collected from a local abattoir, and cumulus–oocyte complexes (COCs) were aspirated from selected follicles. Follicles 2–6 mm in diameter were aspirated using a 21-gauge needle. COCs were cultured in Tissue Culture Medium 199 (TCM199) supplemented with 10% FBS, 0.2 mM pyruvate, 50 µg/mL gentamicin, 0.5 mg/mL FSH and 5 IU/mL hCG (Vetecor, Hertape Calier) for 22 h. The *in vitro* fertilization was performed with frozen-thawed semen from a single Holstein bull. Capacitated sperm were obtained after Percoll gradient (45% and 90%) separation. The IVF was performed in Tyrode/albumin/sodium lactate/sodium pyruvate (IVF-TALP) medium supplemented with 0.6% BSA. Presumptive zygotes were partially denuded after 18 h and cultured in SOF medium in an incubator with a humidified atmosphere of 5% CO₂ and 5% O₂ in air at 38.5 °C for 7 days (insemination was considered as day 0).

Bovine embryonic stem cells isolation and culture (bESC)

Individual whole blastocysts (day 7) were placed in separate wells of a 24-well dish seeded with a monolayer of inactivated MEFs. They were cultured in N2B27 medium supplemented with 1% of BSA, 20 ng/mL bFGF, and 2.5 µM IWR1, and were incubated at 38.5 °C and 5% CO₂. Outgrowths (after 6–7 days in culture) were dissociated and passaged using TrypLE express and reseeded in the presence of the Rho kinase (ROCK) inhibitor Y-27632 (10 µM) into newly prepared wells containing MEFs and fresh medium. Once established, bESC lines were grown on the same conditions and were passed every 3–5 days. To increase cell survival in the replat, the ROCK inhibitor Y-27632 was added to the fresh culture medium for 24 h until the next media change. The medium was changed daily.

Histochemistry and Immunohistochemistry

Alkaline phosphatase activity was detected with an alkaline phosphatase kit (Leukocyte Alkaline Phosphatase kit, cat. # 86R, SIGMA) according to the manufacturer's instructions. For the immunofluorescence staining, cells were fixed with 4% paraformaldehyde (PFA) in DPBS for 10 min, permeabilized with 1% Triton X-100 in Dulbecco's Phosphate Buffered Saline (DPBS) for 20 min, blocked for 1 hour with 1%

BSA and 0.3% Triton X-100 in DPBS, and incubated with primary antibody overnight at 4 °C in blocking solution, anti-Sox2 (Ab97959, 1:300; Abcam), anti-Oct4 (sc-8628, 1:300; Santa Cruz Biotechnology), anti-Nanog (AB80892, 1:250, Abcam), anti-Gata6 (Ab175349, 1:250, Abcam) and anti-trimethyl-Histone H3 (Lys27), also known as Anti-H3K27me3 (07-449, 1:300, Millipore). The next day, cells were washed with PBS and 0.05% tween 20, incubated with secondary antibodies (Alexa Fluor 568-conjugated goat anti-rabbit IgG, Life Tech A-11036, and Alexa Fluor 488-conjugate goat anti-mouse IgG, Life Tech, A-11029) for 1h at room temperature. Then, cells were washed 3x for 10 min each; on the second wash, 10 µg/mL of Hoechst 33342 were included for 10 min. Samples were mounted on microscope slides with Prolong Gold Antifade (Life Tech, P36935). Images were captured using a confocal microscope (TCS-SP5 AOBS; Leica, Germany) using laser excitation and emission filters specific for Alexa 488 and Alexa 568. Digital images were analyzed by evaluating each nucleus fluorescent intensity using ImageJ-Fiji image processing software (National Institutes of Health, Bethesda, MD, USA). The fluorescent intensity (average mean gray value) of each channel was measured by manually outlining each nucleus and adjusting it against the background.

RNA extraction and Reverse Transcription

All samples were submitted to total RNA extraction using Trizol® protocol (Invitrogen). After extraction, total RNA samples were quantified with NanoDrop® (Thermo Scientific). For DNA digestion and reverse transcription, we adjusted the concentration to 1.000 ng RNA per sample. All samples were submitted to DNA digestion using a DNase I - Amplification Grade® (Invitrogen). For reverse transcription (RT) of samples, the High-Capacity cDNA Reverse Transcription kit was utilized (Applied Biosystems Carlsbad, CA-USA).

Real time qPCR

Relative quantification of transcribed genes was analyzed by qPCR using the PowerUP SybrGreen® PCR Master Mix reagent (Applied Biosystems TM) in the Applied Biosystems QuantStudio 6 equipment. qPCR reactions were run in a volume of 12 µL containing 100 nM of each primer, 1X Power UP SybrGreen (Applied

Biosystems), 2.5 μ L H₂O, and 1 μ L template (four-fold diluted cDNA; 25ng). The primers were designed using the software Primer-BLAST (NCBI) based upon sequences available in GenBank (Supplementary data, Table 1). Cycling conditions for amplification were: 95° C for 10 min followed by 45 cycles at 95° C for 15 sec, 57° C for 20 sec, and 60° C for 40 sec. Each sample was analyzed in duplicate for each of the genes. Also, ultrapure DNase and RNase free water were used as a negative control of the reaction. For reference genes, ACTB and PPIA were used. The relative gene expression was performed by $2^{-\Delta Ct}$ [31].

In vitro differentiation

Spontaneous differentiation of bovine iPSCs through embryoid bodies (EBs) formation was performed in plates treated with agarose 0.1% and iPSCs media without bFGF, bFGF2i, or LIF. After 5 days in suspension culture, EBs were plated on 0.1% gelatin-coated wells after 7 days and grown in complete DMEM the cells were collected for RT-qPCR analysis.

Generation of GFP+ lines from bovine iPSCs and ESCs

Briefly, bovine iPSCs or ESCs were splitted into new plates (12 wells plate), in that moment, we added 50 μ L of FUGW lentivirus stock in the medium. The medium was changed daily. After 72 hours of transduction, bovine PSCs were evaluated using EVOS™ digital inverted microscope (Life Technologies). GFP positive colonies were manually picked and transferred into new feeder MEFs plates to obtained GFP biPSCs and GFP bESCs lines.

