UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CARACTERIZAÇÃO MOLECULAR DE CÉLULAS-TRONCO ISOLADAS DE FETOS DE *Gallus gallus*

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ESTRUTURAÇÃO DA DISSERTAÇÃO

Esta dissertação apresenta-se organizada em uma introdução geral, objetivos (gerais e específicos), três capítulos redigidos no formato de artigo, discussões e conclusões gerais, além de um item contendo as perspectivas do trabalho.

A introdução geral apresenta o conceito vigente de células-tronco, enfocando especificamente nas células-tronco mesenquimais e suas principais características. Além disso, uma sessão é dedicada ao organismo modelo-alvo do presente estudo, *G. gallus*.

O capítulo 1 consiste de uma revisão abrangente sobre marcadores moleculares de células-tronco. Especificamente, são discutidos os marcadores clássicos já descritos para células-tronco embrionárias, mesenquimais e hematopoiéticas além da proposição, para cada um dos tipos celulares, de novos marcadores moleculares. Este artigo foi aceito para publicação no periódico *Stem Cells and Development,* cujo fator de impacto atual é 4,459.

O capítulo 2 apresenta uma revisão das características biológicas de células-tronco mesenquimais isoladas de organismos modelo não-convencionais, focando na morfologia, marcadores moleculares expressos e potencial de diferenciação. Além disso, estão listados os principais estudos de pesquisa aplicada nos quais estas células são empregadas. Este artigo será submetido ao periódico *Stem Cell Research & Therapy,* de fator de impacto 3,21.

O capítulo 3 aborda um estudo de isolamento e caracterização de células-tronco mesenquimais oriundas de medula óssea e dos músculos esquelético e cardíaco de fetos de *Gallus gallus*. A caracterização tem enfoque no potencial de diferenciação, nos marcadores moleculares expressos nos estados tronco e diferenciado e também no perfil transcricional das células isoladas. O artigo encontra-se em fase de redação científica.

Os três capítulos estão seguidos por uma discussão geral englobando os conhecimentos discutidos em cada um deles, pelas conclusões geradas com o desenvolvimento deste trabalho de mestrado, bem como pelas perspectivas de continuação da pesquisa científica com células-tronco mesenquimais fetais de *G. gallus.*

SUMÁRIO

LISTA DE ABREVIATURAS

ALP	*Alkaline phosphatase*
BM	*Bone marrow*
bMSC	*Bovine mesenchymal stem cells*
buMSC	*Buffalo mesenchymal stem cells*
cMSC	*Cat mesenchymal stem cells*
CFU-F	*Colony forming units-fibroblasts*
chMSC	*Chicken mesenchymal stem cells*
CM	*Cardiac muscle*
CT	Células-tronco
CTE	Células-tronco embrionárias
CTFG	Células-tronco isoladas de fetos de *G. gallus*
CTMs	Células-tronco mesenquimais
CTH	Células-tronco hematopoiéticas
dMSC	*Dog mesenchymal stem cells*
DMD	*Duchenne muscular dystrophy*
duMSC	*Duck mesenchymal stem cells*
doi	*Days of incubation*
eAE-MSC	*Equine amniotic epithelium mesenchymal stem cells*
eAMSC	*Equine adipose tissue mesenchymal stem cells*
eBMSC	*Equine bone marrow mesenchymal stem cells*
ECM	*Extracellular matrix*
eMSC	*Equine mesenchymal stem cells*
ePB-MSC	*Equine peripheral blood mesenchymal stem cells*
ESC	*Embryonic stem cells*
EPDCs	*Epicardium-derived cells*
EPSC	*Epidermal stem cells*
eTMSC	*Equine tendon mesenchymal stem cells*

eUC-MSC	*Equine umbilical cord mesenchymal stem cells*
FF	*Fetal fibroblasts*
gMSC	*Goat mesenchymal stem cells*
GM-CSF	*Granulocyte/macrophage colony-stimulating factor*
guMSC	*Guinea pig mesenchymal stem cells*
hESCs	*Human embryonic stem cells*
HSC	*Hematopoietic stem cells*
ICM	*Inner cell mass*
ICI	*Intracoronary injection*
iPSCs	*Induced pluripotent stem cells*
mESCs	*Murine embryonic stem cells*
MSC	*Mesenchymal stem cells*
NOD/SCID mice	*Non-obese diabetes/severe combined immunodeficiency mice*
NSC	*Neural stem cells*
NT	*Nuclear transfer*
Oct-4	*Octamer-binding protein 4*
oMSC	*Ovine mesenchymal stem cells*
PGC	*Primordial germ cells*
rMSC	*Rabbit mesenchymal stem cells*
SC	*Stem cell*
SM	*Skeletal muscle*
Sox-2	*SRYsex determining region Y-box 2*
SSEA-1	*Stage-specific embryonic antigen-1*
SSEA-3	*Stage-specific embryonic antigen-3*
SSEA-4	*Stage-specific embryonic antigen-4*
sMSC	*Swine mesenchymal stem cells*
TRA1-60	*Tumor rejection antigen 1–60*
TRA1-81	*Tumor rejection antigen 1–81*
TE	*Trans-endocardial*

RESUMO

Células-tronco mesenquimais (CTMs) estão entre os tipos de células-tronco encontradas a partir da fase fetal até a vida adulta de um indivíduo. As CTMs caracterizam-se pela morfologia similar à de fibroblastos associada à presença das proteínas de superfície celular CD73, CD90 e CD105, não apresentando expressão de marcadores hematopoiéticos, tais como CD34 e CD45. As CTMs são consideradas multipotentes e capazes de diferenciarem-se em osteoblastos, adipócitos e condroblastos. Além de humanos, as CTMs já foram isoladas de uma série de organismos modelo, dentre eles o camundongo e o frango doméstico (*Gallus gallus*). Apesar de pouco difundido nesse campo de estudo, o modelo *G. gallus* apresenta uma série de características que o torna uma alternativa interessante ao modelo murino. Nesse contexto, o objetivo deste trabalho foi isolar e caracterizar molecularmente as CTMs deincubação. Como resultado, obtiveram-se células com as características esperadas para uma CTM clássica. As CTMs isoladas apresentaram morfologia característica, expressão das proteínas de superfície CD73, CD90 e CD105 e ausência de marcadores hematopoiéticos. Além disso, as células mostraram-se capazes de diferenciarem-se em osteoblastos e pré-adipócitos. No entanto, as células isoladas de coração, apesar de apresentarem características de CTMs, foram incapazes de diferenciarem-se em adipócitos. A análise do perfil transcricional destas células, em comparação às obtidas de medula óssea, revelou que as mesmas superexpressam genes relacionados a morfogênese cardíaca, a angiogênese, a diferenciação de células de músculo cardíaco e a coagulação sanguínea. Considerando as características apresentadas pelas células isoladas de músculo cardíaco, existe a possibilidade de ter havido o isolamento de um tipo específico de célula-tronco cardíaca denominada de células derivadas de epicárdio (EDPCs – do inglês *Epicardium derived cells*). Desta forma, os resultados obtidos neste trabalho indicam que é possível isolar CTMs de medula óssea e músculo esquelético de fetos de frango. No entanto, as células obtidas de músculo cardíaco reúnem características de potenciais EPDCs e mais estudos são necessários para determinar a sua identidade.

ABSTRACT

Mesenchymal stem cells (MSC) are found in both fetal and adult individuals. MSC are characterized by their fibroblast-like morfology, the expression of the surface proteins like CD73, CD90 and CD105 and absence of hepatopoietic markers, such as CD34 e CD45. Besides, MSC are considered multipotent stem cells due to their capacity to differentiate into osteoblasts, adipocytes and chondroblasts. MSC have been already isolated from human being and several model organisms, as mice and chicken (*Gallus gallus*). Unfortunately, *G. gallus* is not widely applied for the study of MSC, but it can be an interesting alternative to the murine model. In this context, the aim of this work was to isolate and molecularly characterize MSC derived from bone marrow, cardiac and skeletal muscle of 18-19 days chicken fetuses. Cells isolated from bone marrow and skeletal muscle presented the expected characteristics for MSC. They expressed CD73, CD90 and CD105, but were negative for hematopoietic markers. Moreover, the cells were able to differentiate into osteoblastic and adipogenic lineages. Cells obtained from cardiac muscle presented the same molecular and morphological characteristics, except that they were not able to differentiate into adipocytes. Transcriptional profile analysis of cardiac-derived cells revealed that they overexpress genes related to heart morphogenesis, angiogenesis processess, smooth muscle cells differentiation and blood coagulation. There is a possibility that cells isolated from cardiac muscle are of an specific type named epicardium derived cells (EPDCs). The results obtained in this work point that is possible to isolate MSC from bone marrow and skeletal muscle from chicken fetuses. Nevertheless, cells obtained from cardiac muscle gather characteristics of putative EPDCs and further studies are necessary to elucidate the real identity of cardicac muscle isolated cells.

1. INTRODUÇÃO GERAL

1.1. Características gerais das células-tronco

As células-tronco (CT) possuem particularidades que as colocam em um grupo a parte dos demais tipos celulares caracterizando-se, basicamente, pela capacidade de autorrenovação e de gerar células mais especializadas por meio da sua diferenciação (Watt & Hogan, 2000). Conforme o seu potencial de diferenciação e o período do desenvolvimento em que são obtidas, recebem diferentes denominações.

As células originadas a partir do oócito fertilizado até o estágio de blastocisto são consideradas totipotentes, pois possuem o potencial de gerar um organismo inteiro (Verfaillie et al., 2002). A partir do blastocisto, as células começam a se especializar e são então consideradas pluripotentes. As CT pluripotentes podem gerar células pertencentes aos três folhetos embrionários (endoderme, mesoderme e ectoderme) (Bongso & Richards, 2004), mas já não são mais capazes de gerar um indivíduo. Multipotente seria o termo para as CT capazes de produzir um número limitado de linhagens celulares diferenciadas apropriadas à sua localização (Alison et al., 2002) tal como as CT mesenquimais (CTM).

As CT podem ser isoladas de embriões, fetos e indivíduos adultos (Bianco et al., 2008; Campagnoli et al., 2001; Thomson et al., 1998). No caso das células-tronco fetais, estas apresentam um potencial de diferenciação mais restrito do que as células isoladas de embriões, porém maior do que as encontradas em tecidos adultos (O'Donoghue & Fisk, 2004). Dentre as CT já reportadas na literatura científica estão as CT embrionárias, as células germinativas primordiais, as CT epidermais, as CT hematopoiéticas, as CT neuronais, CT da crista neural e as CT mesenquimais. (Achilleos et al., 2012; Jansen et al., 2005; Pittenger et al., 1999; Schreder et al., 2010; Shamblott et al., 1998; Thomson et al., 1998).

1.2. Marcadores moleculares

Uma das ferramentas utilizadas para caracterizar CT é a da presença de certas moléculas que permitem identificá-las como tal e diferenciá-las dos demais tipos celulares.

As CT embrionárias (CTE) apresentam a expressão de três fatores de transcrição, Oct-4, Nanog e Sox-2, em conjunto com a presença dos antígenos de superfície celular SSEA-4, TRA1-60 e TRA1-81, concomitante com a ausência de SSEA-1 (Gavrilov et al., 2011). Além disso, estas células apresentam a expressão de telomerase e são positivas para atividade de fosfatase alcalina (Thomson et al., 1998).

Nas células-tronco hematopoiéticas (CTH), o marcador CD34 é o mais comumente usado para isolar populações enriquecidas com este tipo celular. Também se observa expressão dos marcadores CD45 e CD133 na fração celular positiva para CD34 (Park et al., 2011). Outra característica das CTH é a não identificação de marcadores de linhagem , ou seja, ausência de marcadores de superfície celular característicos de células sanguíneas maduras como, por exemplo, CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD56, entre outros (Wognum et al., 2003).

No caso das CTM, a Sociedade Internacional para a Terapia Celular (www.celltherapysociety.org) estabeleceu, juntamente com outros critérios considerados mínimos para a sua identificação, a expressão dos antígenos de superfície celular CD73, CD90 e CD105 concomitante com a ausência de expressão de CD14, CD19, CD34, CD45 e HLA-DR (Dominici et al., 2006). No entanto, estas células também são positivas para outros marcadores de superfície, como CD13, CD29, CD44, CD54, CD106, CD 146 e CD166 e negativas para CD10, CD11b, CD14, CD34, CD31, CD45, CD49d e CD116 (Mafi et al., 2011).

1.3. Células-tronco mesenquimais

Dentre os tipos de CT que tem recebido atenção no meio científico encontram-se as CTM, células multipotentes que se caracterizam por apresentar morfologia similar à de

fibroblastos e adesão ao plástico quando cultivadas *in vitro* (Uccelli et al., 2008). Outra característica marcante deste tipo celular é a habilidade de diferenciar-se nas linhagens mesenquimais osteogênica, adipogênica e condrogênica (Pittenger et al., 1999). Porém, há vários relatos de diferenciação de CTM para outros tipos celulares, não originários da mesoderme, como ilhotas pancreáticas (Santos et al., 2010), hepatócitos (Lee et al., 2004; Pournasr et al., 2011) e potenciais neurônios (Dezawa et al., 2004) (Figura 1).

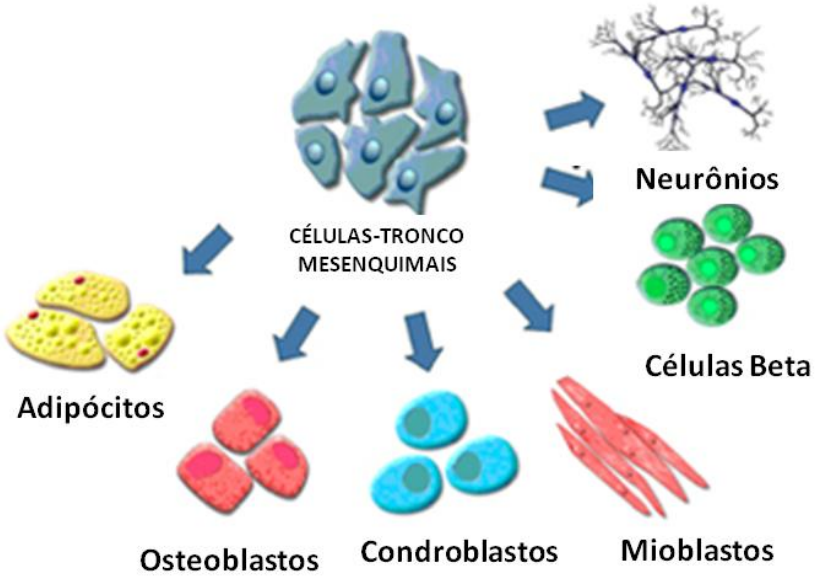

Figura 1. Potencial de diferenciação de células-tronco mesenquimais (adaptado de www.sciencereviews2000.co.uk).

As pesquisas com CTM tiveram seu início na década de 60, com os estudos de A. J. Friedenstein e colaboradores, com células isoladas de medula óssea (Afanasyey *et al*., 2009). Apesar das células originárias desse compartimento terem sido as mais extensivamente estudadas e caracterizadas, outros estudos revelaram a presença de populações de CTM em outros locais do corpo humano, a exemplo de tecidos adultos, como o adiposo (Pierantozzi *et al*., 2011; Zuk *et al*., 2002) e o cardíaco (Pierantozzi *et al*., 2011), fragmentos de osso trabecular (fêmur) (Noth *et al*., 2002), vasos sanguíneos, como a aorta e a artéria femoral (Pasquinelli *et al*., 2010), sangue periférico (Zvaifler *et al*., 2000), pele (Riekstina *et al*., 2009) e a partir fluidos e tecidos de origem fetal, como

líquido amniótico, sangue e veias de cordão umbilical (Kern *et al*., 2006; Santos *et al*., 2010), âmnio e placenta (In 't Anker *et al*., 2004).

Além de seres humanos, há estudos com CTM isoladas de uma série de outros organismos, como cães (Sun *et al*., 2011a), gatos (Martin *et al*., 2002), patos (Li *et al*., 2009), cabras (Cao *et al*., 2012), coelhos (Na *et al*., 2007), galinhas (Khatri *et al*., 2009), cavalos (Del Blue *et al*., 2008), porcos (Ringe et al., 2002), porquinhos–da-índia (Frolich *et al*., 2011), búfalos (Hepsibha *et al*., 2011), ratos (Yoshimura *et al*., 2007), ovelhas (Jager *et al*., 2006) e camundongos (da Silva Meirelles *et al*., 2006), este último o organismo modelo clássico para os estudos da biologia de CT.

As CTMs apresentam grande potencial de emprego na terapia celular uma vez que não apresentam os problemas éticos e o risco de geração de tumores que estão associados ao uso de CTE. Além disso, permitem a realização de transplantes autólogos, uma vez que podem ser acessadas facilmente da medula óssea ou no tecido adiposo do paciente (Connick *et al*., 2012; Sun *et al*., 2011b), e transplantes alogênicos também são facilitados pela capacidade imunossupressora apresentada pelas CTMs (Jones *et al*., 2008). Dentre as áreas em que já se verificou potencial de emprego para as CTMs estão a ortopedia, em defeitos ósseos e de cartilagem, patologias associadas à imunidade, como a artrite reumatoide, isquemias cardíacas e cerebrais e como veículos para tratamentos anti-tumorais (Rastegar *et al*., 2010).

1.4. O organismo modelo *Gallus gallus*

A espécie *Gallus gallus* (Figura 3) é utilizada há aproximadamente dois milênios como modelo em estudos biológicos, principalmente na área do desenvolvimento embrionário (Stern, 2005). Dentre as contribuições deste modelo para a ciência estão a descoberta da circulação sanguínea, a elucidação do mecanismo de transmissão de infecções, a descoberta do oncovírus causador do Sarcoma de Rous, o isolamento do primeiro oncogene, e a descoberta dos linfócitos T e B (revisado em Burt, 2007; Stern, 2004).

Figura 2. Exemplar da espécie *Gallus gallus*, raça *White Leghorn* (Fonte: bluerunbantams. blogspot.com.br).

Apesar de o modelo aviário ser pouco difundido na pesquisa com CT, já há relatos de isolamento de CT neste organismo. As CT embrionárias de frango apresentam expressão de Oct-4, Nanog, telomerase, fosfatase alcalina e SSEA-3, os mesmos marcadores observados em CTE humanas (Lavial *et al*., 2007; Pain *et al*., 1996). Além disso, já foram isoladas CT fetais espermatogoniais, células germinativas primordiais, precursores hematopoiéticos CTM (Cormier & Dieterlen-Lievre, 1988; Khatri *et al*., 2010; Wu *et al*., 2010; Yu *et al*., 2010). As CTM foram isoladas pela primeira vez em 1995, de uma série de compartimentos corporais de fetos com 11 dias de incubação. As células obtidas foram capazes de diferenciarem-se em osteoblastos, adipócitos, condroblastos e mioblastos (Young *et al*., 1995). Recentemente, reportou-se o isolamento de CTM de tecidos adiposo e pulmonar de frangos com 1-2 dias (Gong *et al*., 2011; Khatri *et al*., 2010).

Não obstante o contexto em que se encontra o modelo *G. gallus* no campo das CT, este apresenta características que o qualificam como uma alternativa ao modelo murino,

classicamente utilizado nessa área de estudo. Dentre elas está o fácil manuseio e acesso às células devido ao tamanho do embrião, visivelmente maior do que o de camundongo, e a ocorrência do desenvolvimento *in ovo,* cuja postura ocorre cerca de 20 a 23 horas após a fertilização (Lavial & Pain, 2010). Além disso, o desenvolvimento é rápido, levando em torno de 21 dias (Hamburger & Hamilton, 1951) e o custo de manutenção dos ovos embrionados é baixo, tendo em vista que não é necessário manter a estrutura de um biotério.

Outras características importantes do modelo são a existência de linhagens com pouca variabilidade genética, como a raça *White Leghorn* (Ponsuksili *et al*., 1998) e a disponibilidade genoma totalmente sequenciado (International Chicken Genome Sequencing Consortium, 2004). O genoma do frango caracteriza-se por ser compacto, apesar de possuir um número de genes próximo ao observado em mamíferos (Ellegren, 2005) e apresentar um alto nível de sintenia com este grupo (Stern, 2005). Adicionalmente, há grande similaridade com o genoma humano, evidente pela quantidade de genes ortólogos, cerca de 60%, identificados entre as duas espécies (Cogburn *et al*., 2007; Ellegren, 2005; Stern, 2005).

2. JUSTIFICATIVA

As CTMs, apesar de estudadas desde a década de 60, apresentam vários aspectos da sua biologia não elucidados, de modo que o conhecimento sobre as mesmas ainda está longe de ser esgotado. A espécie *Gallus gallus*, por sua vez, reúne um conjunto de características que tornam este organismo interessante para o emprego na pesquisa com CTMs. Apesar de já terem sido isoladas desse organismo anteriormente, a caracterização das CTMs até o presente trabalho ainda era bastante limitada. Portanto, o isolamento o e a caracterização de CTM de frango realizado neste trabalho consolida um modelo alternativo para o estudo dessas células e torna-se ponto de partida para estudos posteriores de pesquisa básica e aplicada nessa área do conhecimento.

3. OBJETIVOS

3.1. Objetivo geral

Caracterizar as células-tronco mesenquimais isoladas de fetos de *G. gallus* (CTFG) quanto aos marcadores moleculares expressos, ao potencial de diferenciação e o perfil transcricional.

3.2. Objetivos específicos

- Estabelecer culturas *in vitro* de células-tronco mesenquimais a partir dos músculos cardíaco e esquelético e da medula óssea de fetos de *G. gallus*.
- Definir, dentre um conjunto de marcadores moleculares clássicos de estado tronco, aqueles cuja expressão poderia caracterizar as CTFG oriundas das três diferentes fontes.
- Investigar o potencial de diferenciação osteogênica e adipogênica das CTFG isoladas.
- Analisar a expressão de marcadores moleculares de estado tronco e de diferenciações adipogênica e osteogênica antes e depois do processo de diferenciação.
- Estabelecer o perfil de transcritos de CTFG isoladas dos diferentes tecidos e órgãos de *G. gallus* por meio da técnica de microarranjo de DNA.

Capítulo I

Reviewing and updating the major molecular markers for stem cells

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Reviewing and Updating the Major Molecular Markers for Stem Cells

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Stem cells (SC) are able to self-renew and to differentiate into many types of committed cells, making SCs interesting for cellular therapy. However, the pool of SCs in vivo and in vitro consists of a mix of cells at several stages of differentiation, making it difficult to obtain a homogeneous population of SCs for research. Therefore, it is important to isolate and characterize unambiguous molecular markers that can be applied to SCs. Here, we review classical and new candidate molecular markers that have been established to show a molecular profile for human embryonic stem cells (hESCs), mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs). The commonly cited markers for embryonic ESCs are Nanog, Oct-4, Sox-2, Rex-1, Dnmt3b, Lin-28, Tdgf1, FoxD3, Tert, Utf-1, Gal, Cx43, Gdf3, Gtcm1, Terf1, Terf2, Lefty A, and Lefty B. MSCs are primarily identified by the expression of CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166, and HLA-ABC and lack CD14, CD31, CD34, CD45, CD62E, CD62L, CD62P, and HLA-DR expression. HSCs are mainly isolated based on the expression of CD34, but the combination of this marker with CD133 and CD90, together with a lack of CD38 and other lineage markers, provides the most homogeneous pool of SCs. Here, we present new and alternative markers for SCs, along with microRNA profiles, for these cells.

Introduction

Stem cells (SC) are defined as a class of undifferentiated cells capable of self-renewal, perpetuating their population and giving rise to many types of committed or more specialized cells through differentiation [1]. SCs can be found during all stages of development from the embryo to the adult organism, and they consist of cells with varying differentiation potential.

Cells taken from the zygote to as far as the blastocyst stage are considered totipotent because they have the potential to generate a whole organism [2]. By the blastocyst stage, the cells become more specialized and are considered pluripotent. Embryonic stem cells (ESCs) are obtained at this stage and can generate tissues from the 3 germ layers [3], but they are not able to originate a whole individual [2]. Fetal and adult tissues also have several sources of SCs. These cells, however, have a limited differentiation potential that is less than that of ESCs. Mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) are found among this type of SCs.

The discovery of SCs has brought new possibilities to the scientific and clinical area, as they have the potential to be applied in cell replacement therapy, gene therapy, drug discovery, disease modeling, and developmental biology [4–6]. Nevertheless, the pool of SCs obtained from in vivo and in vitro conditions is not homogeneous; rather, the cells are in several stages of differentiation. Therefore, identifying unambiguous markers is essential for isolating the most primitive cells and for clearly identifying the different stages of undifferentiated and committed cells.