Preparation and bovine PSCs contribution to chimeric blastocysts

The embryos produced in vitro with 108 hours after insemination (day 4.5) that have obvious more than 32 cells at were used and defined as morula. Bovine GFP iPSCs or GFP bESCs were added to 80 μ L drop of TCM-199 medium under mineral oil containing the embryos to be injected. Single GFP cells were collected into a 20 μ m injection micropipette. Five-eight cells were introduced into the morula's perivitelline space. Groups of 30 embryos were manipulated simultaneously and each session was

limited to 30 min. After microinjection, the injected embryos were cultured in 1:1 (SOFaa : biPSCs or bESCs medium) medium until the day 7 of the embryos development in the same conditions previously described. The contribution of GFP bovine PSCs was evaluated using EVOS™ digital inverted microscope. Only blastocysts with GFP cells colocalized with ICM were classified as a chimeric contribution.

Statistical analysis

The general linear model procedure in the SAS software program was used to analyze data from all experiments. We also used the Kolmogorov-Smirnov test to check the normality of the data. If the data was anormal, logarithmic transformation was applied to obtain a normal distribution. Significant differences were determined using Tukey's multiple range test for gene expression and Kruskal-Wallis for fluorescence intensity, with probability (P)values < 0.05 considered significant. In the text, values are presented as the means $\pm$ the standard error of the mean (S.E.M.).

Results

Generation and initial characterization of biPSCs

Transduced bovine fetal fibroblasts were plated on the MEF feeder layers and cultured in bFGF and LIF with or without 2i. Morphology changes on bovine cells were detected on day 13-15 after the transduction. On day 21, all colonies were counted, and 5 colonies for each group were expanded by manually picking colonies (except for the LIF2i group, we obtained 3 colonies on the reprogramming plate). bFGF supplementation was more efficient in promoting colony formation than LIF; additionally, 2i plus reduced the number of colonies in both combinations compared to bFGF and LIF (Fig. 1A).

Until the third passage, two LIF2i colonies did not grow, suggesting a loss of the self-renewal ability and the inefficiency of LIF2i to support the reprogramming process. For pluripotency characterization three colonies from each group (bFGF, bFGF2i, and LIF) and one colony from LIF2i were used. In passage 3, all colonies were tested for alkaline phosphatase activity, and they were positive as expected; the bESCs line was also AP positive (Fig. 1B).

The colony cultured in LIF2i did not grow after passage 15, suggesting a loss of the self-renewal ability and inefficiency of LIF combined with 2i to maintain biPSCs pluripotency. All biPSCs lines showed faster proliferation rates, higher clonogenic capacity, indicative of improved self-renewal; additionally, all colonies cultured on bFGF, bFGF2i, and LIF were culture until passage 30. These biPSCs colonies exhibited morphologies typical for naïve stem cell colonies, grew as small, dome-shaped colonies, similar to mESC colonies. Additionally, we observed dome-shaped colonies on the bESCs during the initial passages (passage 3) (Fig. 1C).

Gene expression and Immunohistochemistry profile on biPSCs

To analyze the reprogramming process and identify the differences from the different pluripotency maintenance agents during the biPSCs production, we analyzed all lines isolated on bFGF, bFGF2i, and LIF at different passages (5, 10, 15, and 25) by qPCR using expression of key endogenous pluripotency factors, cell differentiation markers and the mOSKM cassette as targets. Additionally, we included the bESCs line expression in passage 5 for the same genes (Fig. 2).

Interestingly, the expression of SOX2 and OCT4 for bESC and all biPSCs lines on passage 5 were equivalent. After 10 passages, was observed the levels of both genes decreasing with the passages on the biPSCs lines ($p < 0.001$, for both genes), except for the bFGF group on SOX2 levels. This group showed the capacity to maintain higher levels of expression even on late passages. Additionally, we did not observe the expression of NANOG in bESCs. Interestingly, we did identify a low NANOG expression in the biPSCs lines from the bFGF group until the passage 15 ($p = 0.0006$), but at the next analyzed stage (passage 25), was not detected. The LIF group lines showed a higher NANOG expression at passage 10, but the expression did not persist over the following passages.

To better understand the pluripotent profile of the generated biPSCs, we tested our lines for naïve and primed markers, such as ESRRb, STELLA, and LIFr (naïve) and OTX2 and FGF5 (primed state). All lines expressed ESRRb, STELLA, LIFr, and OTX2. However, the ESRRb, STELLA, and OTX2 levels decreased the relative expression on late passages ($p = 0.0267$, $p < 0.0001$, and $p = 0.0002$, respectively). Interestingly, ESRRb

was not detected in bESCs. Also, the LIFr expression differed between the passages ($p=0.0191$), but there was no difference in the groups ($p>0.05$). Additionally, we tested our lines for FGF5, a gene expressed on primed human iPSCs. We detected a low expression in these biPSCs lines.

Finally, we evaluated the exogenous expression of the OSKM vector, observed not only in early passages but also in late passages. However, the levels of mOSKM decreased on late passages ($p<0.001$); these results suggest these biPSCs lines have a persistent expression of the exogenous vector.

Characterizing the pluripotent protein profile of these biPSCs, we tested all lines after passage 25 for pluripotent biomarkers (OCT4, SOX2 and NANOG) and differentiation marker (GATA6) by immunofluorescence. All biPSCs lines showed pluripotency markers such as OCT4 (or POU5F1) and SOX2. As well, the presence of OCT4 and SOX2 were observed on the bESCs. We did not observe the presence of NANOG on biPSCs and bESCs. To identify cells/lines starting the differentiation, we checked for the presence of GATA6. Interestingly, we identified a few colonies expressing GATA6 in one line from bFGF2i culture (Fig. 3A).

The immune assay of the transcriptional silencing associated marker trimethylation of histone H3 lysine 27 (H3K27me3) was performed (Fig. 3A and 3B). All lines of biPSCs were positive for H3K27me3; also, we observed a difference of H3K27me3 levels between the groups ($p < 0.0001$). These results corroborate with RT-qPCR data, suggesting these biPSCs were not fully reprogrammed and that the bFGF lines were closer to the primed state of pluripotency than the other groups.

Differentiation potential of bovine iPSCs and bESCs

Induced pluripotent stem cells provide an excellent opportunity for the differentiation of any tissue. For this purpose, we cultured our lines on low-adhesion culture dishes for 5 days allowing them to form EBs to investigate if the cells can differentiate into three germ layers (ectoderm, mesoderm, and endoderm). All biPSCs lines could form spherical structures with defined borders within 24 hours of being placed in suspension culture (Fig. 4A).

Thus, EBs were cultured in coated-gelatin dishes, after 7 days of culture in IMDM medium different cells morphology were identified growing around the EBs (Fig. 4B). Additionally, these cells were evaluated the in vitro differentiation potential by RT-qPCR using specific markers such as Vimentin (VIM) and Tubulin beta 3 (TUBB3) for ectoderm, Platelet endothelial cell adhesion molecule (PECAM1), and Bone morphogenetic protein 4 (BMP4) for mesoderm, Alpha-fetoprotein (AFP) and Forkhead box A2 (FOXA2) for endoderm. It was possible to identify ectoderm (VIM) expression, mesoderm (PECAM1 and BMP4) markers and endoderm markers (AFP and FOXA2). Interestingly, two lines from bFGF and all LIF lines did not express AFP (Fig. 4C).

We tested biPSCs supplemented with bFGF and bESCs lines on the gold standard assay to PSCs, whether our biPSCs and bESCs had potential to contribute on chimeric embryos. To track our PSCs, we generated biPSCs and bESCs lines expressing green fluorescence protein (GFP) (Fig. 5A). Around 5-10 bovine GFP biPSCs and GFP bESCs were injected in each early embryo (day 4.5) (Figure 5B) and the contribution was evaluated on day 7 of the embryo development. For GFP bESCs, we observed only two blastocysts with GFP bESCs contributing on the ICM, around 3% of contribution in all injected early embryos (Fig. 5D). Additionally, for biPSCs we observed 38 blastocysts with GFP biPSCs on the ICM region (Fig. 5C), around 20% of contribution (Fig. 5D).

Discussion

Here, we presented the first factorial design of biPSCs derived from fetal fibroblasts reprogrammed with mOSKM lentivirus cultured with bFGF or mLIF combined or not with 2i (PD0325901 and CHIR99021). Additionally, we characterized the generated iPSC for EB formation, gene expression, and immunofluorescence staining and compared it to bESC.

Using the polycistronic STEMCCA cassette into bovine fetal fibroblast's genome, was possible generate colonies after 15 days using DMEM F12, KO and KSR supplemented with bFGF or mLIF combined or not with 2i. When we compare the efficiency of colony generation, it is possible to identify that the best efficiency was with bFGF during the reprogramming process (Supplementary data, Fig. 1). Using different

reprogramming factors and delivery systems, Heo et al. [3] and Kawaguchi et al. [18] demonstrated the same window, around 14 days to identify colonies, similar to our results. Contrarily, Huang et al. [4] using bovine OSKM and KMOS transcriptional factors, identified colonies 8-10 days after the transfection. We suggest the bovine transcriptional factors (bOSKM) can be more efficient in promoting the bovine fibroblast reprogramming and colony formation. Additionally, in both experiments, here using lentivirus and by Huang and collaborators [4] the medium supplemented with LIF2i was not efficient on colony formation, expansion, and self-renewal.

To identify if our biPSCs lines were comparable with naïve or primed state, they were characterized by RT-qPCR and immunostaining. Interestingly, testing the endogenous SOX2, OCT4, and NANOG mRNA abundance, we observed in all groups a relative expression decreasing overtime after continuous passages (Fig. 3). We analyzed these lines with immunostaining after passage 25. At that moment, we detected SOX2 and OCT4 presence. These results corroborate with other reports in biPS like cells [4,19,20,34]. However, we only detected NANOG mRNA presence by RT-qPCR on passage 5 (bFGF treatment); after that, mRNA expression and protein were not observed. Bogliotti and collaborators [28] described an extremely low expression using RNAseq of NANOG in bovine embryonic stem cells isolated in customized mTsER-1 medium supplemented with bFGF and IWR-1, corroborating with our RT-qPCR and immunostaining results. However, Cao et al. [33], Heo et al. [3] and Bressan et al [14] demonstrated NANOG mRNA expression and the protein presence by immunostaining in bovine iPSCs. Also, recently Zhao et al. [35] demonstrated bovine ESCs isolated and cultured in mTsER-1 medium supplemented with small molecules and cytokines (CHIR99021, WH-4-023 and XAV939 or IWR-1, Vitamin C, LIF and Activin A).

Interestingly, the nuclear receptor ESRRb is an important player in the maintenance of naïve pluripotency. Working on the Tcf3 repression and the direct target of Nanog, its overexpression could maintain Nanog-null ESC's self-renewal [26,36,37]. Kawaguchi et al [18] reported the ESRRb and STELLA expression on early and late passages of biPSCs; these cells were classified as naïve. In this experiment, the

ESRRb and STELLA expression were detected in the biPSCs; however, the expression decreased on late passages.

Independent of the experimental design used, livestock iPSCs cells frequently show persistent expression of exogenous transcription factors to maintain the undifferentiated state (review by Soto and Ross [22]). We observed the same characteristic in our lines when we observed the exogenous vector expression until passage 25. The incapacity to maintain livestock iPSCs without the reprogramming factor expression has been considered a challenge to report fully reprogrammed cells.

Other important demands involved in reprogramming genuine iPSCs are the differentiated cells' epigenetic signatures. These changes on epigenetic marks are necessary to promote the interaction between transcriptional pathways, cellular metabolism, and the epigenetic regulation of pluripotency [38–40]. The histones modifications decline in abundance on the 8th-cell stage before increasing the blastocyst stage during the embryo development [41]. In iPSCs, the H3K27me3 reconfiguration is crucial for silencing inappropriate gene expression required for cell lineage specification. Using the H3K27me3 profile in our lines, it was possible to identify bFGF treatment efficiency on the reprogramming process, decreasing the H3K27me3 levels when compared to the other groups. Kawaguchi et al. [18] also reported the H3K27me3 presence in biPSCs and, interestingly, those cells could contribute to chimeric embryos. These results emphasize the difficulty in promoting epigenetic reprogramming of differentiated cells.

Self-renewal is a crucial characteristic to stem cells, ESCs or iPSCs, and essential during replating and the frozen-thawed moments. Analyzing our results, we observed an interesting connection between frozen-thawed moments. We started frozen-thawed the lines after passage 10; from this moment on, all groups had downregulation of pluripotent markers expression (Fig. 3). We did not find in the literature explanations or other reports of the same results; however, we suggest that the frozen-thawed process can affect the reprogramming process, especially on intermediate iPSCs.