In this context, the aim of this review is to construct a molecular profile, including classical and new candidate molecular markers, of the 3 most studied human SCs: ESCs, MSCs, and HSCs.

Molecular Markers for ESC Characterization

ESCs are commonly isolated from the inner cell mass (ICM) during the blastocyst stage and possess the capacity to self-renew and to originate all cell types of an organism [7]. Since the first cultures of ESCs were established [8,9], considerable effort has been made to characterize a unique ESC-associated molecular signature. In 2007, the International Stem Cell Forum created the so-called "International Stem Cells Initiative" to establish an ESC molecular identity [10]. A total of 59 human ESC (hESC) lines were analyzed for cell-surface antigens and gene expression as potential markers

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for ESCs [10]. In the same year, a consensus ESC gene list and a consensus differentiation gene list were proposed by Assou and coworkers [11] based on 38 publications regarding ESC transcriptomes. They also created an online database [http://amazonia.montp.inserm.fr] where the transcriptome dataset is available.

The set of molecular markers commonly applied to identify ESCs consists of cell-surface proteins and genes specifically expressed in ESCs (Table 1). The characteristic cell-surface markers of ESCs were first detected in human embryonic carcinoma [12–14]. Among them are stage-specific embryonic antigen-3 (SSEA-3) and 4 (SSEA-4) and the tumor rejection antigens (TRA-1-60 and TRA-1-81) [9,15]. These surface markers are observed in the ICM, but they are absent in the 2–8 cell and morula stages [16]. When ESCs are induced to differentiate, these antigens are downregulated, and SSEA-1 is upregulated [16,17]. Moreover, GCTM2, GCTM343, alkaline phosphatase, CD90, CD24, and CD9 are other surface molecules identified in hESCs [9,10,15,16, 18,19].

In addition to surface molecules, there are some genes whose expression is characteristic of ESCs. Classically, the 3 transcription factors Nanog, Oct-4, and Sox-2 are used as indicators of the uncommitted status of an ESC [15,20]. Alternatively, other molecules (Table 2) are cited in the scientific literature as putative markers of ESCs, and all of them have their expression downregulated when these cells are induced to differentiate [9,15,18,19,21–26]. Below, we discuss the genes most commonly used to confirm ESC identity. It should be noted that some of the genes listed in Table 2 are not discussed because there are none or very few studies about their roles in ESCs.

Classical Molecular Markers for ESC

Nanog

Named after the mythological Celtic land of the ever-young *Tir nan Og*, Nanog was first described in 2002 by 2 groups independently [27,28]. This transcription factor is a homeodomain protein whose expression is observed in the morula and ICM but is absent from unfertilized oocytes, 2- to 16-cell embryos, early morula, and trophectoderm [27,29]. Nanog is downregulated when organogenesis is initiated at the time of embryo implantation [27]. The silencing of the Nanog gene leads to the differentiation of ESCs into trophoectoderm and extraembryonic endodermal lineages, along with a downregulation of Oct-4 [29]. In murine ESCs (mESCs), the overexpression of Nanog can maintain these cells in an undifferentiated state even without LIF, likely by the inhibition of Gata4 and Gata6 [28]. The expression level of Nanog seems to be regulated by the inhibitor of differentiation 1 (Id1) protein [30], which acts as a negative regulator of helix-loop-helix DNA-binding proteins [31]. ESCs in which Id1 is knocked down display Nanog expression levels that are 35% lower than wild-type ESCs and exhibit a loss of the capacity to self-renew [31].

Oct-4

Oct-4, also known as Oct-3, Oct-3/4, POU5f1, OTF3, or NF-A3 [32], is another transcription factor that has roles in controlling the pluripotency of ESCs. It is expressed in unfertilized oocytes [7,32] and after fertilization as far as the 10-cell stage the observed transcripts are mainly of maternal origin and were expressed before zygote formation [32]. After the 10-cell stage, Oct-4 expression stabilizes, indicating the beginning of the embryonic production of Oct-4. During the blastocyst stage, Oct-4 can be observed in both the ICM and trophoectoderm, with Oct-4 levels higher in the former [32]. However, Oct-4 is highly expressed in the ICM of the early blastocyst but is absent from the trophoectoderm in mice [33]. The levels of Oct-4 determine the fate of ESCs because its downregulation leads to ESC differentiation into trophoectoderm [33,34], and an upregulation of less than 2-fold leads to ESC differentiation into extraembryonic endoderm and mesoderm [33].

An important point that Oct-4 alone is not sufficient to maintain an undifferentiated phenotype. The withdrawal of LIF from mouse ESCs leads to their differentiation despite the expression of Oct-4 [33].

Sox-2

Sox-2 is included in the SOX B1 group of transcription factors and has a single high-mobility group DNA-binding domain [35]. Together with Oct-4 and Nanog, Sox-2 plays a role in the maintenance of ESC pluripotency [36]. Its expression is first observed during the morula stage, followed

Table 1. The Most Common Molecular Markers Used for Embryonic Stem Cells, Mesenchymal Stem Cells, and Hematopoietic Stem Cells Characterization

SC	*Molecular markers*
ESCs	
Positive markers	SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, Nanog, Oct-4, and Sox-2.
Negative markers	SSEA-1.
MSCs	
Positive markers	CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166, and HLA-ABC.
Negative markers	CD14, CD31, CD34, CD45, CD62E, CD62L, CD62P, and HLA-DR.
HSCs	
Positive markers	CD34, CD90, and CD133.
Negative markers	CD38 and lineage markers[a].

[a]A detailed list of negative lineage markers can be found on Table 8.

SC, stem cell; ESCs, embryonic stem cells; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; SSEA, stage-specific embryonic antigen; TRA, tumor rejection antigens.

Table 2. The Most Cited Candidate Embryonic Stem Cell Maker Genes in Literature

Gene abbreviation	*Gene name*	*Biochemical function*[a]
Cx43	Connexin 43	Component of connexons.
DNMT3B	DNA (cytosine-5) methyltransferase 3β	Required for genome-wide de novo methylation and is essential for the establishment of DNA methylation patterns during development.
FOXD3	Forkhead box D3	Required for maintenance of pluripotent cells in the pre-implantation and peri-implantation stages of embryogenesis.
GAL	Galanin	Contracts smooth muscle of the gastrointestinal and genitourinary tract, regulates growth hormone release, modulates insulin release, and may be involved in the control of adrenal secretion.
GDF3	Growth differentiation factor 3	Control differentiation of ESCs in mice and humans. This molecule plays a role in mesoderm and definitive endoderm formation during the pre-gastrulation stages of development.
PODXL	Podocalyxin-like	Pro-adhesive molecule, enhancing the adherence of cells to immobilized ligands, increasing the rate of migration and cell-cell contacts in an integrin-dependent manner.
LEFTYA	Left-right determination factor A	Required for left-right (L-R) asymmetry determination of organ systems in mammals.
LEFTYB	Left-right determination factor A	Required for left-right axis determination as a regulator of LEFTY2 and NODAL.
LIN28	Cell lineage protein 28	Acts as a "translational enhancer," driving specific mRNAs to polysomes and thus increasing the efficiency of protein synthesis. Its association with the translational machinery and target mRNAs results in an increased number of initiation events per molecule of mRNA and, indirectly, in stabilizing the mRNAs.
NANOG	Nanog	Transcription regulator involved in inner cell mass and ESCs proliferation and self-renewal. Imposes pluripotency on ESCs and prevents their differentiation towards extraembryonic endoderm and trophectoderm lineages.
OCT4	Octamer binding protein 4	Forms a trimeric complex with SOX2 on DNA and controls the expression of a number of genes involved in embryonic development
REX1	Zinc finger protein 42	Involved in self-renewal property of ESCs.
SOX2	SRY-related HMG box 2	Transcription factor that forms a trimeric complex with OCT4 on DNA and controls the expression of a number of genes involved in embryonic development.
TDGF1	Teratocarcinoma-derived growth factor 1	Play a role in the determination of the epiblastic cells that subsequently give rise to the mesoderm.
TERF1	Telomeric repeat binding factor 1	Component of the shelterin complex (telosome) that is involved in the regulation of telomere length and protection.
TERF2	Telomeric repeat binding factor 1	Component of the shelterin complex (telosome) that is involved in the regulation of telomere length and protection.
TERT	Telomerase	Catalytic component of the teleromerase holoenzyme complex whose main activity is the elongation of telomeres by acting as a reverse transcriptase that adds simple sequence repeats to chromosome ends by copying a template sequence within the RNA component of the enzyme.
UTF-1	Undifferentiated embryonic cell transcription factor-1	Acts as a transcriptional coactivator of ATF2, a transcriptional activator, probably constitutive, which binds to the cAMP-responsive element.

[a]Extracted from GeneCards (www.genecards.org).

by the ICM, epiblast, and cells from the extraembryonic ectoderm [36]. In addition, Sox-2 was also detected in the precursor cells of the developing central nervous system and in both male and female germ cells [37]. An up to 2-fold increase in the expression of Sox-2 leads to the differentiation of ESCs to ectoderm (mainly neuroectoderm), endoderm, and trophoectoderm lineages, likely due to an observed downregulation of genes (Oct-4, Nanog, FGF-4, UTF-1, Lefty-1) controlled by Sox-2 [36].

Sox-2-disrupted mice embryos fail to survive after implantation. Cultured cells from the entire blastocyst or ICM of these embryos differentiate into trophoblast cells and extraembryonic endoderm [37], thus suggesting that Sox-2 has a role in the maintenance of the undifferentiated status of epiblast cells.

The transcription factors detailed above act together in the maintenance of the SC status of ESCs. The knockdown of Sox-2 downregulates Oct-4 and Nanog, indicating that these transcription factors act together to maintain SC status [38]. Additionally, Sox-2 is unable to activate its target genes alone and must have a partner protein, which in ESCs is Oct-4 [35]. These 2 transcription factors bind to DNA as a heterodimer [33], and their targets include their own coding genes and Nanog [39–41]. Nanog also regulates the expression of Sox-2 and Oct-4 [37,38] in a feed-forward manner.

Rex-1

Rex-1, also named zinc finger protein 42 (Zfp-42), is a transcription factor that was first identified in mice teratocarcinoma [12]. It has been studied primarily in mESCs, but its expression has also already been reported in hESCs [16,25]. In mice, Rex-1 is downregulated when the cells from the ICM differentiate into embryonic ectoderm, but it remains expressed in trophoblast cells [42]. In ESCs, Rex-1 appears to inhibit their differentiation, which is evidenced by the increased susceptibility of Rex-$1^{-/-}$ cells to differentiate after exposure to retinoic acid [43]. The expression of this transcription factor is regulated by Sox-2, Nanog, and Oct-4 [44]. An Oct-4-binding site has already been reported in the promoter of Rex-1, and its expression seems to be regulated by the levels of this transcription factor [45].

Dnmt3b

Dnmt3b is a de novo methyltransferase detected in oocytes, 2- to 4-cell embryos, and in the blastocyst stage in humans [46]. In mice, it is expressed in the ICM, epiblast, and embryonic ectoderm in a pattern similar to that observed for Oct-4 [46]. It presents 4 splicing variants, but only the Dnmt3b1 isoform is observed at these stages. This variant is observed in ESCs and, upon differentiation, its expression shifts to the Dnmt3b3 variant [47]. In mESCs, Dnmt3b interacts physically with Dnmt3a and stimulates its reciprocal activities [48]. Dnmt3a$^{-/-}$/3b$^{-/-}$ mESCs show a progressive decrease in the levels of methylation together with an increasing inability to differentiate [49]. The impairment in the methylation levels affects the promoters of Oct-4 and Nanog; consequently, abnormal expression of these transcription factors during differentiation is observed [48]. In contrast, Dnmt3b does not seem to have a role in ESC self-renewal [50].

Foxd3

Foxd3, initially termed *Genesis*, belongs to the HNF-3/Forkhead transcriptional regulatory family [51]. In mice, Foxd3 is first detected during the blastocyst stage. It is not observed in either oocytes or during the first cleavage stages [52]. After ESCs differentiate, Foxd3 can still be detected in neural crest cells [53]. A lack of Foxd3 in mESCs increases the number of apoptotic cells [54]. Foxd3$^{-/-}$ mice embryos die during the gastrulation stage because of a loss of epiblast cells and the expansion of extraembryonic tissues [52]. However, the expression of the genes necessary for ESC maintenance (Oct-4, Sox-2, and Nanog) is not altered in Foxd3$^{-/-}$ embryos [52,54]. Thus, Foxd3 seems to be important for the survival and self-renewal of ESCs and to repress their differentiation [54]. There are studies reporting the interaction of Oct-4 and Nanog with Foxd3, although mRNA levels are not altered in cells lacking Foxd3 [54]. Oct-4 was shown to act as a corepressor of Foxd3 [55], and it is regulated by Foxd3 together with Nanog, which counterbalance the inhibitory effect of Oct-4 on its own promoter [56].

Tdgf1

Tdgf1, also termed Cripto, is a member of the EGFP/TGFα growth factor family [57]. It is first detected at the blastocyst stage; during later stages of embryonic development, it is observed in the developing heart in mice [58]. It appears to have a role in blocking neural differentiation and in allowing ESCs to differentiate into cardiac cells [59]. Cripto$^{-/-}$ embryos die before birth and exhibit aberrant development and a lack of cardiogenic differentiation. Tdgf1 suppression in ESCs leads to their differentiation into neuronal lineages [60]. However, the proliferation levels of Crypto$^{-/-}$ ESCs are not different from wild-type ESCs [61].

Lin-28

Lin-28 is an RNA-binding protein that is highly expressed in hESCs [62] and is important for their growth and survival [63]. ESCs lacking Lin-28 show a decrease in their proliferation and a higher number of apoptotic cells when compared to wild-type ESCs [63,64]. High levels of Lin-28 expression at low cell densities slow the cell cycle and leads to differentiation toward an extraembryonic endoderm lineage [62]. Regarding its roles in ESCs, Lin-28 is involved in enhancing mRNA translation and the inhibition of some microRNA (miRNAs). Lin-28 acts on the let-7 miRNA family to block the processing of pri-let-7a and 7g in vitro. When Lin-28 is knocked down, the levels of mature let-7 family members are increased and are accompanied by decreasing in Oct-4 and Nanog expression. [65]. Lin-28 also regulates Oct-4 at the translational level, as its knockdown leads to a reduction in Oct-4 protein levels but not of its mRNA [63,64,66]. Oct-4 is also observed in Lin-28-associated polysomes, indicating that Lin-28 might be involved in the active translation of this transcription factor [66]. Other targets for translational activation are Cdk4 and cyclins A and B [64].

UTF-1

UTF-1 is a transcription factor that is stably associated with chromatin and acts as a transcriptional repressor

[67,68]. During embryonic development in mice, UTF-1 cannot be observed in the morula but is upregulated at the blastocyst stage, specifically in the ICM. Recently, it has been observed in the primitive ectoderm and extraembryonic ectoderm [69]. ESCs with reduced levels of UTF-1 were delayed in differentiation and experienced perturbed EB formation [67,68], but their self-renewal was not affected, which resulted in increased expression levels of several genes. The explanation for this phenotype is that UTF-1 promotes chromatin condensation of its target genes, preventing their aberrant expression [68]. Moreover, it has been suggested that UTF-1 might maintain an ESC chromatin state that is susceptible to differentiation stimuli [67].

UTF-1 is bound by Oct-4 and Sox-2 in regulatory regions located at 3′ position of its gene, as demonstrated by in vitro assays [70,71]. There is an overlap between genes regulated by UTF-1 and those that are targets of Nanog, Sox2, Dax1, Nac1, Oct-4, Klf4, Zfp-281, Rex1, and c-Myc [69].

Novel Cell Markers for ESCs

Applying ESCs to cellular therapy is not feasible for many reasons. First, ESCs display a high potential for generating tumors in vivo. Moreover, the isolation of a pool of ESCs requires the destruction of human embryos, which raises ethical concerns about their use in cell therapy.

For clinical applications, the determination of markers that identify undifferentiated ESCs from a pool of cells ready for transplantation is desirable because this would allow tumor induction to be avoided [72]. Additionally, it is important to achieve a homogeneous pool of ESCs for basic and applied studies in vitro, allowing for the better characterization of cellular and molecular properties of those cells [72].

In addition to the classical ESC markers discussed above, surface proteins and highly expressed genes have been proposed as new ESC markers. Regarding surface proteins, ESCs have been reported to be positive for CD24, CD30, CD49f, CD50, CD90, CD133, CD200, and CD326. From this set of markers, CD133 and CD326 have been proposed as ESC markers due to their downregulation after the induction of neuronal differentiation [73,74]. On the other hand, CD24 may not be proposed as a marker due to its detection in differentiated cells [75]. CD30, although expressed in ESCs and downregulated under differentiation, was reported to be present on ESCs plasma membrane as a consequence of the culture media, specifically in serum-free condition [76]. Together with those proteins, a new surface marker was recently added to the list of the SSEA-associated membrane molecules of ESCs. Named SSEA-5, its expression is detected in the ICM of human blastocist and it was reported to be 5-fold lower when ESCs were induced to differentiate. Moreover, SSEA-5 was suggested as a marker for removing remaining undifferentiated ESCs in conditions of differentiation induction [74]. Additionally, the combined analysis of SSEA-5 together with CD9, CD50, CD90, and CD200 was more effective in detecting potential teratoma cells within differentiating ESCs [74].

In this sense, it is important to note that additional surface markers can be discovered from plasma membrane proteomics studies (for more details on ESCs plasma membrane proteomics, see the Ref. [77]).

Within ESCs, other highly expressed genes and putative new markers include line-type transposase domain containing 1 protein (L1TD1), Forkhead box O1 (FOXO1), and E1B-AP5. L1TD1 is highly expressed in ESCs and is absent from most adult tissues. *In silico* analysis revealed that it is restricted to the blastocyst stage, where its expression is downregulated during differentiation in a pattern similar to that observed for Oct-4, Nanog, and Sox-2. In addition, L1TD1 is a downstream target for Nanog protein [78].

FOXO1 is also expressed at higher levels in ESCs, is downregulated during ESC differentiation, and has been implicated in the regulation of ESC pluripotency. Knockdown of FOXO1 does not alter the self-renewal of ESCs but is accompanied by the downregulation of Oct-4, Nanog, and Sox-2, leading to the spontaneous differentiation of ESCs into mesoderm and endoderm lineages. Moreover, FOXO1 appears to act by activating Sox-2 and Oct-4 expression [79].

Adenovirus early region 1B-associated protein 5 (E1B-AP5) is a nuclear RNA-binding protein observed in the nucleus and cytoplasm, where its phosphorylated form is presented at the ESC surface and can be used as a specific marker for ESCs. Cells positive for this marker are also positive for the classical ESC markers (ie, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, Nanog, Oct-4, and Sox-2). Moreover, this surface protein was not detected after cell differentiation [72].

Transcriptome studies are also a potential source of new ESC markers [18,19,21–26]. These reports have revealed many genes that are enriched in ESCs and also downregulated during cell differentiation. All of these genes can be considered putative markers of an undifferentiated state, but only a few have had their roles in ESCs investigated. Table 3 summarizes the results from transcriptome studies comparing the expression profiles of ESCs and differentiated cells.

miRNAs associated with ESCs

Studies of mESCs have revealed that null Dicer cells display a pronounced loss in proliferative capacity [84]. The lack of DGCR8, another important member of the miRNA generation pathway [85], appears to cause alterations in mESCs; these cells exhibited extended population doubling, increased numbers of cells in the G_1 phase, and differentiation impairment [86]. These results indicate that miRNAs possibly have important roles in the biology of ESCs, and several have been identified as being exclusive to those cells, such as clusters miR-302-367, miR-520, and miR-371/372/373.

The miR-302-367 cluster is located on chromosome 4 and is composed of 9 miRNAs disposed in a polycistronic manner: miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d, miR-367, and miR-367* [87]. This cluster is highly expressed in ESCs [88,89], more than 20-fold when compared to adult cells or hMSCs, and its expression is rapidly downregulated when cells are induced to differentiate. It has also been detected in induced pluripotent cells [90]. In addition, putative binding sites for Oct3/4, Nanog, Rex1, and Sox-2 have been identified within its promoter sequence, proving its function in ESCs [88]. Indeed, Card et al. [91] showed that Oct-4 and Sox-2 bind to the promoter of this cluster.

Table 3. Genes Expressed at Higher Levels in Embryonic Stem Cells When Compared to Differentiated Cells Based in Transcriptomic Data

ESC lineage	*Differentiated cells*	*ESC-associated transcripts differentially expressed*	*Reference*
HES3, HES4	Cells from fetal brain, fetal liver, adult brain, placenta, adult testis, adult kidney, adult lung, adult heart	CCNB1, CFL1, CGI-48, CHK2, CKS1B, CLDN6, Cx43, DNMT3B, ERH, FAM60A, FAS, FLJ10713, FZD7, GDF3, GTCM1, HMGA1, LIN-28, NCL, Numatrin, PCNA, PNF1, POU5F1, REX-1, SFRP1, SOX-2, TDGF-1, TERF-1, TMSB15A.	[25]
BG02	Embryoid body	ARL8, BRIX, C15orf15, C20orf1, C20orf129, C20orf168, CCNB1, CCNC, CCT8, CDC2, CER1, CRABP1, CRABP2, CYP26A1, DDX21, DNMT3B, EIF4A1, ELOVL6, EPRS, FABP5, GAL, GDF3, GJA1, GSH1, GTCM-1, HDAC2, HMGB2, HMGIY, HNRPA1, HNRPAB, HSSG1, IDH1, IFITM1, IMP-2, IMPDH2, Jade-1, KIAA1573, KIF4A, KPNA2, Laminin receptor, LAPTM4B, LDHB, LEFTB, Lin-28, LRRN1, MAD2L2, MGST1, MTHFD1, MTHFD2, Nanog, NASP, NBR2, NME2, NPM1, NS, Numatrin, POU5F1, PPAT, PSMA2, PSMA3, PTTG1, RAMP, RPL24, RPL4, RPL6, RPL7, SEMA6A, SET, SFRP2, SLC16A1, SMS, SNRPF, SOX2, SSB, STK12, TD-60, TDGF1, TK1.	[26]
GE01/GE07/GE09 (pooled RNA)	Embryoid body	C20orf129, CCNB1, CCNC, CRABP1, CYP26A1, ELOVL6, FABP5, FLJ12581/Nanog, HDAC2, HSPA4, JADE-1, KIAA1573, KPNA2, LEFTB, MGC27165, GST1, NASP, NS, PSMA3, PTTG1, RAMP, RPL17, SEMA6A, SFRP2, SLC16A1, TDGF1, TNNT1, ZNF257.	[80]
H1	Embryoid bodies	AK3, DUSP6, E2IG5, FLJ10713, FRAT2, GAL, LEFT B, MYO10, PLP1, POU5F1, PROML1, PSIP2, SPS, STRIN, VRK1, VSNL1.	[81]
CH3, CH4	Embryoid bodies	A2ML1, AASS, ADCY2, ADD2, AK5, ARTN, C14orf115, C1orf182, C9orf61, CABYR, CACNA1G, CAMKV, CDCA7L, CHST4, CKMT1, CNTN1, CRABP1, CTGF, CXCL6, DCAMKL1, DDX25, DEPDC2, DNMT3B, DPPA2, DPPA4, FBXL16, FGF2, FLJ12505, FLJ12684, FLJ30707, GABRA5, GABRB3, GAP43, GPC4, GPR19, GPR23, GRPR, HESX1, INA, INDO, INHBE, ITGB1BP3, LEFTY1, LOC168474, LOC283174, MDN1, NALP4, NANOG, NAP1L2, NEF3, NEFL, NELL2, NMNAT2, NMU, NPTX2, OLFM1, OSBPL6, PCSK9, POU5F1, PTHB1, PTPRB, PTPRZ1, RAB39B, RARRES2, RASL11B, RDH12, RET, RNF182,SAMHD, SCG3, SCGB3A2, SEPHS1, SLC10A4, SLC7A3, SOX2, SYT1, TAC1, TAF4B, TDGF1,TERF1, TIMP4 TNFRSF8, USP44, WIF1, ZIC3.	[82]
H1, H7, H9	Embryoid bodies, immature hepatocytes and putative neural cells	FLJ35207, FOXH1, FOXO1A, GABRB3, GAP43, GRPR, PHC1, PODXL1, POU5F1, PRDM14, PTPRZ1, SALL1, SALL2, SZF1, THY1, ZIC2, ZIC3, ZNF206.	[21]
BG01, BG02, BG03	Embryoid bodies	CKMT1, DIAPH2, DNMT3B, EBAF, GABRB3, GDF3, GYLTL1B, IFITM1, LCK, LIN28, MIBP, NTS, PMAIP1, POU5F1, TDGF1, UTF1, ZNF206, ZPF42.	[83]

FOXO1, forkhead box O1.