To test the differentiation capacity of our lines, we submitted all biPSCs to embryonic bodies assay and chimeric embryos contribution. All tested lines were

competent to produce EBs after culturing onto non-tissue-culture-treated plates and medium without bFGF and LIF. We submitted the EBs to RT-qPCR analysis to evaluate the expression of ectoderm, mesoderm, and endoderm markers. We detected the expression of ectoderm, mesoderm markers and endoderm markers expression. The absence of AFP endoderm markers expression in few lines can be due to the timing of the analysis (endoderm tissue needs more time to start the differentiation) or the incapacity of our lines to differentiate into endoderm tissue.

Additionally, we evaluated the ability of our bovine PSCs lines in chimeric embryos contribution. We observed the contribution of both bovine PSCs (biPSCs and bESCs) in chimeric embryos, additionally, the GFP biPSCs when injected in early morulas produced more chimeric blastocysts than GFP bESCs. These results indicate our biPSCs have an increased ability to contribute to chimeric blastocysts, consequently, our biPSCs have a pluripotent state closer to naïve than our bESCs. This suggestion can be supported by our RT-qPCR datas, when we observed STELLA and ESRRb expression upregulated in biPSCs than bESCs. Bovine ESCs contribution on chimeric embryos were reported few times [42–45]. In those reports, the authors reported the ability of the bovine embryonic like stem cells in contribute on extra-embryonic and placenta tissues. Recently, Zhao et al. [35] described the efficiency of bESCs on contribute to chimeric fetuses. In, our bESCs culture conditions, we observed a low inefficiency of the bESCs in contributing to chimeric blastocyst.

We infer that the culture conditions, especially the base medium supplemented with small molecules and cytokines, influenced the pluripotency state of these bESCs (reported here and Zhao et al. [35]) and reflected on the capacity to contribute to chimeric embryos. Additionally, Kawaguchi et al. [18] reported for the first time bovine iPSCs with capacity to contribute to embryonic and extraembryonic tissues, still, that iPSCs were classified as naïve-like cells. In the conditions of this work, we observed the higher rates of contribution using bovine iPSCs in blastocysts. These results, encourage us to keep using these cells to produce chimeric embryos and, especially, to for classify them in primed-intermediate, like reported by Yu et al. [23]. The authors reported the intermediate state of pluripotency in mammalian PSCs cultured in vitro. Those cells lines showed the capacity to differentiate in all embryonic tissues and primordial germ

like cells. More interestingly, those lines do not show the naïve markers expression but could be contributed to chimeric embryos and health offspring. Our work is a new chapter on the PSCs field, especially for open new opportunities and goals to apply the livestock PSCs on chimeric embryos models.

Conclusion

In conclusion, our results showed that bovine iPSCs could be generated by mOSKM on bFGF and mLIF combined with/or not 2i. However, the biPSCs on LIF2i treatment were not viable in our conditions. Also, the bFGF supplementation had an increased efficiency in promoting the reprogramming of bovine fetal fibroblasts. This is the first report to compare the gene expression between genuine bESCs and biPSCs. Additionally, we demonstrated the ability of our lines (biPSCs and bESCs) in contribute to chimeric blastocysts.

Abbreviations

iPSCs: Induced pluripotent stem cells; OSKM: Oct4, Sox2, KFL4, and c-Myc; biPSCs: Bovine induced pluripotent stem cells; bESC cells: bovine Embryonic stem cells; bFF: Bovine fetal fibroblasts; IMDM: Iscove's Modified Dulbecco's Media; STEMCCA: Stem cell cassette; MEFs: Mitotically inactivated mouse embryonic fibroblasts; DMEM/F12 KO: Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 knockout; KSR: Knockout serum replacement; bFGF: Basic fibroblast growth factor; pen/strep: Penicillin and streptomycin; LIF: Leukemia inhibitor factor; mLIF: Mouse inhibitor factor; 2i: iGSK3+iMEK; iGSK3: Glycogen synthase kinase 3 inhibitor; AP: Alkaline phosphatase; PFA: Paraformaldehyde; DPBS: Dulbecco's Phosphate-buffered saline buffer; BSA: Bovine serum albumin; RT-qPCR: Reverse transcription quantitative polymerase chain reaction (real-time PCR); EBs: Embryoid bodies;

Declarations

Ethics approval and consent to participate

Protocols were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (protocol number 2913/2013), and by the Ethics Committee on the Use of Animals of the Faculty of Animal Science and Food Engineering, University of São Paulo, Brazil (protocols number 3526250717 and 2192250918).

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Authors' contributions

RCB carried out the design of the study, its execution, and writing. NCGP, BB, LSM, AFS, KR, and PFN participated in its execution and helped to draft the manuscript. PJR, FFB and MFGN participated in its design and coordination. The authors read and approved the final manuscript.

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Figures

Figure 1

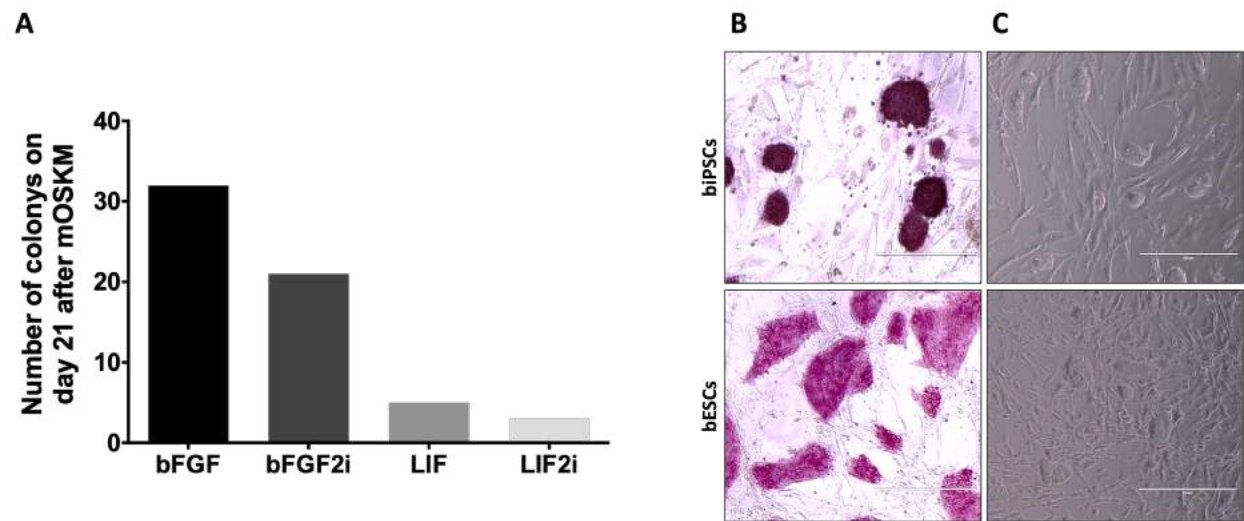


Figure 2

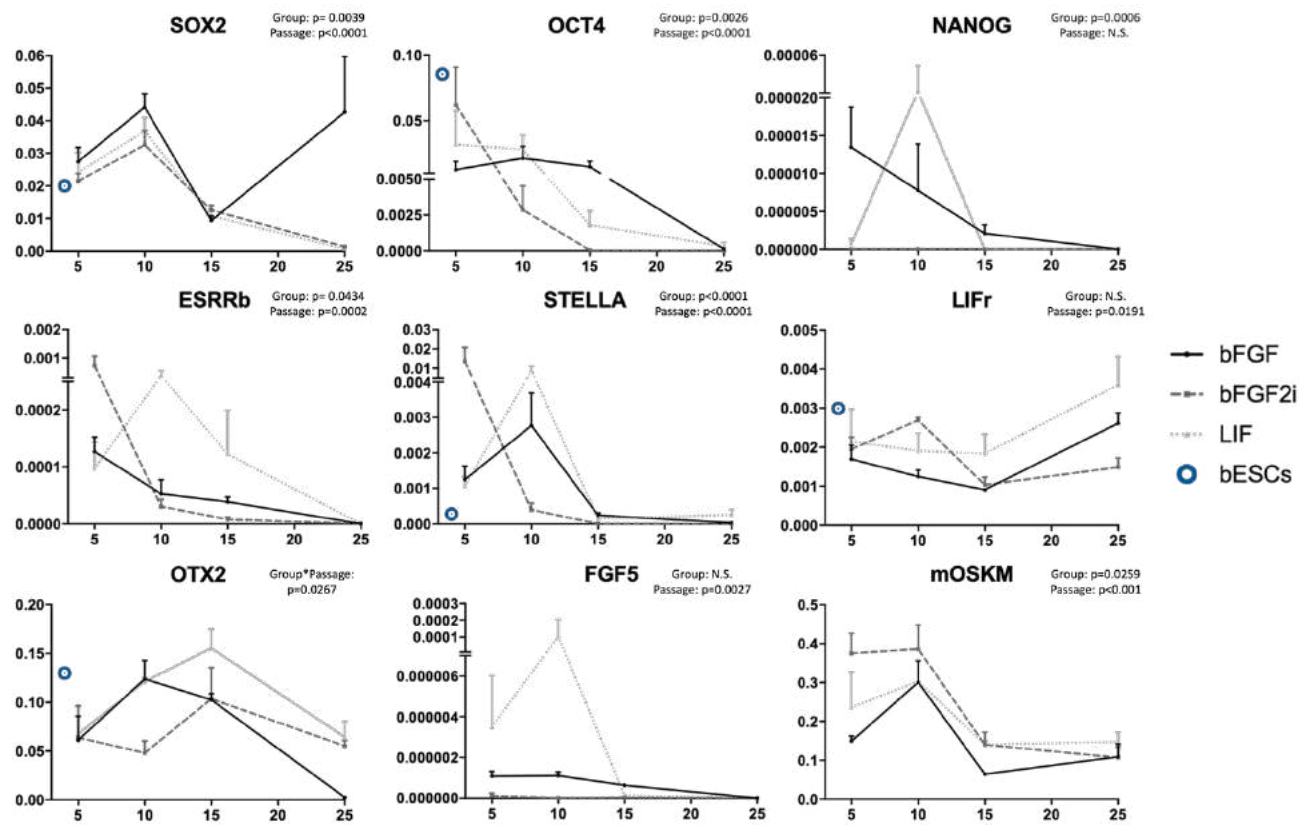


Figure 3

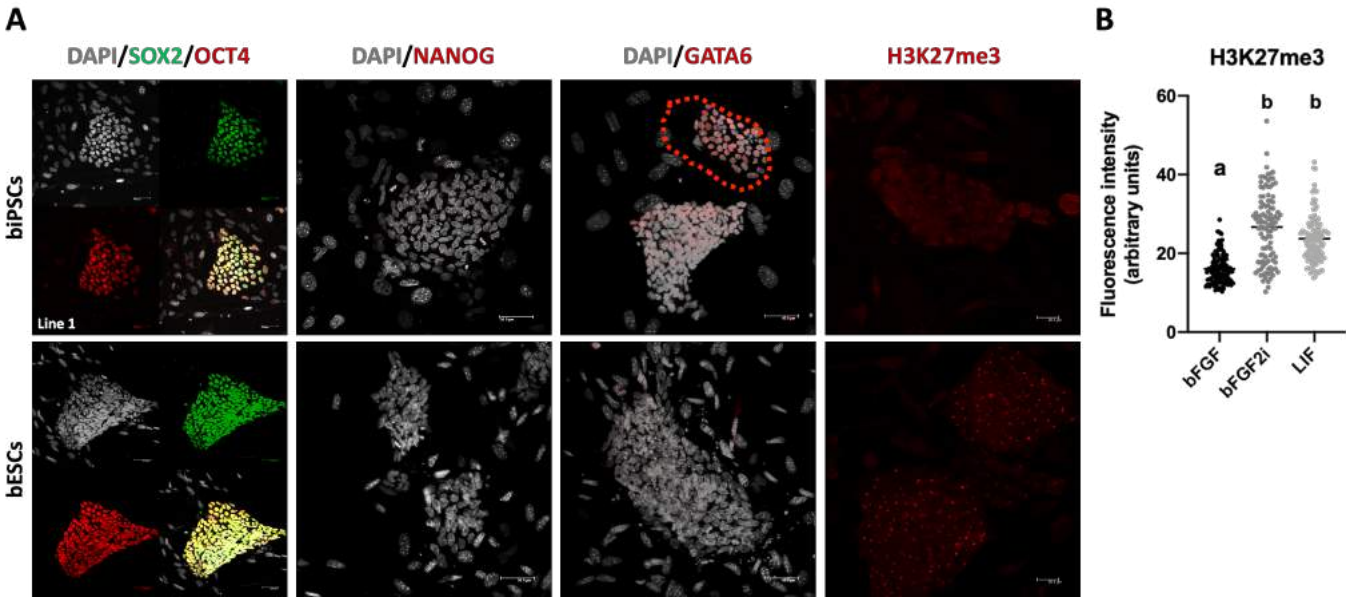


Figure 4

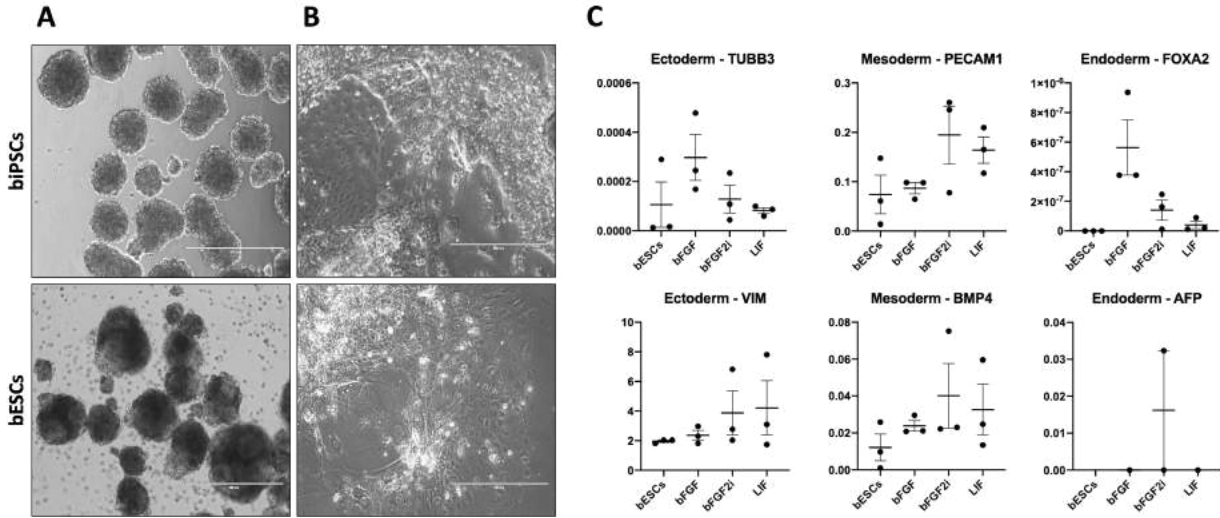
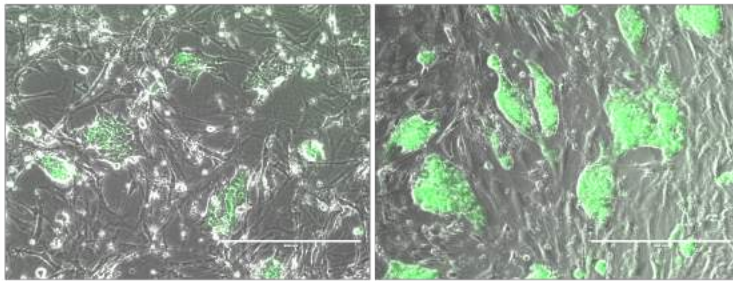


Figure 5

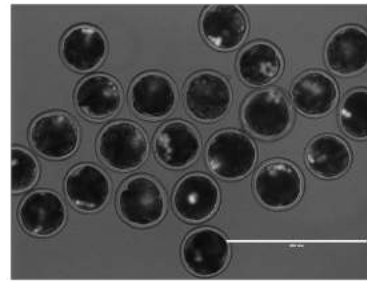
A



bESC FUGW

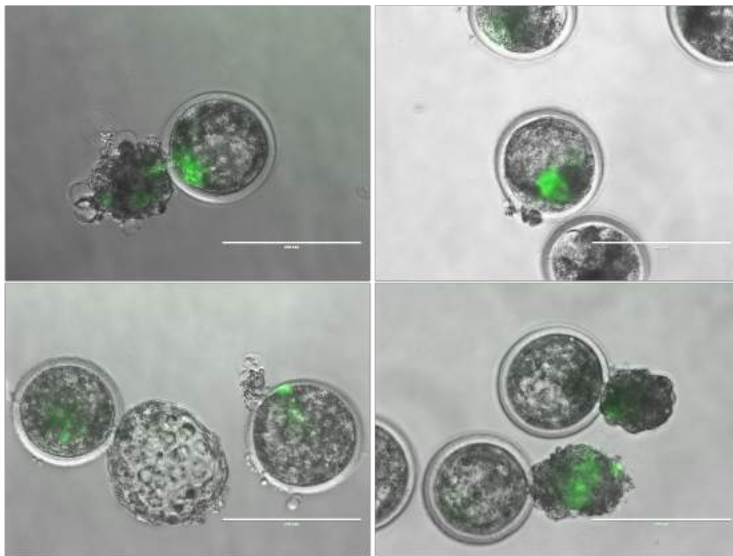
biPSCs FUGW

B

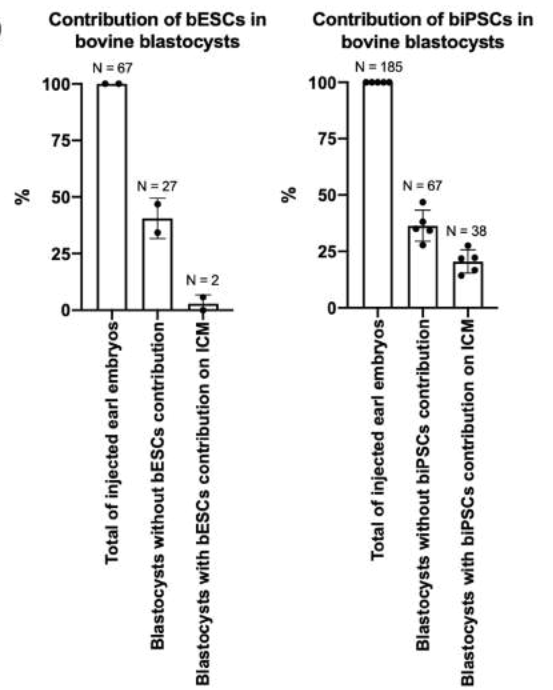


biPSCs FUGW injected in early bovine embryos

C



D



Legends of figures

Figure 1. A, Number of colonies observed 21 days after the mOSKM transduction cultured with different pluripotent maintenance agents. B, Alkaline phosphatase detection in bovine induced pluripotent stem cells derived on bFGF and mLIF, supplemented or not with 2i and in bovine embryonic stem cells. C, Morphology biPSCs colonies derived on bFGF and mLIF, supplemented or not with 2i and in bESCs colonies on the passage 25.