Another typical ESC miRNA cluster is miR-520. It is located on chromosome 19 and is composed of 21 miRNAs: 515-5p, miR-517a, miR-517b, miR-517c, miR-518b, miR-518c, miR-519b, miR-519c, miR-519e, miR-520a, miR-520b, miR-520c, miR-520d, miR-520e, miR-520f, miR-520g, miR-520h, miR-521, miR-524*, miR-525-3p, and miR-526b* [89]. The roles of these 2 clusters in ESCs include (i) cell growth arrest, (ii) negative regulation of cellular metabolic processes, (iii) negative regulation of transcription, and (iv) small GTPase-mediated signal transduction [89].

The miRNAs miR-371-372-373 also comprise a cluster located on chromosome 19. This cluster is highly expressed in ESCs [88], and as observed for other miRNA clusters, its expression is rapidly downregulated when the cells are induced to differentiate [92].

Moreover, miRNAs initially observed in MSCs were also identified in ESCs, such as miR-9, miR-28, miR-29, miR-42, miR-63, and miR-89 [93]. These miRNAs are downregulated during the transition from ESCs to endothelial cells. Interestingly, miR-9 and miR-28 act by inhibiting CDH5 and

endoglin translation, respectively [94]. In addition to the miRNAs identified in ESCs, low levels of piRNA, small noncoding RNAs that interact with Piwi proteins, were also observed in these cells [94]. The occurrence of 3′ modifications and RNA editing in miRNAs observed in ESCs have also been reported [94].

The roles of miRNAs in ESCs are still not fully understood. Research in mice has revealed that miRNAs typical of mESCs have roles in cell proliferation, being implicated in promoting G1 to S phase transition through the suppression of the G1/S inhibitors [95]. Similar results were observed in hESCs; the miR-302 cluster was shown to be involved in the progression from G1 to S phase. Cell cycle regulators, cyclin D1, and possibly Cdk4 are targets of the miRNAs from this cluster [91]. Recently, NR2F2 (nuclear receptor subfamily 2, group F, member 2) was reported as a target for miR-302 [96]. Oct-4 induces the miR-302 expression and it is inhibited by NR2F2 [96].

Although little is known about the function of the miRNAs that are enriched in ESCs, some of them have already been reported as good reprogramming factors for the induced pluripotent stem cells (iPSCs) generation. Among the miRNAs successfully applied on reprogramming of fully differentiated cells into iPSCs, there are the cluster miR-302-367 [97], the miR-302b alone [96,98], the combination of miR-302, miR-369, and miR-200c [99], the miR-302b associated with miR-372, and the miR-372 alone [98]. These miRNAs are generally associated with factors as Oct-4, Sox-2, Klf4, Nanog, and c-Myc [96–98].

Published miRNA profiles of hESCs are summarized on Table 4. The data displayed correspond to the highly expressed miRNAs as listed by the authors of each article.

Interestingly, a comparison between ESCs and iPSCs showed that both cells have similar groups of upregulated miRNAs. Among them there are the clusters of miR-302-367 and miR-17-92 (composed by miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92). However, members of the miR-371-372-373 and miR-520 clusters were downregulated in iPSCs [90]. For more details on iPSCs miRNA profile, see Ref. [90].

Molecular Markers for MSC Characterization

MSCs are multipotent, adherent SCs capable of differentiating into osteoblasts, adipocytes, and chondroblasts [104] and also to nonmesenchymal lineages such as pancreatic islands [105], hepatocytes [106,107], and neuron-like cells [108]. MSCs were first isolated from bone marrow by A.J. Friedenstein [109]; nevertheless, many researchers have been able to isolate them from other regions of the human body, including adipose tissue [110,111], heart [111], trabecular bone [112], vessels [113], peripheral blood [114], skin [115], deciduous teeth [116], and many others. MSCs are also found in fetal tissues and fluids, such as amniotic fluid, umbilical cord blood [117], amnion, and placenta [118].

When MSCs are isolated from different tissues and organs, a heterogeneous pool of cells with several differentiation potentials can be obtained [119]. In addition, MSCs are not present in high quantities in tissues. For example, in the bone marrow, MSCs comprise 0.01% to 0.001% of the total cell number [96]. Therefore, the definition of markers that make it possible to isolate the most primitive MSCs and to identify those subpopulations with different potentials to generate mature cells is necessary to improve the cellular and

Table 4. microRNAs Highly Expressed in Embryonic Stem Cells, When Compared to Embryoid Bodies or Differentiated Cells

Lineage	*Highly expressed miRNA profile*	*Reference*
hES-T3	miR-20b, miR-26b, miR-200c, miR-302a*, miR-302b*, miR-302c*, miR-302d, miR-367, miR-371, miR-372, miR-373.	[100]
H9, I6,BG01v	miR-96, miR-127, miR-141, miR-200b, miR-200c, miR-299-3p, miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302d, miR-324-3p, miR-367, miR-369-3p, miR-372, miR-515-5p, miR-517a, miR-517b, miR-517c, miR-518b, miR-518c, miR-519b, miR-519c, miR-519e, miR-520a, miR-520b, miR-520c, miR-520d, miR-520e, miR-520f, miR-520g, miR-520h, miR-521, miR-524*, miR-525, miR-525*, miR-526b*, miR-550-2, miR-612.	[89]
WA09, WA01, HSF6, HUES7, HUES13	miR-302 cluster, miR-371/372/373 cluster, primate-specific placenta associated cluster (54 miRNAs), miR-17, miR-106a.	[101]
SNU-hES3	miR-154*, miR-200c, miR-302a*, miR-302a, miR-302b*, miR-302b, miR-302c, miR-302c*, miR-302d, miR-371, miR-372, miR-373, miR-373*, miR-368.	[97]
Cyt25, Cyt203, HES2, HES 3, HES4	miR-21, miR-200c, miR-222, miR-296, miR-302a, miR-302c, miR-367, miR-371, miR-372, miR-373, miR-320d, miR-494.	[102]
H1	miR-18b, miR-20b, miR-92b, miR-154, miR-184, miR-187, miR-302a, miR-302b, miR-302c, miR-302d, miR-324-3p, miR-363*, miR-512-3p, miR-518b, miR-518c, miR-519d, miR-520g, miR-524*, miR-1323, miR-1901, miR-1908, miR-1910, miR-1911.	[103]
H9	Let-7a, Let-7b, Let-7c, Let-7d, Let-7g, Let-7i, miR-7, miR-92, miR-106b, miR-155, miR-181d, miR-184, miR-185, miR-187, miR-211, miR-222, miR-296, miR-302a, miR-302d, miR-331, miR-424, miR-484, miR-486, miR-503, miR-519c, miR-520, miR-518c, miR-519a, miR-574, miR-594, miR-744, miR-760, miR-766, miR-766*, miR-874, miR-877, miR-941, miR-1298, miR-1308, miR-1246, miR-1254, miR-1261, miR-1266, miR-1268, miR-1272, miR-1275, miR-1301, miR-1306, miR-1307, miR-1308.	[98]

Asterisk indicates miRNA strands less commonly found associated with Argonauta complex.
miRNA, microRNA.

molecular characterization of MSCs. Thus, the focus of this section will be on information about classical markers for MSCs, recently reported or alternative markers, and the miRNA profile of MSCs.

In 2006, The International Society for Cellular Therapy published the minimal criteria to identify a human SC as an MSC [120]. Among these are the expression of the surface proteins CD73, CD90, and CD105 together with the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR [120]. However, many other markers have been identified and used by researchers (Table 5).

Some of the markers listed above appear to be dependent on the original tissue where the MSCs were isolated, but many are common among all MSCs. Based on the scientific literature, we suggest a list of common positive and negative surface markers found in MSCs (Table 1).

Together with these surface markers, several articles have reported the expression of some ESC-associated markers in MSCs from different sources (Table 6). The expression levels of some of these markers are downregulated when MSCs are induced to differentiate followed by an increase in SSEA-1 [122,124]. These changes in MSC marker expression recapitulate what is observed during ESC differentiation.

The real function of the ESC-associated markers in MSCs is not completely understood, and their presence has been considered as a primitive phenotype and an indication of the stem potential of the cells [141]. On the other hand, the expression of Nanog in MSCs could be due to a transition from in vivo to in vitro conditions, from the quiescent to the proliferative state [111]. In fact, Nanog seems to have roles in the maintenance and differentiation of MSCs in vitro. Studies with murine MSCs reported that the expression of this transcription factor is downregulated during differentiation. In addition, Nanog overexpression or knockdown leads to an increase or a reduction in cell proliferation, respectively [152]. In vitro, the knockdown of *NANOG* also resulted in the elevation of osteocalcin expression, a marker of osteogenic differentiation. In vivo, during the healing of an induced bone injury, Nanog expression was detected early in the process, preceding the expression of osteogenic differentiation markers. The timing of Nanog expression can be explained by the necessity of MSC population expansion, whose cells will be recruited for the healing process [152]. When the same healing experiment was repeated and Nanog expression was blocked, osteogenic differentiation was impaired, and adipogenic cells were observed [152]. In fact, Nanog seems to be related to favoring MSC differentiation to an osteogenic rather than an adipogenic fate. A decrease in Nanog expression is observed during adipogenic differentiation [153], and when Nanog is overexpressed in MSCs induced to adipogenic differentiation, there is a decrease in the expression of adipogenic markers and weaker Oil red staining [154].

Novel and Alternative MSC-Associated Markers

Although great progress has been made regarding the definition of MSC markers, we are still far from defining a specific molecular signature for these cell types. Here, we discuss novel and alternative markers reported for MSCs.

A study of plasma membrane surface proteins of MSCs derived from bone marrow detected 113 transcripts, including 20 CDs, expressed by MSCs but not by hematopoietic cells. From this group, 8 markers (CD49b, CD73, CD90, CD105, CD130, CD146, CD200, and integrin $\alpha V/\beta 5$) allowed for the isolation of MSCs from bone marrow mononuclear cells. CD200 has been proposed as a molecular marker to isolate bone marrow MSCs because cells isolated using this marker display a high enrichment in colony-forming units-fibroblasts when compared to the total mononuclear fraction before sorting and were able to differentiate into osteogenic, adipogenic, and chondrogenic lineages [155].

Battula et al. [151] have proposed FZD9 (Frizzled-9 or CD349) as a marker for primitive MSCs. The cells isolated from placenta that display FZD9 exhibited high clonogenic potential, which was enhanced when FZD9 was combined with CD10 and CD26 [156]. Additionally, this fraction expressed high levels of Oct-4, Nanog, and SSEA-4 [147]. However, Tran et al. [157] proposed that FZD9 is a good marker for the isolation of MSCs specifically for arterio/angiogenic therapy but not for discriminating between MSCs and non-MSCs. The expression of this marker was also observed in MSCs isolated from the periodontal ligament and was downregulated after the second passage [157].

Kaltz et al. [158] reported NOTCH-3 as a marker for the enrichment of MSCs capable of both osteogenic and adipogenic differentiation from bone marrow. In addition, the same research group demonstrated that ITGA11 and MSCA-1 could be used as markers for bone marrow MSC-derived cells that are mainly unipotent: osteogenic or adipogenic, respectively.

Using an antibody against the nerve growth factor receptor (NGFR or CD271), Quirici et al. [159] were able to isolate highly proliferative MSCs that were prone to osteogenic and adipogenic differentiation. Moreover, the authors observed that these cells were able to support the growth of hematopoietic progenitors [159]. The $CD271^{bright}$ subpopulation had been reported to contain a fraction of highly clonogenic bone marrow MSCs [160]. The $MSCA\text{-}1^{+}CD56^{+}$ fraction of this subpopulation demonstrated a high clonogenic potential and osteogenic, chondrogenic, and pancreatic differentiation, but it was unable to generate adipose cells [161]. These cells also present the capacity to inhibit T-cell proliferation and the differentiation of monocytes to dendritic cells [161]. Bühring et al. [160] observed that combining CD271 with CD140b, W8B, HEK-3D6, FZD-9, and CD56 makes it possible to isolate highly clonogenic MSCs.

GD2, a disialoganglioside, is another proposed MSC marker. It is detected in $CD45^{-}$ and $CD73^{+}$, $CD90^{+}$ MSCs isolated from bone marrow, but it is not observed in other cells from the same compartment, such as leukocytes, myeloid cells, T-lymphocytes, B-lymphocytes, or hematopoietic progenitors [162]. MSCs isolated from adipose tissue also present GD2 at the same levels as detected in MSCs from the bone marrow [162]. MSCs isolated from the umbilical cord also present GD2, and it can be detected as far as the 10th passage [163]. Cells isolated based on this marker were able to differentiate into adipocytes, osteoblasts, chondrocytes, and neuronal cells [162–164]. However, it has been reported that the $GD2^{-}$ and $GD2^{+}$ fractions have the same MSC marker profile and a similar potential to differentiate. Moreover, $GD2^{-}$ cells show higher proliferation rates than $GD2^{+}$ cells [164]. Nevertheless, the inhibition of GD2 synthesis leads to a block of neuronal differentiation [164].

Table 5. Surface Markers for Different Mesenchymal Stem Cells Reported in Scientific Literature

Source	*Positive markers*	*Negative markers*	*References*
	Adult origin		
Bone marrow	CD29, CD44, CD71, CD73, CD90, CD105, CD106, STRO-1.	CD13, CD14, CD34, CD45, CD133, CD144.	[106,117,121]
Deciduous teeth	CD13, CD73, CD105, CD146, STRO-1.	CD34, CD43, CD45.	[116,122]
Permanent teeth	STRO-1.	No negative markers reported.	[122]
Periodontal ligament	CD9, CD13, CD29, CD44, CD49a, CD49b, CD49c, CD49d, CD49e, CD51/61, CD71, CD73, CD90, CD105, CD106, CD119, CD120a, CD146, CD166, STRO-1, HLA-ABC.	CD2, CD3, CD8a, CD11a, CD14, CD16, CD18, CD19, CD20, CD24, CD25, CD31, CD33, CD34, CD38, CD41a, CD45, CD50, CD54, CD56, CD62E, CD62L, CD62p, CD66b, CD104, CD117, CD121a, CD123, CD124, CD126, CD133, CD235a, CD318, HLA-DR, STRO-1.	[124–126]
Apical papilla	CD146, STRO-1.	No negative markers reported.	[127]
Skin	CD13, CD29, CD44, CD49d, CD71, CD73, CD105, CD166.	CD10, CD11b, CD14, CD31, CD34, CD45, CD133, CD106, HLA-DR.	[128–130]
Endometrium	CD29, CD44, CD73, CD90, CD105, CD146.	CD31, CD34, CD45, STRO-1.	[131]
Adipose tissue	CD9, CD10, CD13, CD29, CD34*, CD44, CD49d, CD49e*, CD54, CD55, CD59, CD71, CD73, CD90, CD105, CD166.STRO-1, HLA-ABC.	CD11a, CD11b, CD11c, CD14, CD16, CD18, CD31, CD34, CD45, CD50, CD56, CD61, CD62e, CD104, CD106, CD133, CD144, HLA-DR.	[110,117,132]
Periferal blood	CD44, CD105, STRO-1.	CD3, CD14, CD20, CD45, CD68, CD106, HLA-DR.	[114]
Menstrual blood	CD9, CD29, CD41a, CD44, CD59, CD73, CD90, CD105.	CD14, CD31, CD34, CD38, CD45, CD133, STRO-1.	[133]
Saphenous vein	CD13, CD29, CD44, CD54, CD90, HLA-I	CD14, CD31, CD34, CD45, CD133.	[134]
Limbal stroma–eye	CD29, CD54, CD71, CD90, CD105, CD106, CD166.	CD11a, CD11c, CD14, CD31, CD34, CD45, CD138.	[135]
Lung	CD13, CD73, CD90, CD105, CD166, HLA-I, STRO-1.	CD14, CD34, CD45, HLA-DR, HLA-DQ.	[136,137]
	Fetal origin		
Amniotic fluid	CD13, CD29, CD44, CD49e, CD73, CD90, CD105, CD166, HLA-ABC.	CD1a,CD10,CD11a,CD11b,CD14, CD31,CD34,CD45, CD49d,CD50, CD54,CD62E,CD62P,CD117,CD133, HLA-DR,HLA-DP,HLA-DQ.	[138,139]
Bone marrow	CD29, CD44, CD49e, CD54, CD58, CD73, CD90, C105, CD106, CD123, CD166, HLA-ABC.	CD14,CD25,CD31,CD34,CD40,CD45, CD49d,CD50, CD62E,CD62L,CD62P, CD68,CD80,CD86,CD106, CD120a, CD120b,CD127,HLA-DR.	[140,141]
Peripheral blood	CD29, CD44, CD73, C105, CD106.	CD14,CD31,CD34,CD45,CD68,HLA-DR	[140]
Liver	CD29, CD44, CD49e, CD54, CD58, CD73, CD90, CD105, CD106, CD123, CD166, HLA-ABC.	CD14,CD25,CD31,CD34,CD40,CD45, CD49d,CD50, CD62L,CD62P,CD68, CD80,CD86,CD106,CD120a, CD120b, CD127,HLA-DR.	[140,141]
Lung	CD44, CD49e, CD54, CD58, CD73, CD90, CD105, CD123, CD166, HLA-ABC.	CD14,CD25,CD31,CD34,CD40,CD45, CD49d, CD50,CD62E,CD62L,CD62P, CD80,CD86,CD106, CD120a,CD120b, CD127,HLA-DR.	[141]
Pâncreas	CD13, CD29, CD44.	CD34,CD45,HLA-DR.	[142]
Placenta	CD29, CD44, CD49e, CD54, CD73, CD90, CD105, CD166, HLA-ABC.	CD31,CD34,CD45,CD49d,CD123, CD133,HLA-DR.	[118,143]
Spleen	CD44, CD49e, CD54, CD58, CD73, CD90, CD105, CD123, CD166, HLA-ABC.	CD14,CD25,CD31,CD34,CD40,CD45, CD49d, CD50,CD62E,CD62L,CD62P, CD80,CD86,CD106, CD120a,CD120b, CD127,HLA-DR.	[141]
Umbilical cord vein	CD29, CD73, CD90, CD105, CD166.	CD14,CD31,CD34,CD45.	[105]
Umbilical cord blood	CD29, CD44, CD49b, CD49d, CD51, CD58, CD71, CD73, CD90, CD105, CD106, HLA-ABC.	CD3,CD7,CD11a,CD11b,CD14,CD19,CD33, CD34, CD45,CD62L,CD62P,CD117, CD133,CD135,CD31, CD144, LA-DR.	[118,144,145]
Wharton's jelly	CD10, CD13, CD29, CD44, CD49e, CD51, CD68,CD73, CD80, CD90, CD105, CD117, CD166, HLA-ABC.	CD14,CD31,CD33,CD34,CD45,CD56, CD86,CD163, HLA-DR.	[146]

*Less than 28% of positive cells for this marker. The authors considered this percentage as positive.

Table 6. Major Embryonic Stem Cells Markers Observed in Mesenchymal Stem Cells

ESC marker detected	*Source of cells*	*Reference*
Oct-4, Nanog, Sox2, SSEA-4	Bone marrow, adipose tissue, dermis, and heart	[115]
OCT-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, low level of cells were TRA-1-80	Pulp of deciduous teeth	[122]
OCT-4, Nanog, SSEA-4	Amniotic fluid	[139,147]
OCT-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81	Fetal bone marrow, liver, and blood	[148]
SSEA-4	Dermis	[149]
SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4, Nanog, Sox-2, REX-1	Periodontal ligament	[124,150]
Nanog	Bone marrow, adipose, and cardiac tissue	[111]
Oct-4	Menstrual blood	[133]
Oct-4, Nanog, SSEA-4	Bone marrow and placenta	[151]

SSEA-4, a classical ESC marker, has been demonstrated to be useful for isolating MSCs with potential to differentiate into adipocytes, chondroblasts, osteoblasts, pancreatic, and neuronal cells, together with the capacity for forming organized bone tissue in vivo [124,151,165].

miRNAs associated with MSCs

Little is known about the miRNAs involved in the regulation of MSCs. The majority of the reports about miRNAs expressed in MSCs have focused on molecules with roles in the osteogenic [166–168], adipogenic [169–171], and chondrogenic [172,174,175] differentiation pathways. There are few reports exploring miRNA profiles and their functions associated with the maintenance of the stem state of MSCs (Table 7).

Molecular Markers for the Characterization of HSCs

HSCs are adult SCs found in the bone marrow [178], umbilical cord blood [179], fetal liver [180], and peripheral blood after mobilization [181,182]. A true HSC must be able to self-renew and give rise to all of the mature cells that comprise the hematopoietic system [183]. One approach to check the stemness of a candidate HSC is to test its capacity for performing a long-term reconstitution of hematopoiesis in recipient animals subjected to myeloablative treatment [183].

The identification of molecular markers that can characterize true primitive HSCs will make their isolation from the heterogeneous pool of cells where they are located easier. Below, we list the classical molecular markers reported by the scientific literature for the enrichment of highly primitive HSCs (Table 1), together with new candidate markers and the miRNA profiles of these cells.

Classical and Alternative HSC-Associated Markers

$CD34^+$

$CD34^+$ is a member of the sialomucin family of surface molecules [184]. It is the classical marker for HSCs, although its functions are not completely understood. It has been speculated that CD34 plays roles in cell adhesion and/or HSC differentiation [184]. Murine cells constitutively expressing this surface protein failed to completely

Table 7. microRNA Profile Reported for Mesenchymal Stem Cells from Different Sources

MSC source	*miRNAs*	*Reference*
Bone marrow	miR-15b, miR-16, miR-21, miR-22, miR-23a, miR-23b, miR-24, miR-26a, miR-27a, miR-27b, miR-145, miR-29a, miR-30d, miR-99a, miR-100, miR-103, miR-107, miR-125a, miR-130a, miR-143, miR-181a, miR-191, miR-193a, miR-193b, miR-199a, miR-199a*, miR-210, miR-214, miR-221, miR-222, miR-320.	[166]
Bone marrow	miR-18b, miR-21, miR-122a, miR-132, miR-140, miR-143, miR-145, miR-181a*, miR-181a-2, miR-181c, miR-335*, miR-337, miR-340, miR-409-5p, miR-431, miR-491, miR-519b, miR-520f, miR-520e, miR-520g, miR-652.	[173]
Adipose tissue	miR-16, miR-19b, miR-20a, miR-21, miR-24, miR-26a, miR-26b, miR-29a, miR-30c, miR-31, miR-92, miR-93, miR-99a, miR-119a, miR-125a, miR-125b, miR-127, miR-140, miR-146a, miR-146b, miR-152, miR-106b, miR-181d, miR-186, miR-190, miR-19, miR-214, miR-221, miR-270, miR-320, miR-339, miR-342, miR-365, miR-376a, Let-7a, Let-7b, Let-7g, Let-7i.	[176]
Bone marrow	miR-16, miR-21, miR-22, miR-23a, miR-23b, miR-24, miR-26a, miR-27a, miR-27b, miR-29a, miR-30a-5p, miR-31, miR-100, miR-125a, miR-125b, miR-143, miR-145, miR-152, miR-191, miR-199a, miR-199a-AS, miR-214, miR-221, miR-222, miR-320, Let-7a, Let-7c, Let-7d, Let-7e, Let-7f, Let-7i, Lrt-7b.	[177]

Asterisk indicates miRNA strands less commonly found associated with Argonauta complex.

differentiate [185]. Nevertheless, Nielsen and McNagny [186] argue that this blockage of differentiation may be due to inhibitory effects of CD34 on cell adhesion.

The bone marrow fraction positive for this molecule is known to be capable of hematopoietic reconstitution in recipients that underwent myeloablative therapy [187,188], indicating the presence of primitive HSCs. Interestingly, this marker is also expressed in vascular endothelial cells [189].

Nevertheless, there is no consensus regarding whether the most primitive HSCs are located in the $CD34^+$ fraction. In 1996, Osawa et al. [190] observed that murine cells negative for this marker were able to engraft and reconstitute hematopoiesis in irradiated animals more efficiently than $CD34^+$ cells. Further studies using human HSCs confirmed that cells lacking CD34 are able to engraft and differentiate into multilineage hematopoietic cells in vivo [191–193]. These cells can be isolated from the fetal liver, fetal blood, umbilical cord blood, peripheral blood, and bone marrow [191]. Analysis of $CD34^-$ cells isolated from the bone marrow and umbilical cord blood revealed that they lack the HLA-DR and CD90 antigens and are rarer than $CD34^+$ cells [191].