Figure 2. Relative gene expression of *bOCT4*, *bSOX2*, *bSTELLA*, *bESRR β* and *bLIFr* (pluripotent and naïve markers), *OTX2* and *FGF5* (primed markers) and mOSKM cassette in bovine induced pluripotent stem cells derived on bFGF, bFGF2i and LIF between passage 5-25 and in bovine embryonic stem cells (passage 5).

Figure 3. A, Immunofluorescence to SOX2 and OCT4, NANOG, GATA6 and H3K27me3 in bovine induced pluripotent stem cells derived on bFGF, bFGF2i and LIF and in bovine embryonic stem cells. Also, grey cells are DAPI staining of cells nucleus. Scale bars, 50 μ m and 20 μ m only to H3K27me3 images. B, Graphical representation of fluorescence intensity to H3K27me3 in bovine induced pluripotent stem cells derived on bFGF, bFGF2i and LIF and in bovine embryonic stem cells

Figure 4. A, Morphology of embryoid bodies produced from biPSCs colonies derived on bFGF and mLIF, supplemented or not with 2i and bovine induced pluripotent stem cells. Scale bars, 400 μ m. B, Cells with different morphology from EBs cultured for 5 days on suspension plus cultured during 7 days in IMDM. C, Relative gene expression of VIM and TUBB3 (ectoderm markers), PECAM1 and BMP4 (mesoderm markers) and AFP and FOXA2 (ectoderm markers) in embryoid bodies.

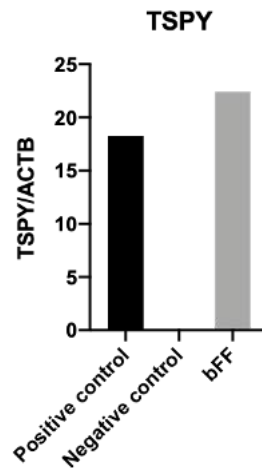
Fig 5. A, Colonies of bovine iPSCs and ESCs expressing green fluorescent protein. Scale bars, 400 μ m. B, 5-10 bovine GFP iPSCs injected into each bovine early embryo (day 4.5). Scale bars, 400 μ m. C, Bovine blastocysts with contribution of bovine

GFP iPSCs on the inner cell mass region on the day 7 of the embryo development. Scale bars, 200 μ m. D, Graphical representation of contribution of GFP bESCs and GFP biPSCs on the inner cell mass region on the day 7 of the embryo development.

Supplementary materials

Table 1. The general list of primers used to characterize pluripotency of biPSCs, cell differentiation of EBs and sexing.

Primer	Transcript id	Foward (5' → 3')	Reverse (5' → 3')	Product size
bACTB	NM_173979.3	CAGCAGATGTGGATCAGCAAGC	AACGCAGCTAACAGTCCGCC	89
bPPIA	NM_178320.2	CATACAGGTCCTGGCATC	CACGTGCTTGCCATCCAA	107
bNANOG	NM_001025344.1	CTCAGCTACAAGCAGGTGAAGA	ACACCCCTGGTGGTAGGAAT	153
bOCT4	NM_174580.2	GCAAACGATCAAGCAGTGACTAC	GGCGCCAGAGGAGAGGATACG	93
bSOX2	NM_001105463.2	ATGGGCTCGGTGGTGAAGT	TGGTAGTGCTGGGACATGTGA	178
bESRRβ	XM_010809669.2	AAATACAAGCGACGGCTGGA	GAGAAGCCTGGGATGTGCTT	245
bLIFr	NM_001192263.1	TGGTGGACCGCAAAAGAATG	AAGTACGGGACCGCTTTTCA	228
bSTELLA	NM_001111109.2	AGTGAGCGGAGGTACAGGAT	TCGCACTCTTGATCGAATCTCA	132
bFGF5	NM_001078011.2	GCAGTAGCACCGCGACTT	TCCACTGGAAGCTGCTCTG	102
bOTX2	NM_001193201.1	ACCCAGACATCTTCATGCGG	AAATGGCTGGGACTGAGGTG	243
bTUBB3	NM_001077127.1	GGATAGACCCCAAGTGGCAAT	TTGTGTGAAGAAGCCTCGTTG	88
bVIM	NM_173969.3	CTCCTACCGCAGGATGTTCCG	TGGATGTGGTCACGTAGCTC	140
bPECAM1	NM_174571.3	AATCAGAGCGTGGGCTCAAA	ATCCACTGGGGCTATCACCT	147
bBMP4	NM_001045877.1	AGCTTCCACCACGAAGAACAT	CACCTCGTTCTCTGGGATGC	102
bAFP	NM_001034262.2	CGGACCTTCCGAGCCATAAC	CTCTTTCCCCATCCTGCAGAC	154
bFOXA2	XM_025001047.1	CGAGCCCAGAGGGCTACTC	GTACGTGTTTCATGCCGTTCA	92
bTSPY	281554	CCCAGAATCGAACAGGATTG	TTGTCTCTCACGGACGAACC	215



SM Figure 1. TSPY gene expression to sexing the bovine fetal fibroblast line.

Chapter 4: Final Remarks

The induced pluripotent stem cells have massive potential for regenerative therapies, *in vitro* embryology studies, cellular agriculture and *in vitro* breeding. However, some aspects of the cellular reprogramming process have not yet been fully elucidated, especially with regard to livestock species. In recent years, some research has clarified the role of cellular metabolism and pluripotent profiles in reprogramming to pluripotency. In this context, the environment (oxygen tension) and small molecules (maintenance of pluripotency) are important elements during the acquisition and the maintenance of pluripotent state.

Firstly, low oxygen tension in the acquisition of pluripotency have been carried out in humans and in mice, however, studies on the influence of low oxygen tension on the bovine iPSC cells (biPSC) have not yet been reported. Thus, the hypothesis that cultivation at low oxygen tension (5% of O₂) increase the reprogramming efficiency of fetal fibroblasts to a state of pluripotency, contributes to the generation of biPSC with characteristics related to pluripotency was tested in the present thesis.

The results described on the Chapter 2, demonstrated for the first time biPSCs generated in low oxygen tension. However, low oxygen tension do not improve the reprogramming efficiency compared with normoxia environment in biPSCs, thus, negating the first part of our hypothesis. Also, the set of bFL2i supplements improved the reprogramming efficiency and generated higher colonies numbers than only bFGF. Interestingly, the low oxygen tension combine with bFL2i set do not support the self-renewal ability in biPSCs after few passages. Low oxygen tension was linked with the maintenance of pluripotency in mice and human stem cells, in our conditions, we do not observe the same effect in biPS, the hypoxia affected only glycolytic genes.