Some authors have raised the possibility of $CD34^-$ cells being a more primitive precursor than $CD34^+$ [191] based in the fact that $CD34^+$ cells were identified in myeloablated receptors that received $CD34^-$ cells [190–193]. Dao et al. [194] proposed that cells can interconvert between $CD34^+$ and $CD34^-$ based on their observation that $CD34^+$ grafts give rise to $CD34^-$ cells, and when these cells were transplanted to secondary recipients, they were able generate $CD34^+$ cells. However, the issue about what kind of cell ($CD34^+$ or $CD34^-$) is the most primitive HSC is a long way from being solved. In contrast to the previously mentioned observations, Gao et al. [195] utilized 3 different sources of HSCs and 4 purification methods and observed no engraftment in mice by the $CD34^-$ fraction. The same results were observed by Bhatia et al. [196], who reported that no engraftment was achieved even when 2.6×10^6 $CD34^-$ cells were injected into recipient mice [196].

Nevertheless, some interesting findings can possibly help to explain these contradictory results. Nakamura et al. [197] observed that, when cultured with murine stromal cells as feeders, $CD34^-Lin^-$ cells turn from nonproliferative to proliferative, generate $CD34^+$ cells, and exhibit colony-forming activity. Additionally, these cells were only able to engraft into nonobese diabetes/severe combined immunodeficiency (NOD/SCID) mice when cultured under these conditions. Interestingly, the range of engraftment was positively related to the level of $CD34^+$ cells. In the reports showing $CD34^-$ engraftment in irradiated recipients, the cells or the mice were exposed to human interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor [191–193]. Moreover, Gallacher et al. [198] isolated different subfractions of $CD34^-CD38^-Lin^-$ and observed that only $CD133^+$ cells were capable of engrafting in NOD/SCID mice. This fraction represents only 0.2% of the $CD34^-CD38^-$ cells. It is possible that the $CD34^-$ fraction contains primitive HSCs, and the negative results for hematopoietic multilineage differentiation in vitro and lack of engraftment in vivo may be due to the low levels of the true primitive $CD34^-$ cells and/or lack of pre-stimulation.

Although a consensus has not been established, HSCs are still being commonly obtained based on their expression of CD34. However, the enrichment of HSCs based on this marker results in a heterogeneous pool of cells [199] in which only a small number of cells can be considered SCs [200]. Therefore, other markers must be applied to subdivide this fraction and identify the most primitive HSCs.

CD133

CD133, also known as AC133, is an HSC surface marker that is restricted to the $CD34^+$ fraction. The percentage of $CD133^+$ cells is generally lower than that of $CD34^+$ cells, comprising 20%–60% of the $CD34^+$ cells isolated from the adult bone marrow, umbilical cord blood, peripheral blood, fetal liver, and bone marrow [201]. The $CD133^+$ fraction of the $C34^+$ HSC pool exhibits a high capacity for expansion, is enriched for megakaryocytic progenitor cells, and is able to differentiate into a larger number of erythroid cells [202]. These cells can home to and engraft in the bone marrow of myeloablated mice and sheep, primary and secondary recipients in the last case [201,203].

However, CD133 is not a marker that is exclusive to HSCs. In mice ESCs, CD133 is upregulated in committed and early progenitor cells and has been proposed to be a molecular marker of this stage of differentiation [204]; CD133 is also found in fetal and adult hepatic SCs [205] and in neural SCs [206]. Moreover, CD133 has been proposed to be a common marker for 2 cell fractions, $CD34^+$ and $CD34^-$, which may be enriched for the most primitive SCs [199]. The engraftment of the latter fraction in NOD/SCID mice is only observed when they are enriched for CD133.

Thy-1

Thy-1, also known as CD90 or CDw90, is observed in cells lacking or with low levels of expression of Lin^-, *c-kit*, CD38, CD45RA, CD71, and HLA-DR [207, 208].

Studies using umbilical cord blood [209], peripheral blood [210], and fetal [207] and adult bone marrow-derived cells have shown that the $CD34^+Thy\text{-}1^+$ subset is capable of generating long-term cultures and can give raise to multilineage differentiated cells in vitro. This type of cells is also able to engraft in radiation-ablated recipients and generates myeloerythroid and lymphocyte B lineages more effectively than the $Thy\text{-}1^-$ fraction [207]. However, it has been suggested that both $Thy1^+$ and $Thy1^-$ subsets have self-renewal potential and are capable of engrafting in recipient mice and that the unique difference between them is the HSC frequency. The levels of chimerism of these 2 subset fractions are similar under nonlimiting cell transplantation conditions, but a limiting dilution analysis revealed that HSCs are 5 times more abundant in the $Thy\text{-}1^+$ fraction [211].

There are also reports demonstrating the generation of $Thy\text{-}1^+$ cells from $Thy\text{-}1^-$ cells [211,212]. However, Majeti et al. [213] were able to establish a cell hierarchy, where $Thy\text{-}1^+CD45RA^-$ cells give rise to $Thy\text{-}1^-CD45RA^-$ cells that are, in turn, upstream of $Thy\text{-}1^-CD45^+$ cells.

Although it is known that the $Thy\text{-}1^+$ fraction includes primitive HSCs, the function of this surface protein has not yet been elucidated. A reduction in the number of hematopoietic colonies was observed when an anti-Thy-1 antibody was added to cultures of $Thy\text{-}1^+$ cells, suggesting that it may be involved in the development of HSCs [209]. Thy-1 has

also been proposed as a cell cycle status indicator based on the observation that only the Thy-1$^+$ fraction enters the S/G2/M-phases when cells are stimulated by cytokines [212].

Kinase insert domain receptor

Kinase insert domain receptor (KDR), also known as vascular endothelial growth factor receptor 2 (VEGFR2) or fetal liver kinase-1 (Flk1) in the mouse, is a less-known HSC marker. In vitro and in vivo studies have revealed that primitive HSCs are enriched in the CD34$^+$KDR$^+$ fraction, whereas the KDR$^-$ subset is composed mainly of lineage-committed hematopoietic progenitor cells. KDR$^+$ cells are very rare in the CD34$^+$ fraction, comprising 0.1 to 0.5% of the cells [214]. It has also been proposed that the CD34$^+$KDR$^+$ fraction comprises 5% to 6% of hemangioblasts, cells with bilineage differentiation potential, that are capable of generating hematopoietic and endothelial cells [215].

Cub domain protein 1

Cub domain protein 1 (CDCP1) is co-expressed with CD34 and CD133 in HSCs and is absent from mature cells. The HSC fraction isolated based on this marker and subsequently injected into the NOD/SCID mouse is able to engraft and generate various mature hematopoietic lineages [216].

HSC-Associated Negative Markers

CD38

This surface molecule is considered a negative marker for HSCs. It is expressed in differentiated erythroid, myeloid, B-lymphoid precursors, and T-lymphoid lineages [199]. The CD34$^+$CD38$^-$ fraction consists of a highly primitive set of cells that are able to generate progeny in long-term cell culture [199,217] and can engraft NOD/SCID mice to produce differentiated hematopoietic cells [196]. These cells are quiescent, and the increase in CD38$^+$ cells correlates with the beginning of cycling and differentiation [199].

Even when the CD34$^-$ cells are enriched, the fraction that demonstrates a greater engraftment and proliferation capacity is CD38$^-$ [191].

HSC-Associated Lineage Markers

Uncommitted HSCs lack characteristic markers that distinguish them from differentiated lineages [196] (Table 8). When HSCs are purified, it is usually by negatively selecting for these markers.

Table 8. Differentiated Hematopoietic Lineage Markers Absent from the Surface of Hematopoietic Stem Cells[a]

Cell type	*Marker*
Lymphocytes T	CD2, CD3, CD4, CD5, CD7, CD8.
Lymphocytes B	CD10, CD19, CD20, CD24.
Myeloid	CD14, CD15, CD16, CD33, CD41.
Erythroid	CD71, glycophorin A.
Natural killer	CD56.
Granulocyte	CD66b.

[a][200,208,198,220, http://pathologyoutlines.com].

New HSC-Associated Markers

Many molecular markers have been established for HSCs. However, HSCs exist within a highly heterogeneous pool of cells, which makes it difficult to isolate the most primitive SCs. Therefore, the discovery of new HSC markers will help to obtain a more homogeneous pool of HSCs. Below, we discuss some of the potential new markers for these cells.

CD49f, also known as integrin α6, yielded positive results when applied together with CD34 and Thy-1 to sort HSCs. The CD34$^+$ Thy1$^+$/Thy1$^-$CD49f$^+$ fractions showed high levels of chimerism in receptor mice [211].

Complement component 1 q subcomponent receptor 1 (C1qRp), or CD93 and a human homolog of the murine protein AA4, has been proposed to be a marker for the simultaneous isolation of primitive HSCs found in both CD34$^+$ and CD34$^-$ fractions. In vitro and in vivo assays have demonstrated that sorting cells using C1qRp leads to the isolation of primitive hematopoietic progenitors. This molecule has also been proposed to be a positive sorter for the HSCs found in the CD34$^-$ fraction, as the isolation of these cells is difficult due to the lack of a characteristic positive marker [219].

The isolation of primitive HSCs based on the activity of aldehyde dehydrogenase (ALDH) has also been reported. ALDH consists of a group of enzymes that are involved in the oxidation of aldehydes to carboxylic acids [220]. Cells positive for ALDH are negative for lineage markers but enriched for CD34$^+$ cells and for cells with short- and long-term activities [221,222]. This is a simple and inexpensive method for isolating HSCs; the fluorescent substrate for ALDH can also be combined with other markers [223].

Some additional HSC markers have been established in mice and are interesting for future studies to confirm their status as HSC markers in humans. Among them are ecotropic viral integration site 1 (Evi1), endothelial cell-selective adhesion molecule 1 (Esam1) and Flk2. Evi1 is a transcription factor exclusively expressed in HSCs and is downregulated during their differentiation [214]. Evi1 expression identifies HSCs with long-term repopulating activity, which are considered the most primitive HSCs [223]. Evi1-positive cells exhibit significant self-renewal and differentiation potential in vitro [223]. In studies in vivo, Evi1$^+$ cells showed the potential to engraft in irradiated recipients and to give rise to differentiated lineages, as evidenced by the presence of myeloid, B, and T cells in the peripheral blood of recipients [223]. The same was observed in secondary transplantations, demonstrating the in vivo long-term multilineage repopulating potential of Evi1-positive HSCs, suggesting that Evi1 is needed to maintain long-term HSC activity [223]. Evi1 overexpression blocks the differentiation of HSCs and induces their expansion. Therefore, Evi1 appears to regulate the transition from HSC to a more committed progenitor. In other words, it controls the balance between self-renewal and differentiation [223].

Esam1 is a transmembrane protein that is highly expressed in both human and murine HSCs and is downregulated when these cells become committed to differentiate [224]. Mature hematopoietic cells, with the exception of megakaryocyte progenitors, do not express this marker. Higher levels of chimerism were observed when mouse cells were sorted using an Esam1 antibody [224]. It has been

proposed that Esam1 may be a more effective marker and could be a substitute for other markers in the isolation of HSCs [224].

Flk2 has been proposed as a negative marker for HSCs with long-term repopulating capacity. Murine cells isolated from bone marrow and fetal liver that are negative for this marker show a greater capacity to repopulate irradiated recipients. Flk2 is present in short-term HSCs and is upregulated as the cells become more mature [225].

miRNAs Associated with HSCs

It is known that miRNAs also have a role in SC mechanisms [226]. However, little research has focused on the miRNAs related to the most primitive HSCs. Most of the work has focused on miRNAs related to more committed cells or to diseases associated with the hematopoietic system [227]. Below, we summarize the latest reports on miRNAs in HSCs from the adult bone marrow, umbilical cord blood and mobilized peripheral blood (Table 9).

The miRNAs differentially expressed in the $CD133^+$ fraction of the cells isolated from bone marrow (miR-146a, miR-146b-sp, miR-99a, miR-10a, miR-125b, miR-551b, miR-125a-sp) are involved in the inhibition of differentiation, apoptosis, and cytoskeletal remodeling. miRNAs expressed in the $CD34^+CD133^-$ subset (miR-142-3p and miR-425) were observed to have negative effects on cell proliferation [229].

Merkerova et al. [232] detected differences in the miRNAs expressed by HSCs obtained from bone marrow and umbilical cord blood. Bone marrow-derived $CD34^+$ cells differentially express let-7b, miR-1, miR-34a, miR-195, miR-203, miR-214, miR-545, and miR-548d, and umbilical cord blood cells express the set of miRNAs that is clustered on chromosome 19q13: miR-517c, miR-518a, miR-519d, and miR-520 h.

The miRNA miR-155 seems to block myeloid and erythroid differentiation in hHSCs [230]. Moreover, it is thought that miR-17, 24, 146, 155, 128, and 181 may also maintain hematopoietic cells at an early stem-progenitor stage by blocking their differentiation [230].

Table 9. microRNAs Expression Observed in Different Hematopoietic Stem Cells Fractions from Different Tissues

HSC origin	*Cell fraction*	*miRNAs*	*Reference*
Adult bone marrow[a]	$CD34^+CD133^-$	miR-15a, miR-15b, miR-16, miR-17, miR-19a, miR-19b, miR-20a, miR-20b, miR-21, miR-23a, miR-25, miR-26a, miR-26b, miR-30b, miR-92a, miR-92b, miR-101, miR-106a, miR-126-3p, miR-142sp, miR-142-3p, miR-142-5p, miR-144, miR-181a, miR-191, miR-221, miR-222, miR-223, miR-451, miR-663, miR-638, Let-7a, Let-7c, Let 7f, Let-7g.	[228,229]
	$CD133^+$	miR-15a, miR-16, miR-17, miR-19a, miR-19b, miR-20a, miR-20b, miR-21, miR-26a, miR-26b, miR-29c, miR-30b, miR-92a, miR-92b, miR-101, miR-126-3p, miR-142-3p, miR-142-5p, miR-144, miR-221, miR-222, miR-223, miR-451, Let-7a, Let-7f.	[229]
	$CD34^+$	miR-9-3, miR-16a, miR-16b, miR-17, miR-20, miR-23a, miR-23b, miR-24-1, miR-24-2, miR-25, miR-26a, miR-26b, miR-27a, miR-29a, miR-29c, miR-30a, miR-30b, miR-30d, miR-32, miR-33, miR-92, miR-93, miR-95, miR-96, miR-100-1/2, miR-102, miR-103, miR-103-2, miR-106, miR-107, miR-122a, miR-123, miR-128a, miR-128b, miR-130a, miR-135-2, miR-146, miR-155, miR-181a, miR-181b, miR-181c, miR-182, miR-183, miR-190, miR-191, miR-193, miR-192-2/3, miR-194, miR-197, miR-198, miR-199a, miR-202, miR-203, miR-204, miR-205, miR-206, miR-212, miR-213, miR-221, miR-222, miR-223, Let-7b, Let-7c, Let-7d.	[230]
Umbilical cord blood	$CD34^+$	miR-10a, miR-10b, miR-15a, miR-16, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-20b, miR-21, miR-27a, miR-27b, miR-93, miR-99a, miR-99b, miR-100, miR-101, miR-106a, miR-106b, miR-125a, miR-125b, miR-126, miR-129, miR-130a, miR-142-3p, miR-142-5p, miR-144, miR-155, miR-181a, miR-181c, miR-181d, miR-196b, miR-222, miR-451, miR-519d, miR-551b, miR-520h, Let-7e.	[231–233]
	$CD34^+CD38^-$	miR-127, miR-365, miR-452, miR-520h, miR-526b*.Predicted miRNAS: miR-100, miR-105, miR-149, miR-209.	[231]
Peripheral blood	$CD34^+CD133^+$[b]	miR-10a, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-20b, miR-34a, miR-93, miR-106a, miR-126, miR-130a, miR-146a, miR-221, miR-363, miR-599.	[233]
	$CD34^+$	miR-10a, miR-15a, miR-16a, miR-16b3, miR-17, miR-19a, miR-19b, miR-20, miR-20a, miR-23a, miR-23b, miR-24, miR-24-1, miR-25, miR-26a, miR-26b, miR-27a, miR-30b, miR-30c, miR-30d, miR-30e, miR-92, miR-93, miR-103, miR-103-2, miR-106, miR-107, miR-123, miR-126, miR-130a, miR-140, miR-142, miR-143, miR-146, miR-155, miR-181a, miR-191, miR-193, miR-196b, miR-197, miR-213, miR-221, miR-222, miR-223, miR-320, miR-363, miR-424/322, Let-7a, Let-7b, Let-7c, Let-7d, Let-7f, Let-7g.	[230,234]

[a]The miRNAs cited here are the listed by the article's authors as the highly expressed in the cells.
[b]Common miRNAs found in 2 pools of HSCs, either $CD34^+$ or $CD133^+$.
Asterisk indicates miRNA strands less commonly found associated with Argonauta complex.

Conclusions

ESCs are generally identified by a set of surface markers and the expression of 3 transcription factors (Nanog, Oct-4, and Sox-2). However, transcriptome assays have revealed a set of genes that are highly expressed in ESCs and are not found in their differentiated counterparts. The commonly cited markers are Rex-1, Dnmt3b, Lin-28, Tdgf1, FoxD3, Tert, Utf-1, Gal, Cx43, Gdf3, Gtcm1, Terf1, Terf2, Lefty A, and Lefty B. Nevertheless, these putative markers are far less studied than Nanog, Oct-4, and Sox-2. Some lack studies in hESCs, and others have not even been studied in ESCs.

MSCs are usually identified by the expression of CD73, CD90, and CD105 along with the absence of CD34. Although there is variation among the MSCs isolated from many different regions, it is possible to establish a common set of markers for these cells in which the cells are positive for CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166, and HLA-ABC expression and negative for CD14, CD31, CD34, CD45, CD62E, CD62 L, CD62P, and HLA-DR expression. Moreover, many articles have reported the expression of ESC markers; SSEA-4 has been proposed as a new marker for isolating MSCs. Nevertheless, there is no consensus about the expression of these molecules, and little is known about their roles in MSCs.

HSCs are primarily isolated based on the expression of CD34, but the pool of cells obtained is composed of cells with many degrees of differentiation. Therefore, the combination of this marker with other surface molecules, such as CD133 and CD90, along with the lack of CD38 and lineage markers provides the most homogeneous pool of SCs. In addition, the $CD34^-$ fraction has also been reported as containing true HSCs, but there is no consensus regarding this finding.

Regarding miRNAs, profiles are being established for each type of SC. However, their functions in the status of SCs have not been completely elucidated. Although there has been considerable progress in the study of SC markers, it is still far from being fully understood.

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Author Disclosure Statement

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Capítulo III

Isolation and characterization of mesenchymal stem cells from *Gallus gallus* fetuses

Artigo a ser submetido para publicação em revista a ser definida

Isolation and characterization of mesenchymal stem cells from *Gallus gallus* fetuses

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Abstract

Mesenchymal stem cells (MSC) are multipotent plastic adherent cells present in many body compartments of adult and fetal individuals. MSC are able to differentiate in osteoblast, condroblasts and adipocytes, and are characterized by the expression of surface proteins like CD73, CD90 and CD105, but lack CD34 and CD45. MSC were already studied and isolated from several model organisms including *Gallus gallus*. Despite the fact that chicken is not a first choice model employed for MSC studies, chicken display a series of advantages over conventional models to the studies on fetal MSC. The present study reports the isolation and molecular characterization of putative MSC obtained from bone marrow, skeletal and cardiac muscle of chicken fetuses. Cells isolated from bone marrow and skeletal muscle are plastic adherent fibroblast-like cells, expressed CD73, CD90 and CD105 and are able to differentiate into osteoid and adipocytic lineages. Thus, cells isolated from these two sources were considered fetal MSC. Nevertheless, cells obtained from cardiac muscle presented all the characteristics cited above, except that they were not able to differentiate into adipocytes. Transcritptome analysis indicated that these cells are compromised with the cardiac lineage and, considering their molecular and morphological characteristics, they possibly may be epicardium-derived cells (EPDCs).

1. Introduction

Generally, metazoans are constituted by three major groups of cells: (i) germinative cells, which give rise to gametes, (ii) somatic cells, which compose the majority of the body and are terminally differentiated, and (iii) stem cells (SC) (Bongso & Richards, 2004). SC are a group of undifferentiated cells that maintain the SC population through self-renewal and are able to generate more specialized cells by differentiation processes (Bongso & Richards, 2004). Among the SC already described are: (i) embryonic stem cells (ESC), isolated from the blastocyst inner cell mass (Bongso & Richards, 2004); (ii) primordial germ cells (PGC), found in gonadal ridge of 5- to 9-week-old fetuses (Bongso & Richards, 2004; Shamblott *et al.*, 1998); (iii) epidermal stem cells (EPSC), located in hair follicles, sebaceous glands and interfollicular epidermis (Braun *et al.*, 2003); (iv) hematopoietic stem cells (HSC), in bone marrow (Sutherland *et al.*, 1989); (v) neuronal stem cells (NSC), in the central nervous system (Conti & Cattaneo, 2010), and (vi) mesenchymal stem cells (MSC) (Pittenger *et al.*, 1999).

MSC can be found in fetal and adult individuals (Campagnoli et al., 2001; Pittenger et al., 1999). These cells are multipotent and therefore able to differentiate into chondroblasts, osteoblasts, adipocytes (Pittenger *et al.*, 1999) and also into non-mesenchymal lineages such as pancreatic islands (Santos *et al.*, 2010), hepatocytes (Lee *et al.*, 2004) and neuron-like cells (Dezawa *et al.*, 2004). In addition, MSC are characterized by the presence of the surface markers CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166, and HLA-ABC and the absence of CD14, CD31, CD34, CD45, CD62E, CD62L, CD62P, and HLA-DR (Calloni *et al.*, 2013). Besides human beings, MSC have already been isolated from several other organisms, such as mice, rat, dog, cat, cattle, horse, sheep, goat, chicken and others (Bhardwaj & Kundu, 2012; da Silva Meirelles *et al.*, 2006)Calloni et al., manuscript in preparation).

Although MSC are investigated since Friedenstein first isolation on 1960's (Afanasyev *et al.*, 2009) and more closely after Pittenger's publication, in 1999 (Pittenger *et al.*, 1999), there are many aspects of the MSC biology that remain to be elucidated. For example, there is no specific marker to define MSC (Calloni *et al.*, 2013; Keating, 2012).

Moreover, markers to distinguish MSC from more committed progenitors are not available yet and they are not characterized (Keating, 2012; Lindner *et al.*, 2010), the trilineage differentiation potential of MSC was not demonstrated *in vivo* (Keating et al., 2012) and microRNAs are starting to be studied in MSC (Calloni *et al.*, 2013). Additionally, new MSC sources are continuously discovered (e.g., gingival, oral mucosa and renal glomeruli) and novel research fields based on the use of MSC have being created (*e.g.*, MSC as vaccine platform) (Bruno *et al.*, 2009; Tomchuck *et al.*, 2012; Zhang *et al.*, 2012).

G. gallus is not widely used as model organism in the MSC field of research. However, there are reports of isolation of ESC phenotypically similar to human ESC (Lavial & Pain, 2010), fetal spermatogonial stem cells (Yu *et al.*, 2010), embryonic germ cells (Wu *et al.*, 2010), hematopoietic progenitor cells(Cormier & Dieterlen-Lievre, 1988) and MSC (Young *et al.*, 1995) from this organism.

Chicken fetuses gather several characteristics that make them interesting for SC research. Among them are low maintenance cost and short incubation period of eggs, the easy access and handling of fetuses, and the availability of breeds with low genetic variability, as the White Leghorn (Ponsuksili *et al.*, 1998). Moreover, *G. gallus* genome is already completely sequenced (Stern, 2005) and presents a high similarity with human genome, evidenced by the 60% orthologs identified between the two species (Cogburn *et al.*, 2007; Ellegren, 2005; Stern, 2005). In terms of gene organization, many regions of human and chicken genomes are more similar to each other than are those of human and mouse. (Ellegren, 2005). In this context, the present study reports the isolation and molecular characterization of MSC from bone marrow, skeletal and cardiac muscle of chicken fetuses, aiming to propose this organism as a model to study the fetuses MSC biology.

2. Materials and methods

2.1. Cells isolation and cultivation

Cells were isolated from cardiac and skeletal muscle, and bone marrow of White Leghorn chicken fetuses with 18-19 days of incubation (doi). In brief, eggs were opened and the animals were euthanized following Ethical Comitee of Federal University of Rio Grande do Sul (protocol number 18976). Small pieces of heart and lower limb skeletal muscle were incubated in collagenase I (1.5 $mg.mL^{-1}$) at 37° C for 30-60 minutes. Cells from bone marrow were isolated from the tibial bone. The epiphysis were removed and the marrow was collected by inserting a syringe needle into the bone and flushing with Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Saint Louis, MO).

Cells were plated in six-well culture plates and maintained in low glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 3.7 $g.L^{-1}$ sodium bicarbonate, 10.5 mM HEPES (Sigma Aldrich, Saint Louis, EUA), and 1% (v/v) antibiotic solution (Sigma Aldrich, Saint Louis, EUA). The culture plates were incubated at 37° C in a humidified atmosphere containing 5% (v/v) CO_2. The culture medium was changed each two or three days. When the culture became confluent, the cells were detached with trypsin 1.0 $g.L^{-1}$ and expanded to culture flasks or used in experiments.

2.2. Differentiation assays

Adipogenic differentiation. Approximately 4×10^4 cells were plated in six-well plates and, after 24 hours, incubated in adipogenic media (DMEM supplemented with 10% (v/v) FBS, 10^{-8} M dexamethasone, 6.8×10^{-2} $U.mL^{-1}$ insulin, 100 µM indomethacin and 5 µM rosiglitazone) for 21 days. At the end of the experiment, the intracellular lipid inclusions were visualized with Oil Red staining.

Osteogenic differentiation. Approximately 4×10^4 cells were plated in six-well plates and, after 24 hours, incubated in osteogenic media [DMEM supplemented with 10% (v/v) FBS,

10^{-8} M dexamethasone, 1.03 mM β-glicerophosphate (Sigma Aldrich, Saint Louis, EUA) and 17.27 µM L-ascorbic acid-2-phosphate] for 21 days. The extracellular calcium deposition was evaluated by von Kossa and alizarin staining.

Controls consisted of cells not exposed to differentiation culture medium. The culture medium was changed each three days for all cultures.

2.3. Alkaline Phosphatase (ALP) assay

The activity of alkaline phosphatase was determined by a protocol adapted from Widyowati (2011). Cells were lysed in a culture plate with a solution 0.1 mM of sodium dodecyl sulfate and 250 µL of the lysate was incubated with 25 µL p-nitrophenil-phosphate 40 mM (prepared in a solution 1:1 containing 4 mM $MgCl_2$ and 0.4 M Na_2CO_3 pH 10 buffer). The reaction mixture was incubated at 37° C for one hour and stopped with 50 µL 0.5 M NaOH solution. The absorbance was determined at 405 nm. The alkaline phosphatase was expressed in units per µg of protein and one unit of enzyme was defined as the activity causing release of 1 nmol of p-nitrofenol per minute under the standard assay condition used (Widyowati, 2011). The total protein amount of the cell lysates was determined by the method developed by Bradford (1976).

2.4. Molecular analysis

2.4.1. PCR reactions

Total RNA was isolated using the kit Ilustra RNAspin Mini (GE Healthcare, Fairfield, EUA), following the provided instructions. The integrity of the RNA was checked in 2% (w/v) agarose gel, with samples previously denatured by the addition of formamide and heat at 65° C for 10 minutes (Masek *et al.*, 2005).

RNA extracted (150 ng or less, depending on the yield of the extraction) was reversed transcribed using Phusion RT-PCR kit (Thermo ScientificScetific, Waltham, EUA).

The cDNA was amplified using the primer sequences summarized in Table 1. The reaction mixture was elaborated following the kit instructions and contained: 5 µL Phusion HF Buffer, 0.5 µL 10mM dNTPs, 0.5 µL each primer, 100 ng cDNA, 0.5 µL Phusion Hot Start DNA and water to the final volume of 25 µL. The cycling steps were initial denaturation of 30 s at 98° C and 30 cycles of 10 s at 98° C, 10 s at 55° C and 1 min and 35 s at 72° C. The final extention was performed at 72° C for 5 min. The PCR products were visualized in 2% (w/v) agarose gel.

Table 1: Polimerase chain reaction primer sequences.

	Gene	*Primer sequences*	*PCR product (bp)*	*Accession number*
Embryonic stem cells markers	OCT-4	Forward: 5'- TCAATGAGGCAGAGAACACG -3' Reverse: 5'- TTGTGGAAAGGTGGCATGTA -3'	391	DQ867024
	NANOG	Forward: 5'- CTTCCAGCTCTGGGACACTC -3' Reverse: 5'- CCAGATACGCAGCTTGATGA -3'	372	DQ867025.1
	SOX 2	Forward: 5'- CTCTGCACATGAAGGAGCAC -3' Reverse: 5'- CCTTGCTGGGAGTACGACAT -3'	397	NM_205188.1
	TERT	Forward: 5'- CAGCAGAACCAAAGCCTACC -3' Reverse: 5'- GTGCTGTTCCCCTCTCTCTG -3'	330	NM_001031007.1
Hematopoietic stem cells markers	CD31	Forward: 5'- GGACCTGACCTTGAGAGTGC -3' Reverse: 5'- TGGTTTCAGAGCCACTTTC -3'	387	XM_001234535
	CD34	Forward: 5'- CGTGTTCTGCACAGTGAGGT -3' Reverse: 5'- TGCTTGTGTTCTCTGGATGC -3'	371	XM_417984.3
	CD45	Forward: 5'- GCTGCTTCGTAAGGATACGC -3' Reverse: 5'- GGTGTTGTGCAAGGATGTTG -3'	393	NM_204417.1
	CD133	Forward: 5'- AATGTTGGACCTTTGCTTGG -3' Reverse: 5'- GCTGCGTAACCCTTCTGAAC -3'	395	XM_001232164.2
Mesenchymal stem cells markers	CD73	Forward: 5'- CCCATATCCCTTCATGGTTG -3' Reverse: 5'- GGATGCACAGTGAAACATGG -3'	380	XM_419855.3
	CD90	Forward: 5'- GCCGCTATGAGAACAAGACC -3' Reverse: 5'- AAGTCCACAGCTTGCAGGAG -3'	358	NM_204381.1
	CD105	Forward: 5'- AGCATCCAGTGGTCCAAGAC -3' Reverse: 5'- CTCACGGAAGAGGACCTCAG -3'	387	NM_001080887

Adipogenic differentiation markers	FABP4	Forward: 5’- AGACTGCTACCTGGCCTGAC -3' Reverse: 5’- TCCCATCCACCACTTTTCTC -3’	360	NM_204290
	DLK1	Forward: 5’- GTGCAAGTGACCCATGTGAG -3’ Reverse: 5’- ATTTGGCATTAGCCAACCAC -3’	381	NM_001142254
	PPAR-γ	Forward: 5’- TTGCCAAAGTGCAATCAAAA - 3' Reverse: 5’- CTTCTCCTTCTCCGCTTGTG -3’	358	NM_001001460
	Adiponectin	Forward: 5’- TAGGCTTCCTCCTTTGCTCA - 3' Reverse: 5’- AGATCTTGGTGAAGCGGATG -3’	387	NM_206991
Osteogenic differentiation markers	Alkaline phosphatase	Forward: 5’- CTTCCTGCTGGGTCTCTTTG -3' Reverse: 5’- TAACCGCCAAAGGTGAAGAC -3’	312	NM_205360
	BMP2	Forward: 5’- ATTATGAAGCCAGCCACAGC -3’ Reverse: 5’- ATACAACGGATGCCTTTTGC -3’	397	NM_204358
	BMP4	Forward: 5’- GAGCTTCCACCATGAAGAGC -3' Reverse: 5’- TTTGCCCTGATGAGTCTGTG -3’	394	NM_205237
	Osteonectin	Forward: 5’- TGCTGCAAGATGAGAACCTG -3’ Reverse: 5’- CAAAGAAGTGGCAGGAGGAG -3’	373	NM_204410
	Osteocalcin	Forward: 5’- CTGCTCACATTCAGCCTCTG -3' Reverse: 5’- TGCGCTCTGCCTTTATTTCT -3’	366	NP_989866
	Osteopontin	Forward: 5’- TGTGGCATATGGCTTCAGAG -3’ Reverse: 5’- CGTTGTTTTCAATGCTGTGG -3’	359	NM_001201386
	Act β	Forward: 5’- CTCCCTGATGGTCAGGTCAT -3' Reverse: 5’- ACATCTGCTGGAAGGTGGAC -3’	343	NM_205518

Quantitative real time analysis was performed using a Step One Plus (Applied Biosystems, Foster City, CA). The amplification was carried in 96-well plates using TaqMan Universal PCR Master Mix system (Applied Biosystems, Foster City, CA) in a 20 µL reaction volume. The reation mixture was composed of 2 µL of cDNA solution (50 ng of total cDNA), 900 nM of primers, 250 nM of probe (Table 2), 10 µL of master mix and 2 µL of water. PCR samples were incubated for 2 min at 50° C, 10 min at 95° C, followed by 40 cycles of 15 s at 95° C and 1 min at 60° C. Each sample was analyzed in triplicate. The relative quantification of gene expression was determined following the $2^{-\Delta\Delta Ct}$ method, as proposed by Livak and Schmittgen (2001), using at least two housekeepings genes. The housekeepings applied in this study were β-actin, ubiquitin or G6PDH.

2.4.2. Transcriptome analysis

RNA was extracted as described above and the transcriptome analysis was performed by The Center for Functional Genomics, University at Albany. The analysis was made using the Chicken Gene 1.0 ST array (Affymetrix, Santa Clara, CA). Network analyses of differentially expressed genes were realized using the web interface Genemania (www.genemania.org) (Warde-Farley *et al.*, 2010). This tool shows the relationship among the gene list imputed and extends the list with genes related to those initially inserted in the program. Networks were generated based on the biological process and molecular function associated to the genes and the connections indicate co-expression, co-localization, genetic interaction, same reaction participation in a pathway, physical interaction, or predicted functional relationship among genes. Moreover, Genemania provides the functions associated to the genes in the network.

2.5. Statistical analysis

Statistical analysis of alkaline phosphatase enzymatic activity and gene expression analysis through real time PCR was performed by the Kruskal-Wallis non-parametric test. $P < 0.05$ was considered statistically significant.

Table 2: Real time polymerase chain reaction primers and probes sequences.

	Gene	*Primer sequences*	*Probe sequences*	*Product (pb)*
Stem cell markers	CD34	Forward: 5'- ATGGGAACACAGATCCTG-3' Reverse: 5'- AGGGATTTGATTCCGAAC-3'	5'- TGCTCCGGAAGTCTCACTGG-3'	70
	CD73	Forward: 5'- GGCTTTACTGTGGACATA-3' Reverse: 5'- GGGTTCCTGTATAGAGAAA-3'	5'- TCCTCCAATAACAACATCCACTCCTT-3'	94
	CD90	Forward: 5'- ACCAGATAAAGAACATCACTG-3' Reverse: 5'- CGAGGTGTTCTGGATCAA-3'	5'- CGCACTTCTCCAGTTTGTCTTTGAT-3'	80
	CD105	Forward: 5'- CAGCTCTACATTAGCCAG-3' Reverse: 5'- CGTGATGGACACATTCAG-3'	5'- CCTCAGCATCCAGTGGTCCA-3'	88
Osteogenic differentiation markers	BMP2	Forward: 5'- GCCATTGTTCAGACTTTG-3' Reverse: 5'- CAAGGTAGAGCATTGAGATA-3'	5'- CACTCAGTTCTGTCGGCACAC-3'	97
	BMP4	Forward: 5'- CTGTGCTGATATGCCTTG-3' Reverse: 5'- AGGACTTGGCATAGTAGG-3'	5'- CCATCAGCATTCGGTTACCAGG-3'	85
	Ostepontin	Forward: 5'- GGACTTTCCTGACATTCC-3' Reverse: 5'- CAGTGTCATTGGAATCATTG-3'	5'- CCATCATCATCATCATCCACGGC-3'	80
	Osteonectin	Forward: 5'- CAGGACATAGACAAGGATC-3' Reverse: 5'- TGGGGAAAGGTTAAAACA-3'	5'- CTTCCTCCTCTGCTGCCAACTT-3'	83
Adipogenic differentiation markers	FABP4	Forward: 5'- GAGACTGTTATCAAGAGAAAA-3' Reverse: 5'- CATGCTCTTTCGTAAACTC-3'	5'- CTGGTAACATTATTCATGGTGCATTCC-3'	95
	PPARγ	Forward: 5'- GGAACAGAACAAAGAAGTAG-3' Reverse: 5'- TTCGCAAATTCTGTAATCTC-3'	5'- CACTGCCTCCACAGAGCGAA-3'	90

	Adiponectin	Forward: 5’- TGCAGAATTAATTTCCTTCA-3’ Reverse: 5’- GGGAAATAGGAGATGTGA-3’	5’- AATACCAGCAACATCCAGGAGGC-3’	100
Housekeepings	β-actin	Forward: 5’- GAGCAAAAGAGGTATCCTG-3’ Reverse: 5’- TGCCAGATCTTCTCCATA-3’	5’- TGAACACGGTATTGTCACCAACTGG-3’	84
	G6PDH	Forward: 5’- GTTCCGGGCGATATCTTC-3’ Reverse: 5’- TGGTCATCAGTTTGGTGTA-3’	5’ -ACTCTCACCACCAACTCGTTGC -3’	91
	Ubiquitin	Forward: 5’- GATGCAGATCTTCGTGAA-3’ Reverse: 5’- CTTGGCCTTCACATTTTC-3’	5’-CGTTGACTGGCAAGACCATCAC-3’	88

3. Results

3.1. Cell isolation and initial characterization

We successfully isolate cells from bone marrow (BM), skeletal (SM) and cardiac muscle (CM). The cells were plastic-adherent and presented a fibroblast-like morphology. Cells lasted in culture for at least 10 passages. After reached this passage number, cells were observed to slow proliferation and start to detach from the culture plate.

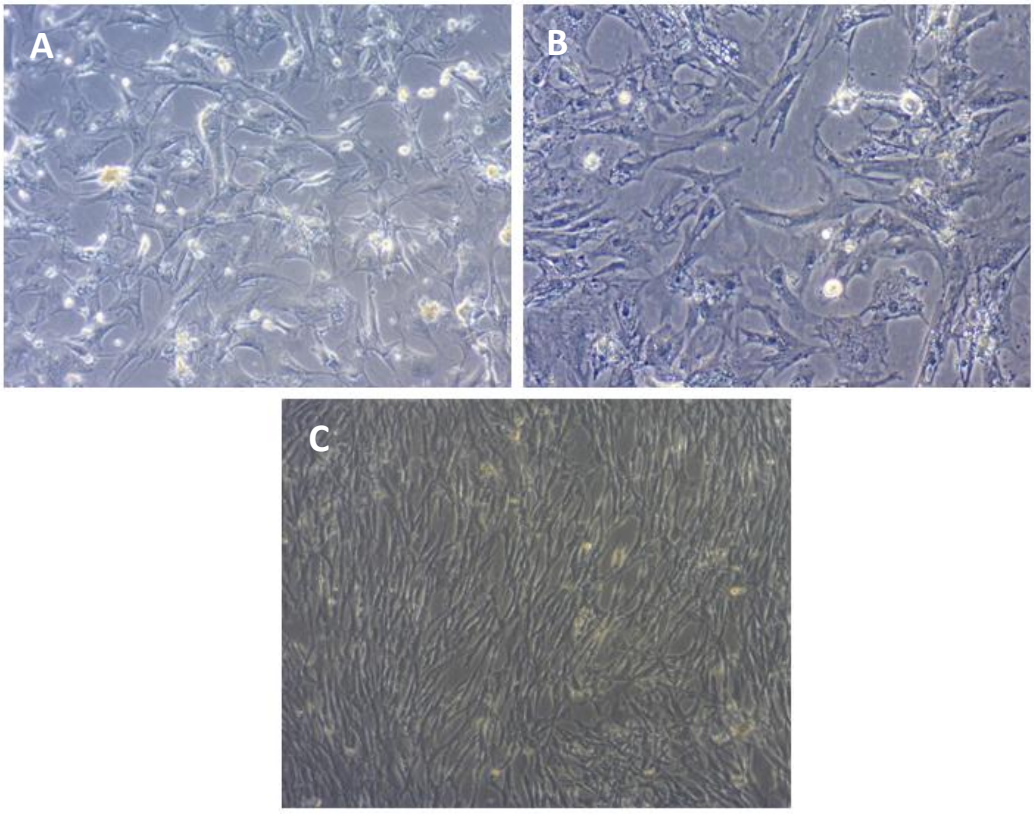

Figure 1. Morphology of the cells isolated from cardiac muscle (A), bone marrow (B), and skeletal muscle (C) of 18-19 doi *G. gallus* fetuses. Magnification: 100×.

Once cultures of each of the isolated cells were established, we proceeded with further characterization. Cells were evaluated regarding to stem cell markers expressed and their potential to differentiate into osteogenic and adipogenic lineages. Stem cells markers expression was analyzed before and after differentiation induction.

Results of these assays are presented below, separated by the tissue source employed for cell isolation.

3.1.1. Bone marrow isolated cells

***Stem cell markers*.** Cells isolated from BM were analyzed by end point PCR and by real time PCR method throughout the differentiation assay regarding the expression of stem cells markers. In this sense, ESC or HSC-associated markers were not detected (Figure 2). Therefore, these markers were not further evaluated by quantitative PCR analysis. End-point PCR only showed the expression of CD90 (Figure 2). However, CD73 and CD105 were detected by quantitative PCR (Figure 3). The expression of MSC markers was observed to downregulate during differentiation assay, although no significant statistical difference was detected (Figure 3).

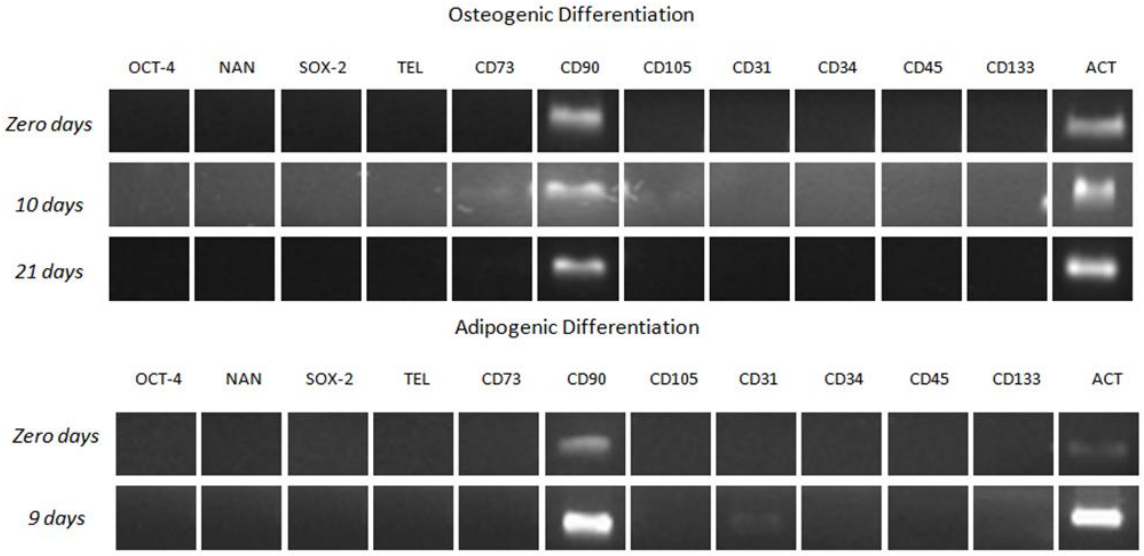

Figure 2: Stem cell markers expressed by the cells isolated from bone marrow of 18-19 days of incubation (doi) *G. gallus* fetuses during osteogenic and adipogênic differentiation. Abbreviations: NANOG (NAN); telomerase (TEL); β-actin (ACT).

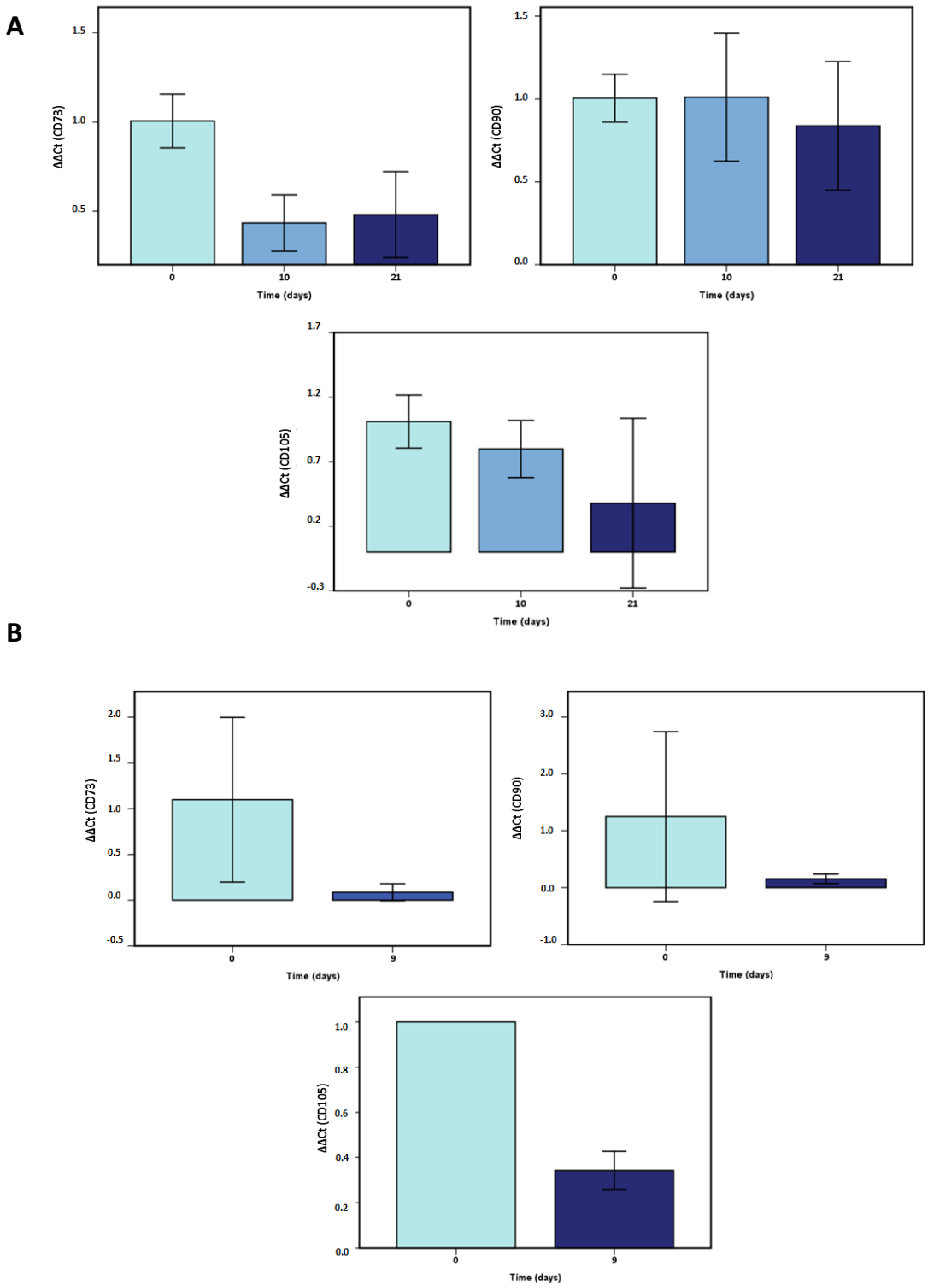

Figure 3: Mesenchymal stem cells associated markers relative expression during osteogenic (A) and adipogenic (B) differentiation of bone marrow cells isolated from of 18-19 doi *G. gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test ($p<0.05$).

Cell differentiation. Potential MSCs isolated from BM were able to differentiate into osteoblasts cells, where extracellular calcium depots were evidenced by von Kossa and alizarin staining (Figure 4). Alkaline phosphatase specific activity (Figure 5) was measured during cells differentiation. Higher enzymatic levels were detected in cells exposed to osteogenic media when compared to cells maintained in DMEM and increased from the beginning to the end of the assay. However, statistic significance was not detected among comparisons.