In the Chapter 3, bovine iPSCs were generated by lentiviral reprogramming on bovine fetal fibroblasts. The reprogramming was conducted in a 2x2 factorial experiment design, sets of supplementations during culture (bFGF, bFGF+2i, LIF and LIF+2i) were tested to improve the reprogramming efficiency and the pluripotent state in biPSCs. Additionally, bovine embryonic stem cells (bESCs) were derived to compare the pluripotent profile and characteristics to biPSCs. The pluripotent profile of our

biPSCs and bESCs were positive for *SOX2*, *OCT4*, *STELLA*, *LIFr* and *OTX2*. Also, our biPSCs expressed *ESRRb* another naïve pluripotent marker. However, many pluripotent markers decreased the expression in our biPSCs lines after the passages. More interestingly, we evaluated the ability of our bovine PSCs to contribute in chimeric embryos. We observed the capacity of both lines in contribute near of ICM in blastocysts, however, the efficiency of our biPSCs supplemented with bFGF were higher than our bESCs.

Thus, we generated biPSCs in different oxygen tension and with different sets to support the acquisition and the maintenance of pluripotency. Our results show the ability of biPSCs supplemented with bFGF to contribute in chimeric blastocysts. These cells lines should facilitate the optimization of culture conditions for generation and maintenance of bovine iPSCs that have the potential to generate chimeric offspring.

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Curriculum Vitae

EDUCATION

PhD student in Pharmacology and Biotechnology, São Paulo State University (UNESP) (2016-current) / University of California Davis (UC Davis)

Master of Science in Animal Science, University of Sao Paulo (USP), Brazil (2011-2014)

Biological Science, Centro Universitário Herminio Ometto de Araras (UNIARARAS), Brazil (2007-2010)

Public defense of PhD: 05/05/2021

VISITING SCHOLAR

University of California Davis, USA

Effective date (Start): August 21, 2019

Effective date (End): May 20, 2020

Title: “Trophectoderm Complementation Assay for pluripotent cells: a proposal for assessing pluripotency in cattle”

Supervisor: Prof Pablo J Ross

Funding: FAPESP-Brazil (19/10751-0)

Instituto Leloir, LELOIR, Argentina.

Effective date (Start): September 4, 2017

Effective date (End): September 17, 2020

Title: “Human induced pluripotent stem cells (hiPSCs), generation, maintenance and basic characterization”

Supervisor: Fernando Pitossi

Funding: CBAB/CABBIO

AREAS OF RESEARCH AND LAB TECHNIQUES

- Stem Cells (iPSCs and ESCs)

Related methods: Isolation and characterization of embryonic stem cells, iPSC generation and characterization, in vitro differentiation.

- Epigenetics and molecular biology

Interest: small molecules on pluripotent state of iPSCs and ESCs, oxygen tension on pluripotent state of iPSCs and ESCs.

- Bovine embryo production

Related methods: General medium production, oocyte collection and maturation, in vitro fertilization, embryo culturing.

- LAB TECHNIQUES

Immunostaining, confocal analysis, gene expression by RT-qPCR, gene knockdown by siRNA, transfection, lentivirus production, micromanipulation of zygotes and embryos, chimeric embryos.

BIBLIOGRAPHY

MANUSCRIPTS IN PREPARATION

Generation of human progenitor primordial germ cell-like in Turner syndrome patients

Aline Fernanda de Souza; Fabiana Fernandes Bressan; Naira Caroline Godoy Pieri; **Ramon Botigelli**; Ester Silveira Ramos; Willian Allan King, Flavio Vieira Meirelles

Generation of porcine primordial germ cells-like (PGCLCs) from induced pluripotent stem cells with different profile phenotypes related of the culture condition.

Naira Caroline Godoy Pieri, Aline Fernanda de Souza; Ramon Cesar Botigelli, Laís Vicari de Figueiredo Pessôa; Kaiana Rechia; Mayra Hirakawa Glória; Raquel Vasconcelos Guimarães de Castro; Diego Leal; Paulo Fantinato-Neto, Daniele dos Santos Martins; Flávio Vieira Meirelles; André Furugen C. Andrade; Fabiana Fernandes Bressan

Bovine induced pluripotent stem cells are affected by oxygen levels during the reprogramming process

Brendon Bessi; Ramon Botigelli; Pamela Moraes; Naira Caroline Godoy Pieri; Aline Fernanda de Souza; Raquel de Castro; Flavio Vieira Meirelles; Fabiana Fernandes Bressan;

Different small molecules and maintenance agents affect reprogramming process and pluripotency state of bovine-induced pluripotent stem cells

Ramon Cesar Botigelli; Naira Carolina Godoy Pieri; Brendon Bessi; Aline Fernanda Souza, Raquel Castro; Flávio Vieira Meirelles; Fabiana Fernandes Bressan; Marcelo Fábio Gouveia Nogueira

SUBMITTED MANUSCRIPTS

Embryonic microRNAs are essential for bovine preimplantation embryo development. **PNAS**
Erika E Paulson; Emily Lillian Fishman; Ramon C. Botigelli; Richard M Schultz; Pablo J Ross

In vitro induced pluripotency from urine-derived cells in swine. **Scientific reports.**

Kaiana Recchia; Lucas S. Machado; Ramon C. Botigelli; Naira C. G. Pieri; Gabriela Barbosa; Brendon W. Bessi; Raquel V. G. Castro; Lais V. F. Pessôa; Paulo Fantinato Neto; Flavio V. Meirelles; Aline F. Souza; Simone M. M. K. Martins; Fabiana F. Bressan

Genomic and Phenotypic Analyses of Antral Follicle Count in Aberdeen Angus Cows. **Livestock Science.**

Gerson Oliveira Junior, Vinícius G. Pinheiro, Pablo Augusto de Souza Fonseca, Camila B. Costa, Elisa Mariano Pioltine, Ramon Botigelli, Eduardo Razza, Ronaldo L. Ereno, Jose Bento Ferraz, Marcelo M. Seneda

FULL LENGTH PEER REVIEWED PUBLICATIONS:

10 in total – h-index 5; Total number of citations currently 38.

Number of citation for each manuscript are given in parentheses [] where applicable.

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AWARD:

2015 - 2^o Students competition of SBTE meeting. Is the NPPC/NPR2 system, described for murine model, applied to bovine antral follicles?