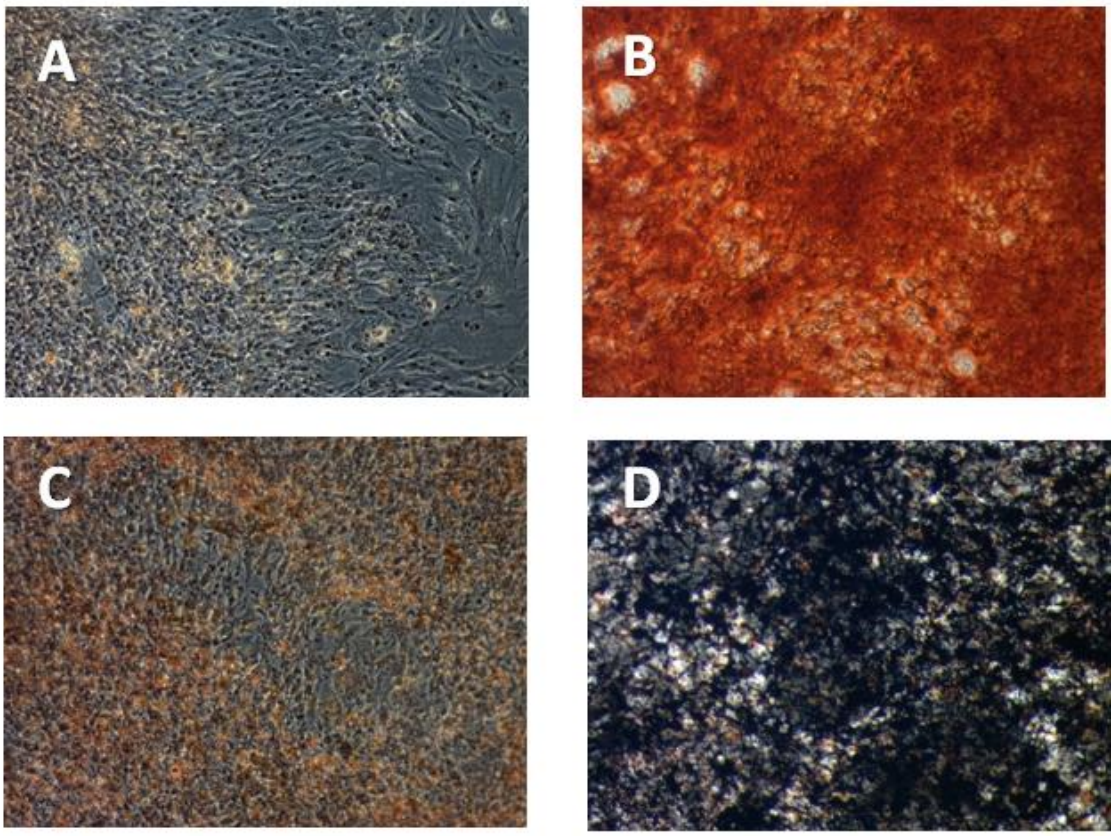

Figure 4: Osteogenic differentiation of cells isolated from bone marrow of 18-19 doi *G. gallus* fetuses. (A, C) controls and (B, D) cells exposed to the osteogenic differentiation media. (A, B) alizarin staining and (C, D) von Kossa. Magnification: 100×.

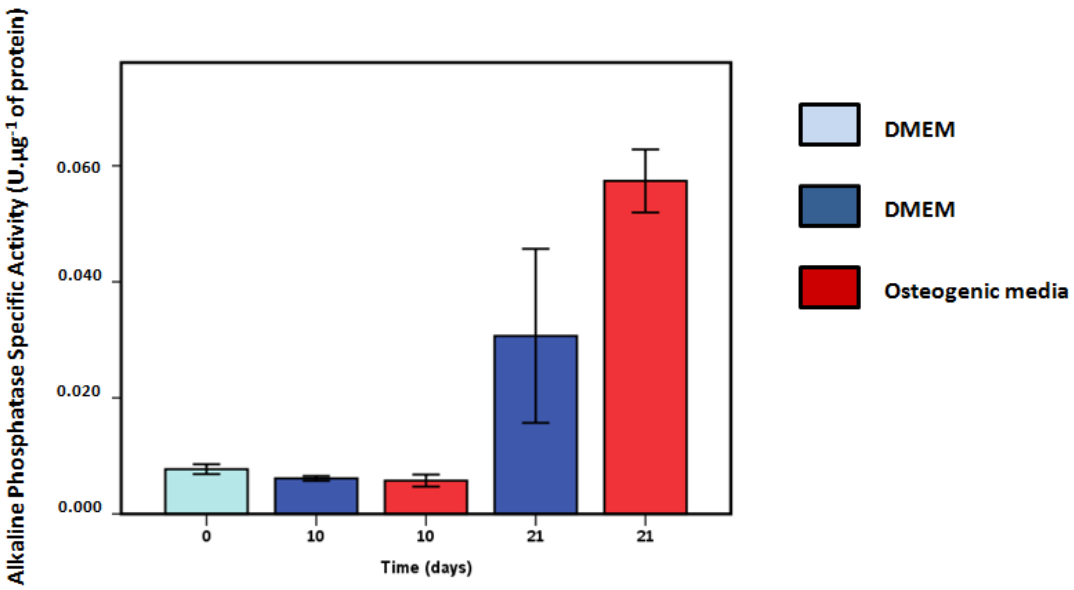

Figure 5: Alkaline phosphatase specific activity during osteogenic differentiation of BM cells isolated from 18-19 doi of *Gallus gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. The symbol (*) indicates expression levels significantly different according to the Kruskal-Wallis non-

The expression of osteogenic markers was evaluated by end-point PCR before, 10 and 21 days after osteogenic differentiation induction (Figure 6). Interestingly, BM cells expressed osteopontin and osteonectin before the exposition to the differentiation media (Figure 6). After osteogenic induction, osteopontin was detected on day 10, together with BMP-2 and BMP-4 (Figure 6). Osteonectin was detected in all time points analyzed (Figure 6). Osteocalcin and ALP expression was not detected (Figure 6).

The expression of some osteogenic markers was measured by real time quantitative PCR (Figure 7). It was observed an increase in BMP-2 and BMP-4 (Figure 7). Osteonectin had its expression reduced during the differentiation assay (Figure 7). ALP, not detected in end point PCR (Figure 7), was detected in real time PCR analysis and also presented an expression reduction during the assay (Figure 7). Osteopontin peaked on 10 days of differentiation (Figure 7). No significant statistical difference among the days analyzed was observed for any of the osteogenic markers.

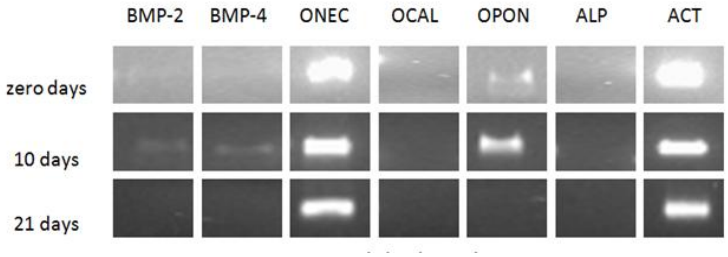

Figure 6: Osteogenic markers expression profile of cells isolated from bone marrow of 18-19 doi *Gallus gallus* fetuses and exposed to the osteogenic media during 21 days. Abbreviations: bone morphogenetic protein-2 (BMP-2); bone morphogenetic protein-4 (BMP-4); osteonectin (ONEC); osteocalcin (OCAL); osteopontin (OPON); alkaline phosphatase (ALP); β-actin (ACT).

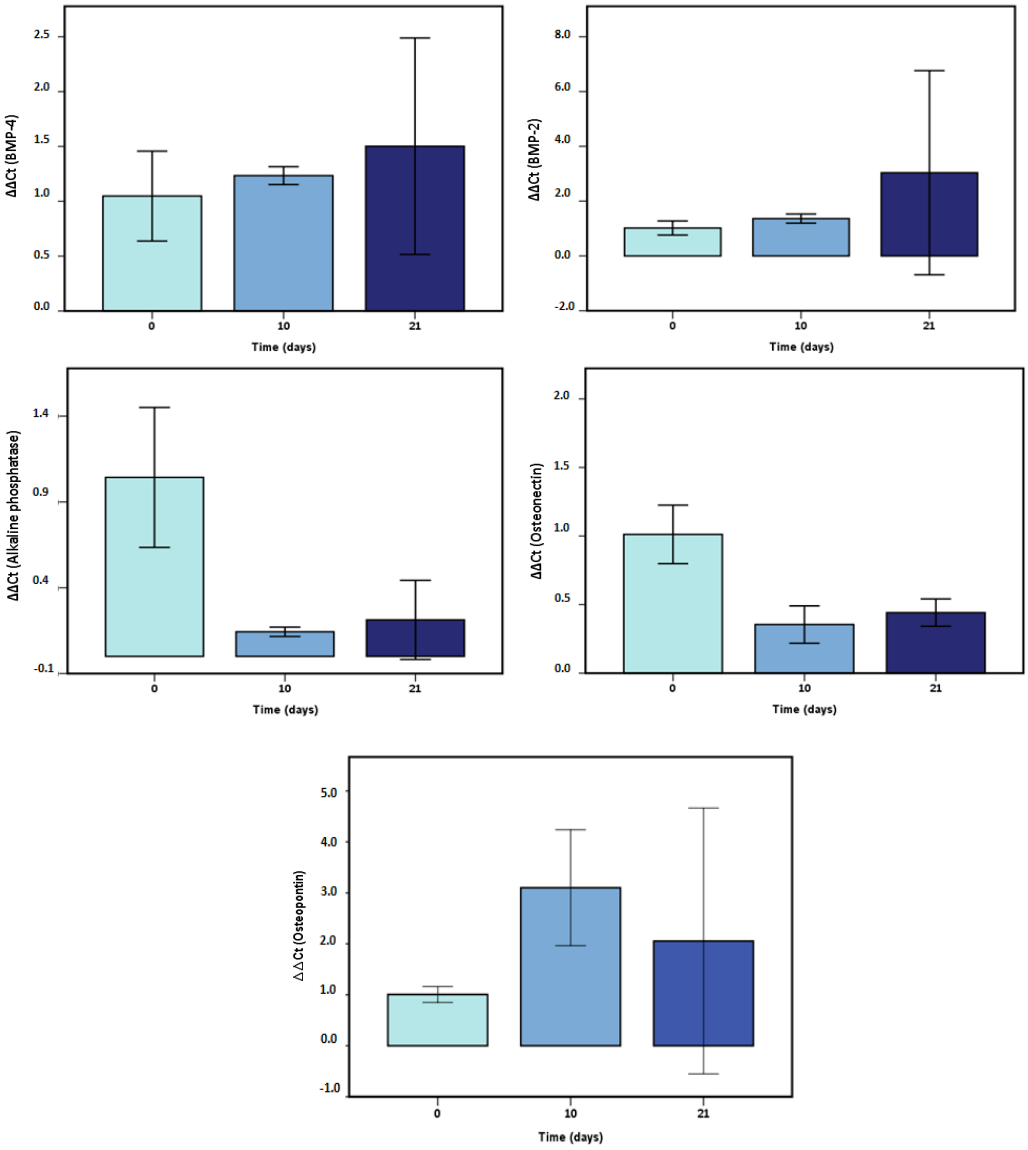

Figure 7: Relative expression of osteogenic markers during osteogenic differentiation induction of bone marrow cells isolated of 18-19 doi *G. gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test ($p<0.05$).

Cells isolated from BM were also able to differentiate into adipocytes and lipid depots were observed after Oil red staining (Figure 8). Cells showed morphology alterations early in the beginning of the assay. Interestingly, it was not detected expression of FBP4, adiponectin and PPAR-γ in end-point PCR (Figure 9). DLK1 expression was detected during the whole assay, even before the adipogenic media exposition (Figure 9).

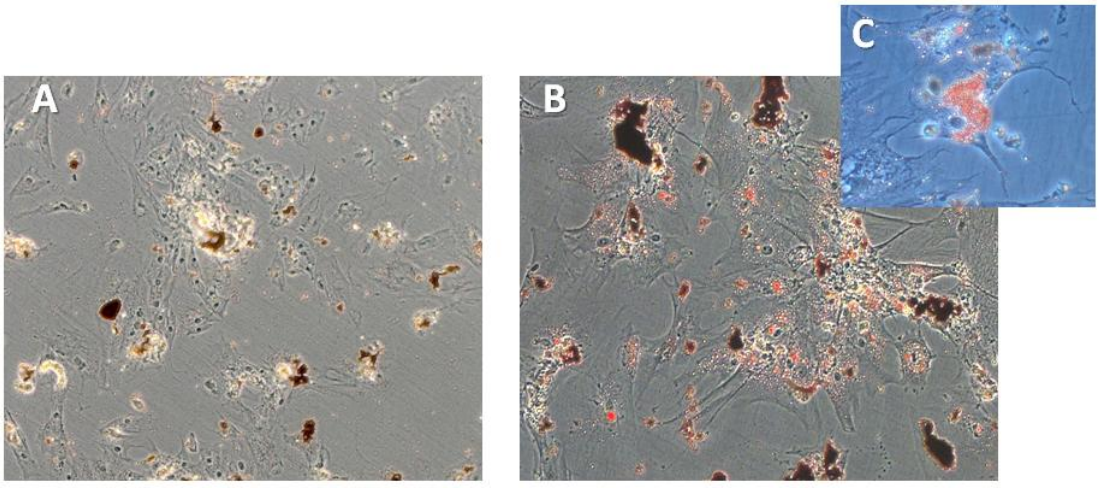

Figure 8: Adipogenic differentiation of cells isolated from bone marrow of 18-19 doi *G. gallus* fetuses. (A) control, (B) cells exposed to the adipogenic differentiation media and (C)detail of lipid depots. Magnification: 100×.

As proceeded for osteogenic differentiation, the expression of some adipogenic markers were evaluated by real time quantitative PCR. FABP4 and PPAR-γ were detected in the cells induced to adipogenic differentiation and have their expression level reduced after adipogenic media exposition (Figure 10). However, no statistical differences were detected in the conditions analyzed. Adiponectin expression was not detected in any time evaluated (Figure 10).

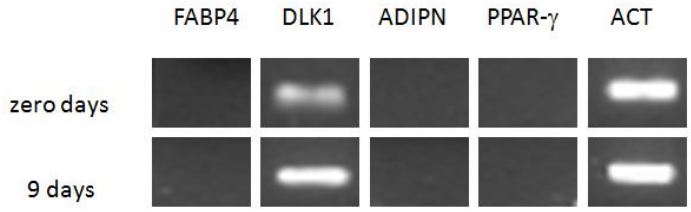

Figure 9: Adipogenic markers expression profile of cells isolated from bone marrow of 18-19 doi *G. gallus* fetuses and exposed to the adipogenic media during 9 days. Abbreviations: adiponectin (ADIPN); β-actin (ACT).

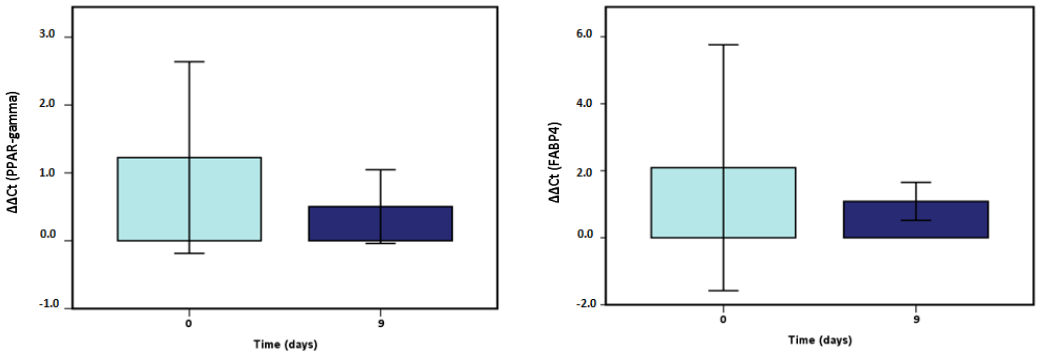

Figure 10: Adipogenic markers relative expression profile of cells isolated from bone marrow isolated of 18-19 doi G. gallus fetuses and exposed to the adipogenic media during 9 days. Bars represent the average of technical replicates. Error bars represent the standart error. No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test ($p<0.05$).

3.2.1. Skeletal muscle isolated cells

Stem cell markers. Stem cells markers expression was investigated throughout the differentiation assay by end point PCR and by real time PCR method for skeletical muscle (SM) stem cells. No ESC and HSC markers were detected on SM isolated cells (Figure 11). Only CD90 was detected through end point PCR investigation (Figure 11). The three MSC markers were detected on quantitative PCR analysis (Figure 12). As observed for BM isolated cells, most of these markers presented a expression decrease during osteogenic and adipogenic differentiation (Figure 12). However, CD73 was detected only on 16 day on osteogenic differentiation and CD105 was observed before

media exposition and on 21 day on adipogenic differentiation (data not shown). No significative statistical difference was observed among the time points analyzed.

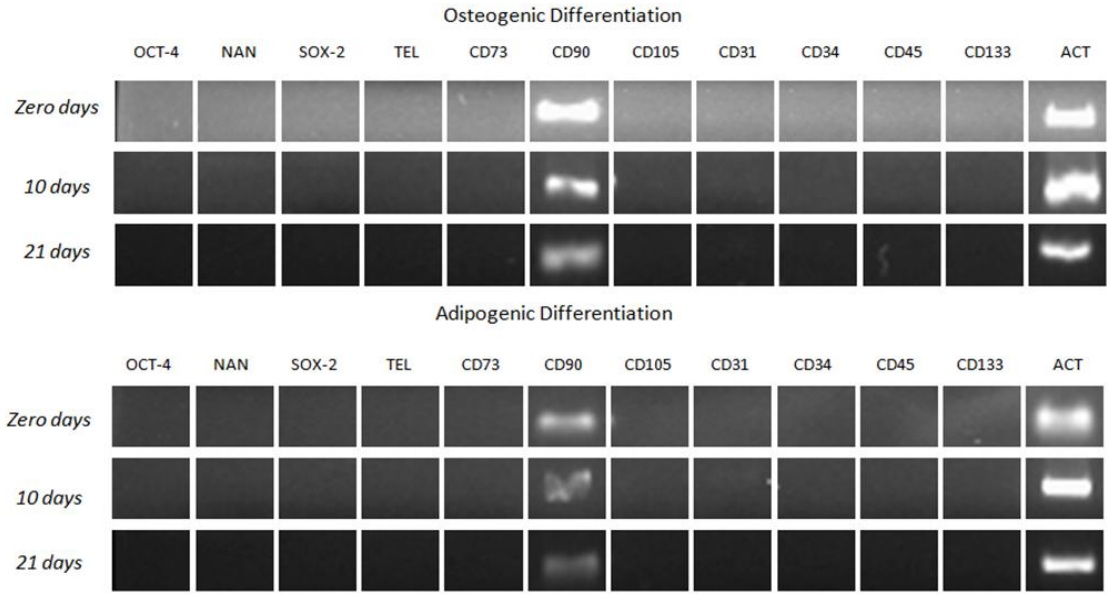

Figure 11: Stem cell markers expressed by the cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses during osteogenic and adipogenic differentiation. Abbreviations: NANOG (NAN); telomerase (TEL); β-actin (ACT).

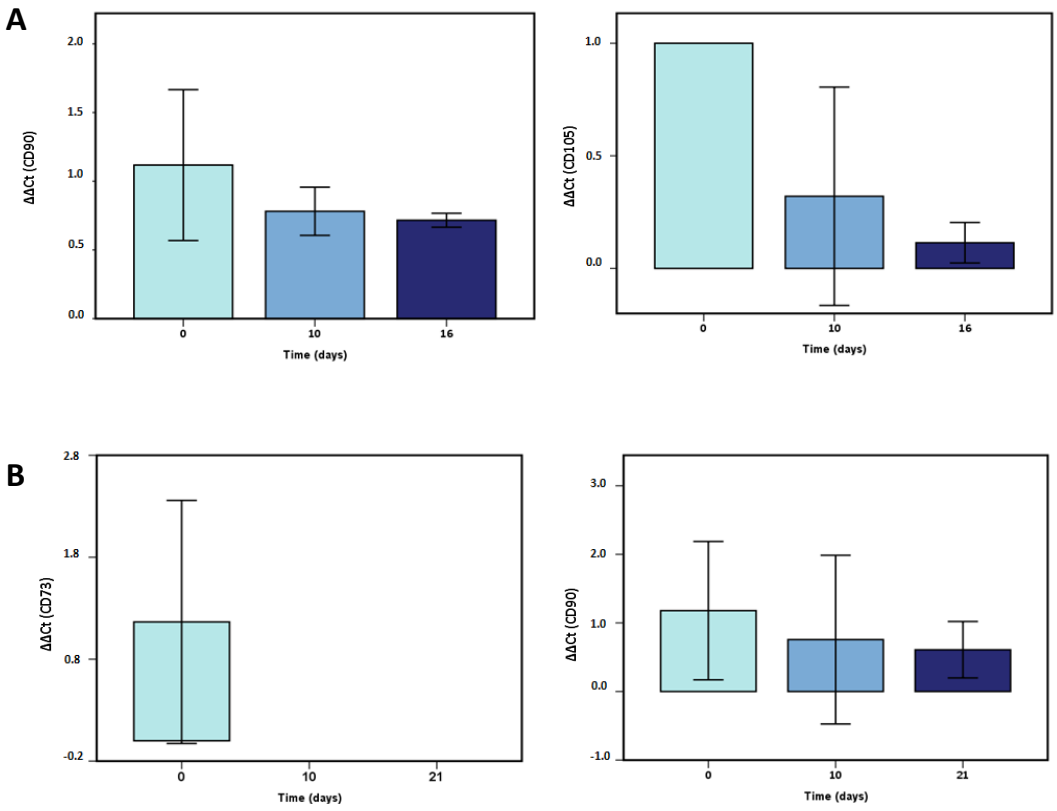

Figure 12: Mesenchymal stem cells associated markers relative expression during osteogenic (A) and adipogenic (B) differentiation of skeletal muscle cells isolated from of 18-19 doi *G. gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test ($p<0.05$).

Cell differentiation. SM isolated cells were able to differentiate into osteoblasts and extracellular calcium depots were detected by von Kossa and alizarin staining (Figure 13). The osteogenic assay could not be maintained up to 21 days because cells started to detach from the culture plate. For this reason, the assay lasted 16 days. The low cell number attached in the culture plate may be the reason of the undetection of ALP on cell lysate.

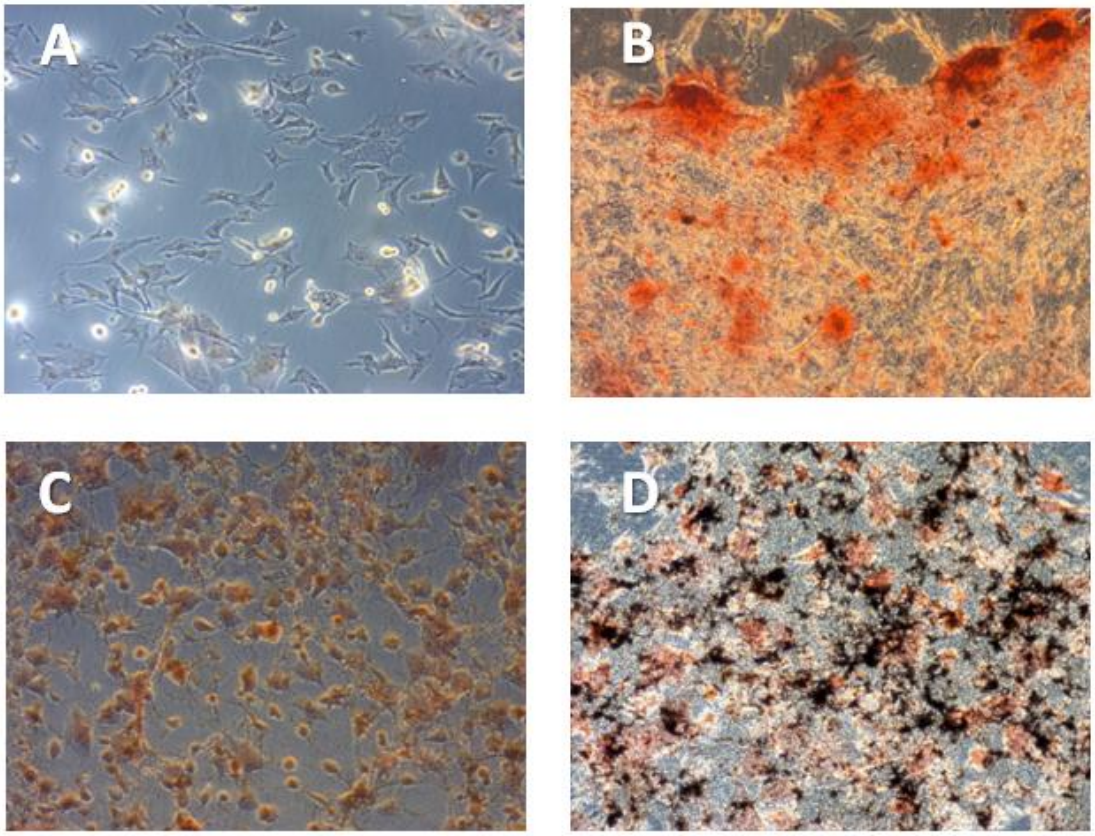

Figure 13: Osteogenic differentiation of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses. (A, C) controls and (B, D) cells exposed to the osteogenic differentiation media. (A, B) alizarin staining and (C, D) von Kossa. Magnification: 100×.

Regarding to osteogenic markers, end point PCR results showed that osteonectin was detected in all times analyzed (Figure 14). Osteopontin was only detected before the osteogenic media exposition. BMP-2 presented a slight expression on days 10 and 16 (Figure 14). Osteocalcin and ALP expression was not detected.

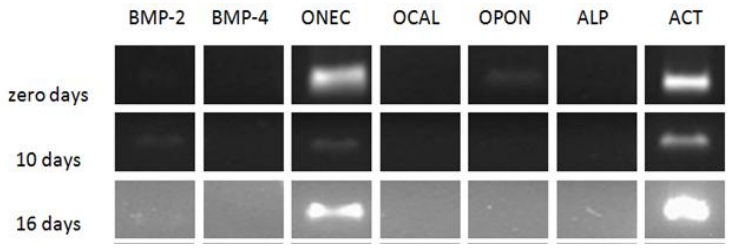

Figure 14: Osteogenic markers expression profile of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses and exposed to the osteogenic media during 16 days. Abbreviations: bone morphogenetic protein-2 (BMP-2); bone morphogenetic protein-4 (BMP-4); osteonectin (ONEC); osteocalcin (OCAL); osteopontin (OPON); alkaline phosphatase (ALP); β-actin (ACT).

Quantitative analysis indicated a decrease in BMP-4 levels, in contrast with higher levels of BMP-2 at the 10 day of the assay (Figure 15). Osteonectin expression levels decreased from the start to the 10 day and become elevated on 16 day (Figure 15). No ALP expression was detected on the control condition, what prevented the calculation of the relative expression through $2^{-\Delta\Delta Ct}$ method. However, ALP was detected in samples from 16 day (data not shown). Osteopontin was not detected. No significant statistical difference was observed among the days analyzed.

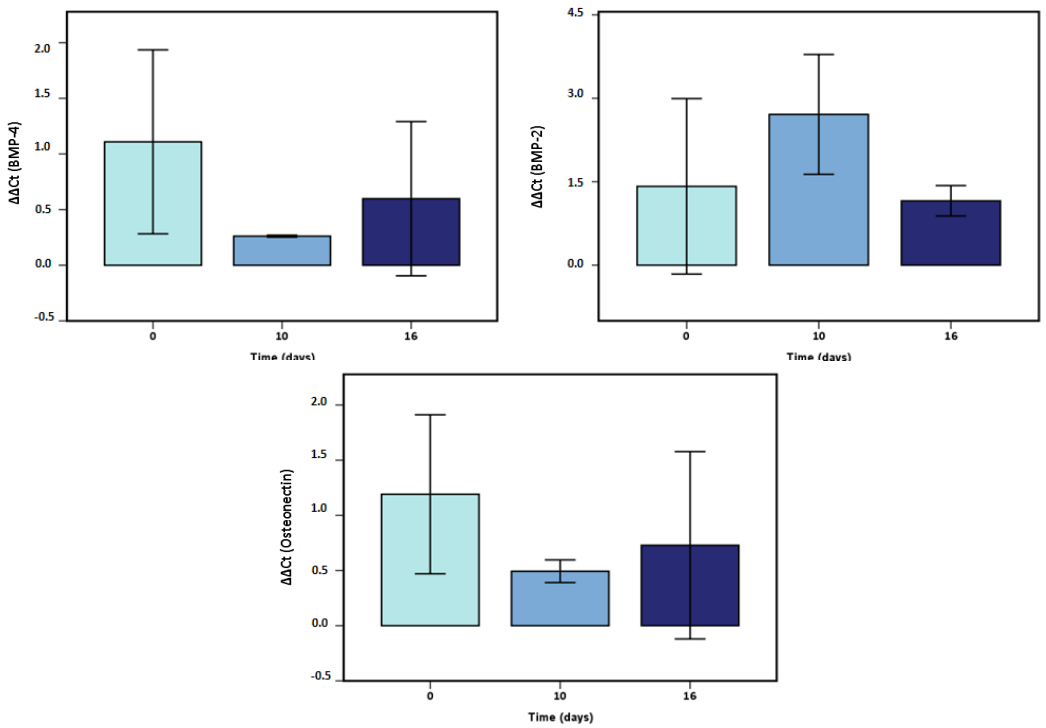

Figure 15: Relative expression of osteogenic markers during osteogenic differentiation induction of skeletal muscle cells isolated of 18-19 doi *G. gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test

After the exposition to adipogenic media, SM isolated cells started to accumulate intracellular lipids and differentiation was confirmed by Oil red staining (Figura 16). As observed for BM isolated cells, end point PCR did not revealed expression of adipogenic markers (FABP4, adiponectin and PPAR-γ). DLK1 was observed before and after the differentiation induction (Figure 17). Quantitative PCR indicated FABP4 and PPAR-γ expression, peaking on 10 day (Figure 18). Adiponectin expression was not detected in any time evaluated. No significant statistical difference was observed among the days analyzed.

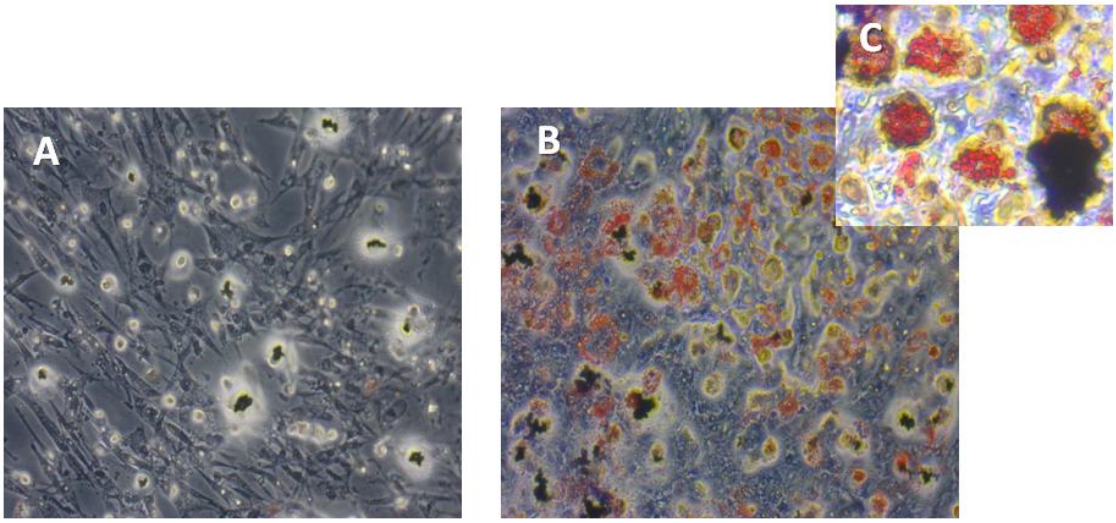

Figure 16: Adipogenic differentiation of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses. (A) control, (B) cells exposed to the adipogenic differentiation media and (C) detail of lipid depots. Magnification: 100×.

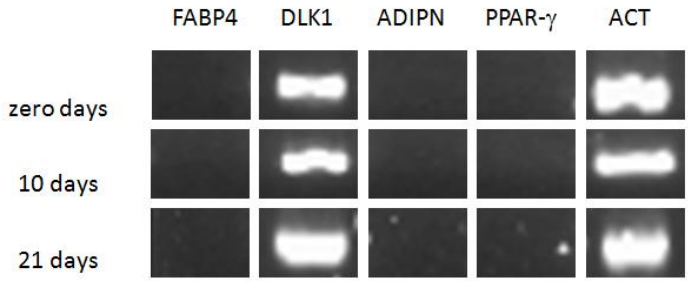

Figure 17: Adipogenic markers expression profile of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses and exposed to the adipogenic media during 21 days. Abbreviations: adiponectin (ADIPN); β-actin (ACT).

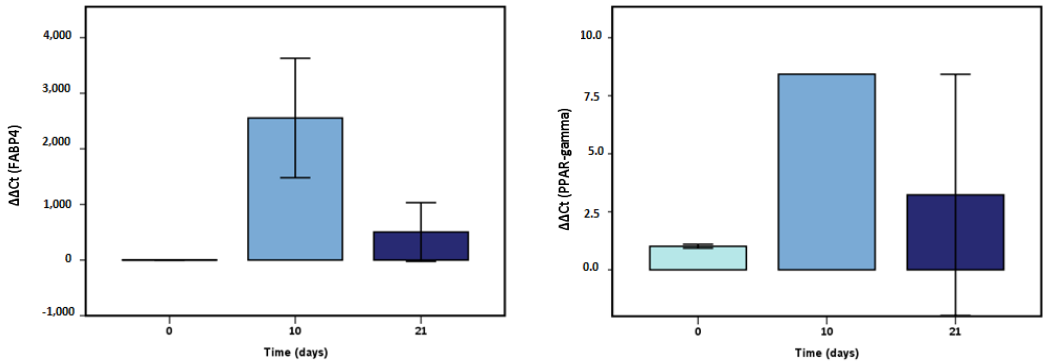

Figure 18: Adipogenic markers relative expression profile of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses and exposed to the adipogenic media during 21 days. Bars represent the average of technical replicates. Error bars represent the standart error.No significant statistical difference was observed according to the Kruskal-Wallis non-parametric test ($p<0.05$).

3.2.3. Cardiac muscle isolated cells

Stem cell markers. CM isolated cells expressed all the MSC markers evaluated (CD73, CD90 and CD105) before and after differentiation induction (Figure 19). Furthermore, a slight expression of CD31 and CD34 was detected on cells before and 10 days after osteogenic induction. Other markers analyzed were not detected (Figure 19). Quantitative PCR revealed an increase in the MSC markers expression during differentiation (Figure 20).

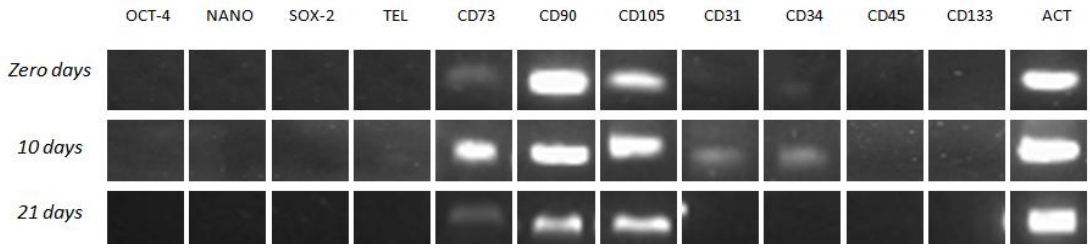

Figure 19: Stem cell markers expressed by the cells isolated from cardiac muscle of 18-19 doi *G. gallus* fetuses during osteogenic differentiation. Abbreviations: NANOG (NAN); telomerase (TEL); β-actin (ACT).

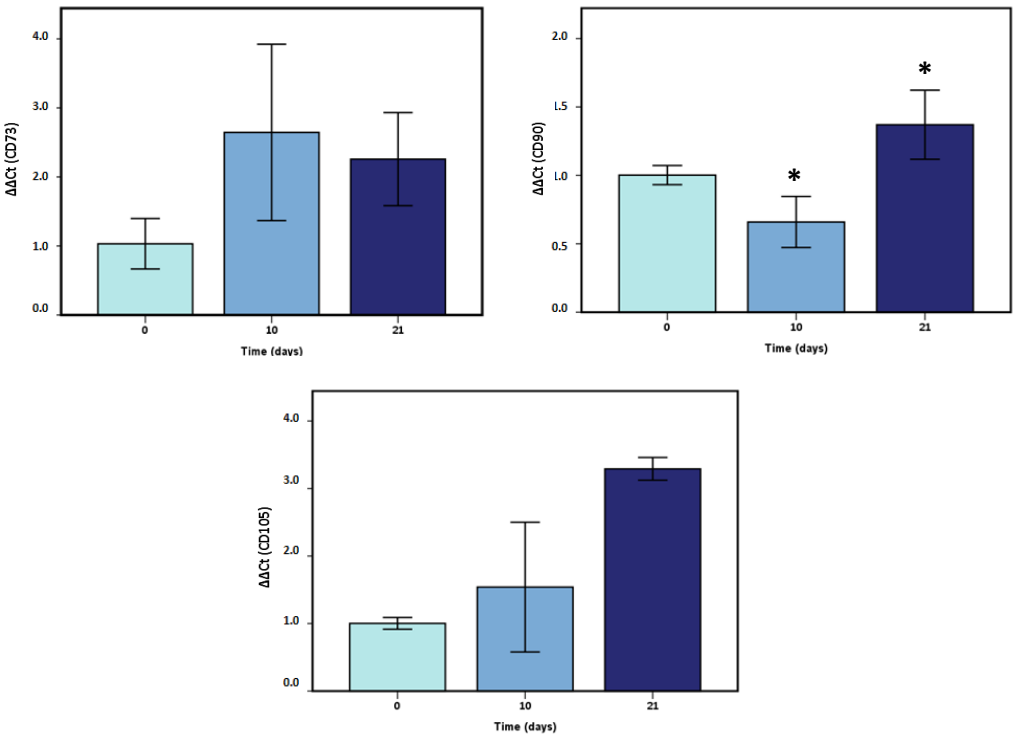

Figure 20: Mesenchymal stem cells associated markers relative expression during osteogenic differentiation of cells isolated from cardiac muscle of 18-19 doi *G. gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. The symbol (*) indicates expression levels significantly different according to the Kruskal-Wallis non-parametric test ($p<0.05$).

Cell differentiation. CM isolated cells were exposed to adipogenic and osteogenic media. Nevertheless, only osteogenic media induced differentiation into the cells (Figure 21). No lipid droplets were detected on CM cells, even when the assay was extended up to 30 days. On the other hand, calcium depots were detected at the end of osteogenic induction (Figure 21).

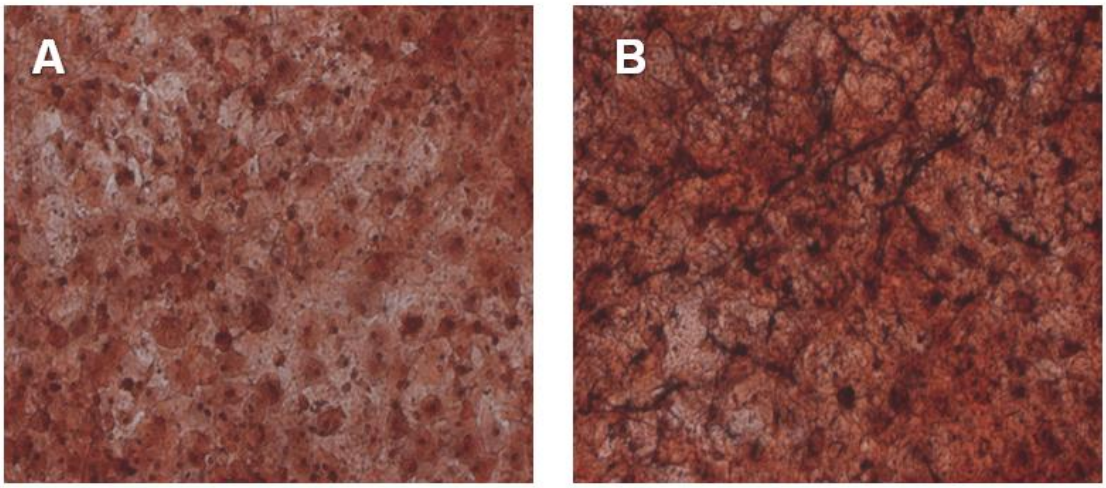

Figure 21: Osteogenic differentiation of cells isolated from skeletal muscle of 18-19 doi *G. gallus* fetuses. (A) control and (B) cells exposed to the osteogenic differentiation media. von Kossa staining. Magnification: 100×.

Alkaline phosphatase activity measurement (Figure 22) revealed an increase in the enzyme activity during osteogenic differentiation. Statistical difference was observed only between 10 day control and 21 day osteogenic media exposition.

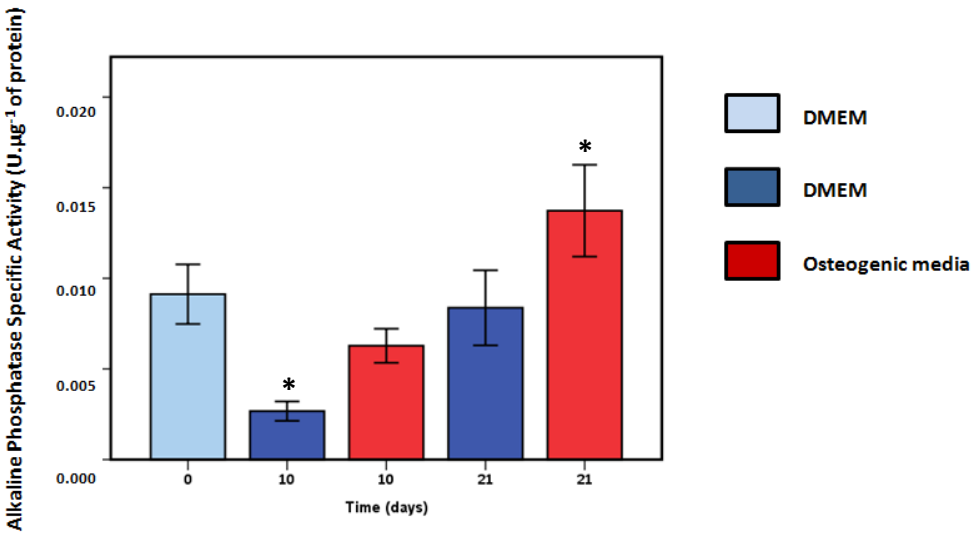

Figure 22: Alkaline phosphatase specific activity during osteogenic differentiation of cardiac muscle cells isolated from 18-19 doi of *Gallus gallus* fetuses. Bars represent the average of technical replicates. Error bars represent the standart error. The symbol (*) indicates expression levels significantly different according to the Kruskal-Wallis non-parametric test ($p<0.05$).

Osteogenic markers were detected only at the end of the assay (Figure 23). As observed for the cells from the other compartments, no osteocalcin and ALP expression was detected on end point PCR (Figure 23). Relative quantification (Figure 24) revealed a significant increase in the BMP-4 and osteonectin expression. However, BMP-2 had a significant decrease in its levels from de beginning to the end of differentiation. Again, ALP was detected, showing higher expression levels on 10 day. However, no significant difference was observed for the ALP levels in the three time points analyzed

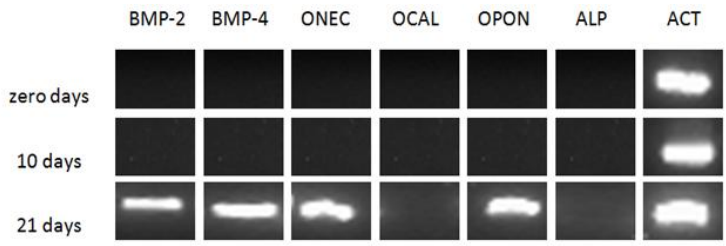

Figure 23: Osteogenic markers expression profile of cells isolated from cardiac muscle of 18-19 doi *G. gallus* fetuses and exposed to the osteogenic media during 21 days. Abbreviations: bone morphogenetic protein-2 (BMP-2); bone morphogenetic protein-4 (BMP-4); osteonectin (ONEC); osteocalcin (OCAL); osteopontin (OPON); alkaline phosphatase (ALP); β-actin (ACT).

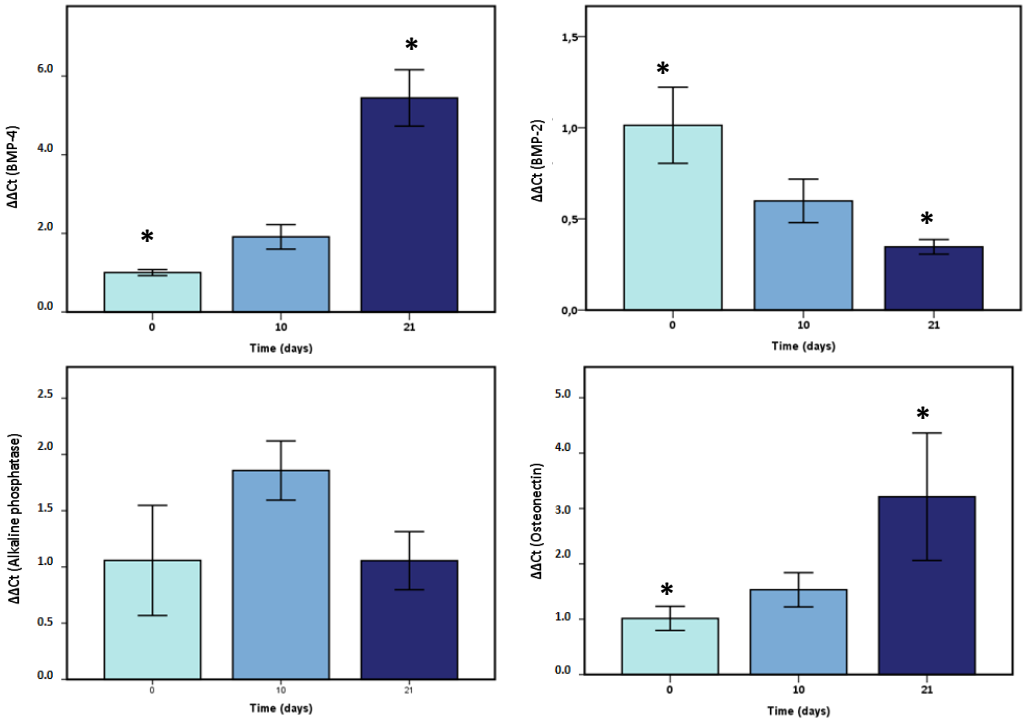

Figure 24: Relative expression of osteogenic markers during osteogenic differentiation induction of cardiac muscle cells isolated of 18-19 doi *G. gallus* fetuses. Error bars represent the standart error. The symbol (*) indicates expression levels significantly different according to the Kruskal-Wallis non-parametric test ($p<0.05$).

3.4. Transcriptome analysis

Cells isolated from CM presented a behavior different from cells from the other two sources. For this reason we performed a comparative transcriptome analysis between RNA samples from BM, a classical source of MSC, and cells isolated from CM , intending to identify possible differences and similarities in terms of gene expression between these two kinds of cells.

In this sense, cells from two sources presented a different transcription profile (Figure 25). Especifically, two gene sets were differentially expressed between the cells analyzed (Figure 25; Table 3). Genes overexpressed in BM isolated cells and downregulated in CM counterparts (Table 4) presented molecular functions related to bone morphogenesis (MEF2C and DLX5), fatty acid transport (PLA2G10 and SLC27A6) and nucleic acids binding (CEBPA, MEF2C and DLX5). These genes are involved in biological processes involved with cellular response to bacterium, to organic cyclic compounds and to lipopolysaccharide presence (MEF2C). Nevertheless, the function analysis performed by GeneMania for this group of genes presented a analysis bias. Some of the genes were not included in the function analysis of the software. Therefore, other biological processes and molecular functions may be related to this group of genes.

Genes highly expressed in CM isolated cells (Table 5) are mainly related to heart morphogenesis (TBX20, GATA-4 and GATA-6), angiogenesis processess (EDN1, TBX20, PRKCB1, GATA-4, GATA-6, AQP1 and LAMA5), smooth muscle cells differentiation (EDN1, IGFBP5, GATA-4, GATA-6, EPAS1 and FHL1) and blood coagulation (EDN1, THBD, and PLEK) (Table 4).

Table 3. A list of genes diferentially expressed between bone marrow and cardiac muscle isolated cells

Genes overexpressed in BM cells	
Gene identification	***Gene name***
DMC1	dosage suppressor of mck1 homolog
MMP10	matrix metallopeptidase 10 (stromelysin 2)
EDIL3	EGF-like repeats and discoidin I-like domains 3
TFCP2L1	transcription factor CP2-like 1
GCHFR	GTP cyclohydrolase I feedback regulator
PGR	progesterone receptor
MARCH11	membrane-associated ring finger (C3HC4) 11
AKR1D1	aldo-keto reductase family 1, member D1
RARB	retinoic acid receptor, beta
SPON2	spondin 2, extracellular matrix protein
DLX5	distal-less homeobox 5
GPR171	G protein-coupled receptor 171
PANX3	pannexin 3
MEF2C	myocyte enhancer factor 2C
SPINK6	serine peptidase inhibitor, Kazal type 6
PLA2G10	phospholipase A2, group X
PPP1R17	protein phosphatase 1, regulatory subunit 17
ICOS	inducible T-cell co-stimulator
MMP13	matrix metallopeptidase 13 (collagenase 3)
FAM70B	family with sequence similarity 70, member B
SLC27A6	solute carrier family 27 (fatty acid transporter), member 6
RNF144	ring finger protein 144ª
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
F13A1	coagulation factor XIII, A1 polypeptide
HOXA7	homeobox A7
GREM1	gremlin 1

Genes overexpressed in CM cells	
Gene identification	***Gene name***
KIAA1324L	KIAA1324-like
FAM83H	family with sequence similarity 83, member H
EGFL6	EGF-like-domain, multiple 6
EDN1	endothelin 1
NOX4	NADPH oxidase 4
HTR2A	5-hydroxytryptamine (serotonin) receptor 2ª
IGFALS	insulin-like growth factor binding protein, acid labile subunit
NPY	neuropeptide Y
ANPEP	alanyl (membrane) aminopeptidase
ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2
MECOM	MDS1 and EVI1 complex locus
PRKCB	protein kinase C, beta
LAMA5	laminin, alpha 5
THBD	thrombomodulin
GEM	GTP binding protein overexpressed in skeletal muscle
SALL3	sal-like 3
TBX20	T-box 20
CCL4	chemokine (C-C motif) ligand 4
SLC9A3	solute carrier family 9 (sodium/hydrogen exchanger), member 3
EBF3	early B-cell factor 3
AQP1	aquaporin 1
ABLIM1	actin binding LIM protein 1
EPAS1	endothelial PAS domain protein 1
SHC3	SHC (Src homology 2 domain containing) transforming protein 3
PLEk	Pleckstrin
GPR56	G protein-coupled receptor 56
SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2
NTN4	netrin 4

DRAM1	DNA-damage regulated autophagy modulator 1
GATA6	GATA binding protein 6
FHL1	four and a half LIM domains 1
TREM2	triggering receptor expressed on myeloid cells 2
NID2	nidogen 2
IL1RL1	interleukin 1 receptor-like 1
VIP	vasoactive intestinal peptide
SMOC2	SPARC related modular calcium binding 2
TMEM211	transmembrane protein 211
CDH6	cadherin 6, type 2, K-cadherin
GATA4	GATA binding protein 4
CCRL1	chemokine (C-C motif) receptor-like 1
CP	ceruloplasmin

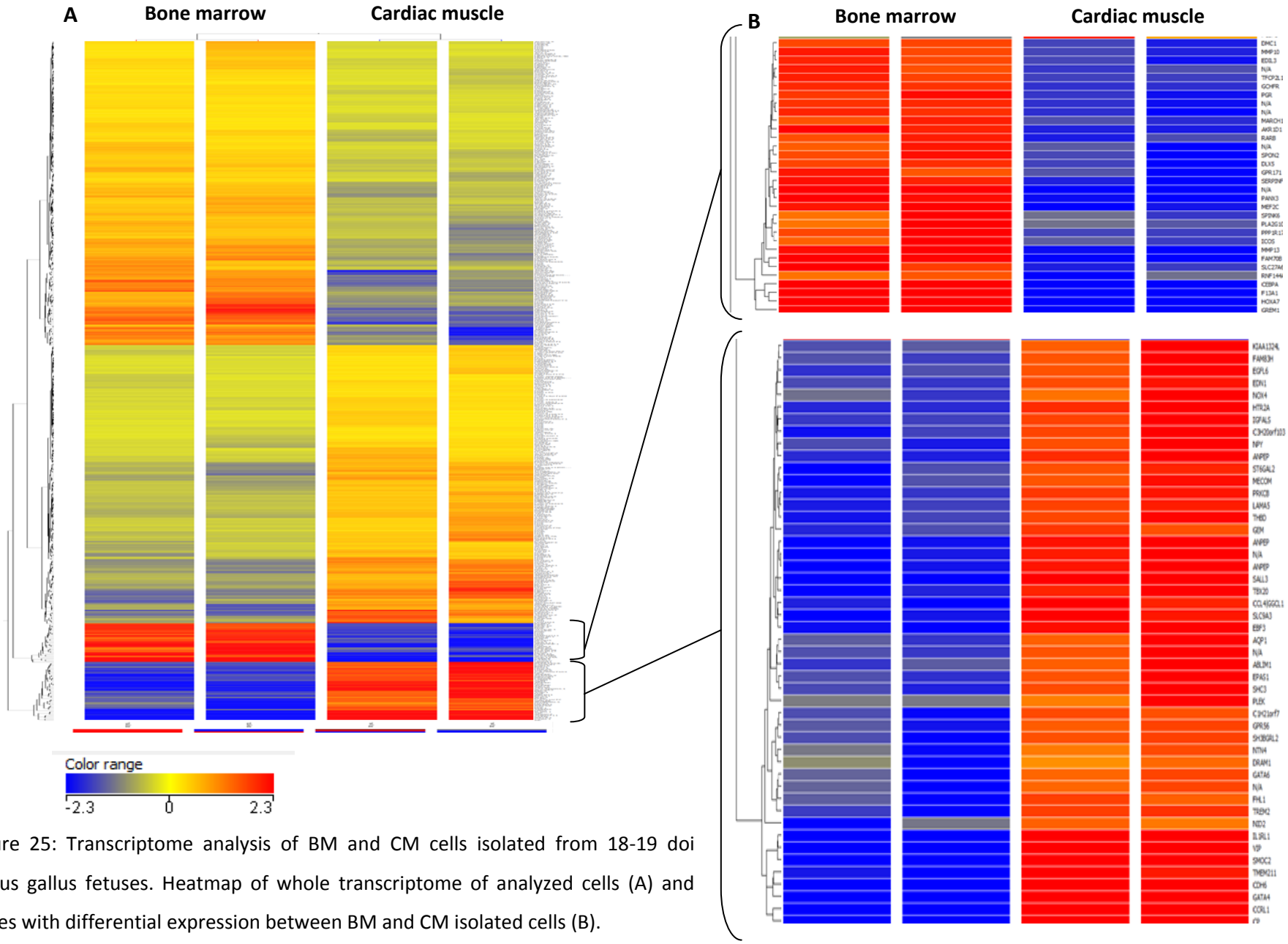

Figure 25: Transcriptome analysis of BM and CM cells isolated from 18-19 doi Gallus gallus fetuses. Heatmap of whole transcriptome of analyzed cells (A) and genes with differential expression between BM and CM isolated cells (B).

Table 4. Biological processes and molecular functions associated to the genes overexpressed in BM isolated cells.

Biological processes	***False discovery rate***	***Molecular functions***	***False discovery rate***
cellular response to organic cyclic compound	8.16×10^{-2}	regulatory region DNA binding	9.21×10^{-2}
response to bacterium	8.16×10^{-2}	bone morphogenesis	9.21×10^{-2}
regulation of cell activation	8.16×10^{-2}	transcription regulatory region DNA binding	9.21×10^{-2}
response to lipopolysaccharide	$8.16v10^{-2}$	regulatory region nucleic acid binding	9.21×10^{-2}
response to molecule of bacterial origin	1.04×10^{-1}	bone development	2.75×10^{-1}
nitric oxide biosynthetic process	1.73×10^{-1}	activating transcription factor binding	3.21×10^{-1}
response to organic cyclic compound	1.80×10^{-1}	RNA polymerase II regulatory region DNA binding	3.26×10^{-1}
regulation of monooxygenase activity	2.14×10^{-1}	RNA polymerase II regulatory region sequence-specific DNA binding	3.26×10^{-1}
cellular response to lipopolysaccharide	2.14×10^{-1}		
secretory granule lumen	2.29×10^{-1}	sequence-specific DNA binding	3.40×10^{-1}
		histone deacetylase binding	3.53×10^{-1}

Table 5. Biological processes and molecular functions associated to the genes overexpressed CM isolated cells.

Biological processes	***False discovery rate***	***Molecular functions***	***False discovery rate***
muscle structure development	7.25×10^{-3}	hormone activity	1.08×10^{-4}
regulation of angiogenesis	7.25×10^{-3}	smooth muscle contraction	1.53×10^{-3}
blood vessel morphogenesis	7.25×10^{-3}	muscle structure development	2.20×10^{-3}
blood vessel development	7.89×10^{-3}	inositol phosphate-mediated signaling	2.20×10^{-3}
axon guidance	9.02×10^{-3}	vasoconstriction	2.41×10^{-3}
vasculature development	9.02×10^{-3}	regulation of response to external stimulus	8.48×10^{-3}
angiogenesis	9.02×10^{-3}	regulation of heart rate	8.48×10^{-3}
muscle cell differentiation	9.02×10^{-3}	muscle cell differentiation	8.48×10^{-3}
basal lamina	1.03×10^{-2}	second-messenger-mediated signaling	9.33×10^{-3}
regulation of hemostasis	2.74×10^{-2}	regulation of blood vessel size	1.23×10^{-2}
regulation of blood coagulation	2.74×10^{-2}	digestive system development	1.23×10^{-2}
regulation of coagulation	2.91×10^{-2}	digestive tract development	1.23×10^{-2}
cardiac septum morphogenesis	2.91×10^{-2}	regulation of tube size	1.23×10^{-2}
regulation of platelet activation	3.24×10^{-2}	blood vessel morphogenesis	1.36×10^{-2}
central nervous system development		positive regulation of multicellular organismal process	

substrate adhesion-dependent cell spreading	3.32×10^{-2}	regulation of systemic arterial blood pressure by hormone	2.11×10^{-2}
positive regulation of angiogenesis	3.32×10^{-2}	blood vessel development	2.11×10^{-2}
regulation of wound healing	3.32×10^{-2}	cardiac septum morphogenesis	2.11×10^{-2}
regulation of response to external stimulus	3.82×10^{-2}	calcium-mediated signaling	2.11×10^{-2}
second-messenger-mediated signaling	4.41×10^{-2}	behavior	2.19×10^{-2}
cardiac septum development	5.05×10^{-2}	vasculature development	2.25×10^{-2}
outflow tract morphogenesis	5.28×10^{-2}	regulation of systemic arterial blood pressure mediated by a chemical signal	2.25×10^{-2}
endoderm development	6.44×10^{-2}	vascular process in circulatory system	2.25×10^{-2}
response to hypoxia	6.92×10^{-2}	positive regulation of angiogenesis	2.25×10^{-2}
basement membrane	7.75×10^{-2}	smooth muscle cell differentiation	2.25×10^{-2}
response to oxygen levels	7.92×10^{-2}	regulation of anatomical structure size	2.25×10^{-2}
negative regulation of hemostasis	7.92×10^{-2}	angiogenesis	2.48×10^{-2}
negative regulation of blood coagulation	8.66×10^{-2}	cardiac septum development	3.02×10^{-2}
negative regulation of coagulation	8.66×10^{-2}	heart looping	3.96×10^{-2}
regulation of smooth muscle cell proliferation	9.76×10^{-2}	embryonic heart tube morphogenesis	4.33×10^{-2}
	9.76×10^{-2}	determination of heart left/right asymmetry	4.57×10^{-2}
		endoderm development	4.57×10^{-2}
		regulation of systemic arterial blood pressure	

	feeding behavior	4.97×10^{-2}
	digestion	5.39×10^{-2}
	endocrine process	5.82×10^{-2}
	epithelium development	6.18×10^{-2}
	embryonic heart tube development	6.38×10^{-2}
	leukocyte chemotaxis	6.38×10^{-2}
	positive regulation of response to external stimulus	6.38×10^{-2}
	response to drug	7.71×10^{-2}
	soluble fraction	8.59×10^{-2}
		9.29×10^{-2}
		9.29×10^{-2}

3. Discussion

MSC were firstly obtained from BM by Friedenstein and co-workers (Afanasyev *et al.*, 2009) and BM is still the most common local of MSC isolation. However, MSC have already been found in several other body compartments, from fetal to adult stage (Campagnoli *et al.*, 2001; Pittenger *et al.*, 1999). Young *et al.* (1995) were able to isolated putative mesenchymal stem cells from 26 compartments of 11 days chicken fetuses, including bone, intestine, skeletal muscle and heart. Following this logic, we isolated potential MSC from bone marrow, skeletal and cardiac muscle of 18-19 doi *G. gallus* fetuses. Cells isolated presented fibroblast-like morphology and were able to adhere to plastic surface. BM, SM and CM isolated cells lasted in culture for at least 10 passages.

MSC must express the surface markers CD73, CD90 and CD105 but lack CD45, CD11b, CD14, CD19, CD34, CD79a and HLA class II (Dominici *et al.*, 2006). There are also reports relating the absence of CD31 and CD133 (Kern *et al.*, 2006; Zuk *et al.*, 2002). BM, SM and CM isolated cell were positive for the surface proteins CD73, CD90 and CD105 and negative for hematopoietic stem cells markers CD31, CD34, CD44 and CD133. Two of the positive markers, CD73 and CD105, were not detected by PCR endpoint method in BM and SM cells. However, we were able to detect them through real time quantitative PCR. These results may be due to low expression levels of these two markers, what could make the detection possible only by applying a more sensitive method, as real time PCR.

Beside the classic MSC surface markers, there are reports of embryonic stem cells markers expression in MSC (Battula *et al.*, 2007; Riekstina *et al.*, 2009; Trubiani *et al.*, 2010). Therefore, we also evaluated the expression of ESC markers in the cells isolated from BM, CM and SM. However, none of the cells expressed any of the markers evaluated. These results are not in line with results from Khatri *et al.* (2009), who reported the presence of Oct-4, Sox-2 and Nanog in MSC isolated from 1- to 14-day-old chicken BM. However, the expression of ESC markers on MSC is not an established consensus. Some studies report no expression of ESC markers on MSC. Pierantozzi *et al.* (2011) did not detect Oct-4 and Sox-2 in MSC isolated from bone marrow, adipose tissue, and cardiac biopsies. Nanog expression was observed, but not

in freshly isolated cells. This marker was detected after the cell cultivation *in vitro* and its presence is attributed to the culture conditions adaptation (Pierantozzi *et al.*, 2011).

Generally, the expression MSC markers are evaluated only in cells in stem status, but not during the differentiation induction. Therefore, we decided to study the expression behavior of embryonic, mesenchymal and hematopoietic stem cells markers also during the cells exposition to the osteogenic and adipogenic medium. Stem cell markers not detected before differentiation induction remained undetected after the differentiation media exposition, but MSC markers expression were detected during the whole differentiation assays. These results agree with Liu *et al.* (2008) observations. These authors reported that the number of cells positive for CD13, CD29, CD44, CD73, CD90, CD105, and CD166 did not changed after osteogenic induction. Real time PCR analyzes confirmed the detection of the markers during the whole process of differentiation. However, it was observed a downregulation of MSC markers during differentiation for BM and SM isolated cells, and upregulation for CM cells. The behaviour of MSC associated markers may be correlated to the differentiation progression. Proteomic analysis of surface markers before and after adipogenic and osteogenic differentiation detected downregulation of CD90 and CD105, but upregulation of CD73 (Niehage *et al.*, 2011). In the case of our study, osteogenic differentiation of BM and SM cells seems to have occurred earlier, when compared to CM isolated cells, and this may explain the non-concordant expression behavior between cells from heart and cells from the other two sources.

Besides the surface markers expression, MSC must be able to differentiate at least into adipocytes, chondrocytes and osteoblasts (Dominici *et al.*, 2006). All the cells isolated were able to differentiate into osteoblasts, what was confirmed by von Kossa and alizarin staining of calcium depots. In addition to the staining, other differentiation markers were evaluated. Alkaline phosphatase specific activity increased in BM and CM isolated cells exposed to the osteogenic media, as expected for this differentiation (Liu *et al.*, 2008). However, no ALP was detected in the cell lysate from SM isolated cell, although ALP has been already reported during osteogenic differentiation of skeletal muscle MSC (Yoshimura *et al.*, 2007). The absence of ALP activity may be due to the SM cells detachment from the cultured plate observed after the differentiation media exposition. This fact probably led to a reduced enzyme levels in the cell lysate,

too low to generate enough colored product to be detected in a spectrophotometer. Moreover, although enzyme activity was detected in two assays, the ALP gene expression was not observed on end point PCR. However, ALP was detected on real time PCR analysis, which may indicated that the end point primers used for ALP cDNA amplification were not effective or the ALP mRNA levels are too low to generate RT-PCR visible bands on agarose gels. Interestingly, the ALP enzymatic assays results do not match with real time PCR ALP gene expression levels, what have been observed before in adult human BM MSC osteogenesis (Frank *et al.*, 2002). For these cells, the higher enzymatic activity occurred between 5 and 10 days of differentiation, but the ALP gene expression remained constant during the whole assay (Frank *et al.*, 2002), revealing that the enzymatic activity does not always reflect the gene expression.

Gene expression profile of osteogenic markers was different among cells from different sources. For BM isolated cells, we observed a decreasing expression of BMP-2, osteonectin and ALP, but BMP-4 and osteopontin was upregulated. SM cells showed a peak on BMP-2 expression together with a decrease on BMP-4 and osteonectin on 10 day. ALP was only detected at the end of the assay and osteopontin was not observed on real time analysis. CM isolated cells presented an increasing expression of BMP-2, BMP-4 and osteonectin. An ALP peak was noticed on 10 day.

BMP-2, and also BMP-4, are growth factors included on the transforming growth factor beta (TGF-β) superfamily (Bragdon *et al.*, 2011). Both BMP-2 and BMP-4 are known to stimulate MSC osteogenic differentiation (Luu *et al.*, 2007). BMP-2 is detected on MSC and its expression is higher during differentiation, presenting two peaks (4-7 and 21-28 days) (Bi *et al.*, 1999). BMP-2 was showed to accelerate and increase the osteogenic differentiation (Chen *et al.*, 1997). BMP-2 super-expression provokes an increase in the BMP-4, BMP-3, ALP, osteocalcin and osteopontin expression (Chen *et al.*, 1997). BMP-4 expression enhancement occurs in the late phase of differentiation, correlated with osteogenic markers, such as ALP, osteocalcin and osteopontin (Chen *et al.*, 1997).

Osteopontin is known to be expressed previous to the osteocalcin during the osteogenic differentiation, and is detected both in immature fibroblastic cells and in post proliferative mature osteoblasts from rat calvaria (Liu *et al.*, 2003; Liu *et al.*, 1994). Osteocalcin generally appears latter stage of the differentiation, only in the post

proliferative stage (Malaval *et al.*, 1999). ALP detection increases during the differentiation progression (Liu *et al.*, 1994; Malaval *et al.*, 1999) and osteonectin was observed to not change its expression after the differentiation media exposition. (Shur *et al.*, 2001).

The osteogenic markers expression pattern observed in our experiments may be explained by the speed of differentiation progression. It was noticed that BM and SM isolated cells differentiate earlier than CM cells. Possibly, BM differentiates as soon after the osteogenic media exposition. Peaks of osteogenic markers might have happened before the 10 day and this may explain the downregulation of some markers on time points analyzed. CM cells started to differentiate close to the end of the osteogenic assay, in line with the markers expression still upregulated on 21 day. SM cells may be in an intermediated state of differentiation, evidenced by the BMP-2 peak on 10 day, followed by an expression increase of osteonectin, BMP-4 and ALP on 16 day.

BM and SM isolated cells were also able to be differentiated into adipogenic lineages and lipid droplets could be seen inside the cell cytoplasm. However, none of the adipogenic differentiation markers were detected by end point PCR, except for DLK1. PPAR-γ and FABP-4 were only detected by real time PCR analysis, which also confirmed the adiponectin absence. Dlk1 expression was detected in cells both before and after the adipogenic differentiation induction. PPAR-γ is considered an early marker of adipogenic differentiation, whereas FAPB-4 is detected in the intermediate/terminal phase (Niemela *et al.*, 2007). Adiponectin is not expressed in preadipocytes and it is restrict to mature adipocytes (Korner *et al.*, 2005), and DLK1 is expressed in MSC (Abdallah *et al.*, 2004) and also in preadipocytes (Smas & Sul, 1993), downregulating during their conversion into mature adipocytes (Smas & Sul, 1993). Experiments overexpressing DLK1 on MSC showed that these cells were unable to differentiate into adipocytes. At the molecular level, DLK1 overexpression reduced the expression of PPAR-γ, FABP4 and adiponenctin. In this line, the presence of DLK1 in our cells may have led to low levels of PPAR-γ, FABP4 and adiponenctin, whose expression could only be detected by a more sensitive technique, as real time PCR. Therefore, the markers expression pattern observed in our cells indicate that they may not have reached the adipocyte status, being in an intermediated stage as preadipocytes.

Quantification of Dlk1 expression should be proceeded to better understand the expression behavior of the other marker genes.

The phenotype presented by the BM and SC isolated cells is in agreement with the minimal defining multipotent mesenchymal stromal cells stated by The International Society for Cellular Therapy in 2006 (Dominici *et al.*, 2006). CM isolated cells, although presenting the surface markers profile characteristic of MSC and being able to differentiate into osteoblasts, were not capable to generate adipocytes, even when the adipogenic media exposition was extended to one month. Indeed, MSC isolated from heart have been already reported as having a slow differentiation into adipocytes (Pelekanos *et al.*, 2012). On the other hand, our cells share many common characteristics with epicardium-derived cells (EPDCs). EPDCs are characterized by the presence of the surface markers CD90 and CD105, and absence of CD31, CD34, CD45 and CD133 (van Tuyn *et al.*, 2007). Moreover, EPDCs, like the cells isolated from CM, are able to differentiate into osteoblasts but not in adipocytes (van Tuyn *et al.*, 2007). Then, CM cells isolated by us might need more exposition time to the differentiation media to turn into adipocytes or they are not MSC, but EPDCs.

Transcriptome analysis of CM isolated cells together with cells isolated from BM, the classical source of MSC, were proceeded to evaluate the similarities and differences between the two cells. Two groups of genes were differentially expressed between the two cell types and some interesting targets are discussed here.

Among upregulated genes on BM isolated cells we identified Gremlin, DLX5 and MEF2C. None of the genes detected in our transcriptome analysis were reported in other articles (Jansen *et al.*, 2010; Pelekanos *et al.*, 2012). The only exception is DLX5, which was reported as superexpressed by BM MSC by two other transcriptional analysis (Jansen *et al.*, 2010; Tsai *et al.*, 2007).

Gremlin is a member of the DAN/Cerberus family seems to be involved in the maintenance of undifferentiated state of cells. In tumor, Gremlin promotes the cells expansion and blocks their differentiation, thus being responsible for maintaining the self-renew of tumor cells (Sneddon *et al.*, 2006). Stromal cells overexpressing Gremlin shows lower proliferation rate, delayed osteocalcin expression and no mineralization after the osteogenic induction (Gazzerro *et al.*, 2005). On the other hand, deletion of Gremlin led to an increase mineral deposition and bone formation. When marrow

stromal cells were exposed to BMP-2, those null for Gremlin presented an increase in alkaline phosphatese activity and enhanced the effect of BMP-2 (Gazzerro *et al.*, 2007).

DLX5 (Distal-less homeobox 5) is expressed in MSC, but its expression increases after osteogenic induction and downregulates on adipogenic stimuli (Lee *et al.*, 2012; Lee *et al.*, 2013) Overexpression of DLX5 suppresses and enhances adipogenic and osteogenic differentiation, respectivel (Lee *et al.*, 2012; Lee *et al.*, 2013). Otherwise, knockdown of DLX5 was showed to enhance adipogenic differentiation of 3T3 cells. (Lee *et al.*, 2013). Maybe MSC isolated from BM are primed to osteogenic differentiation due to the DLX5 expression. However, adipogenic stimuli donwregulates the DLX5 expression and lead to adipogenic differentiation of the cell.

MEF2C is a transcription factor commonly observed in cardiac cells (McDermott *et al.*, 1993). However, MEF2C has also reported roles on bone formation and development. Knockout of this gene on osteoblasts and osteocytes leads to an increase in the appendicular and axial skeleton bone mass and decreased osteoclast bone absorption (Collette *et al.*, 2012; Kramer *et al.*, 2012). MEF2C also plays roles in skeleton development. Its absence impairs chondrocyte hypertrophy, a previous stage for bone formation, and leads to defects on endochondral ossification (Arnold *et al.*, 2007). Although MEF2C importance in bone formation, there are no previous reports about its function on MSC.

CM isolated cells seems to be more compromised with cardiac lineages. Genes upregulated are related to muscle differentiation, heart morphogenesis, angiogenesis and coagulation. Among the upregulated genes with interesting functions are EGFL6, NPY, VIP, TBX20, GATA-4 and GATA-6.

EGFL6 is highly expressed in kidney, lung and bone tissues and promotes endothelial cell migration and angiogenesis, inducing the organization of endothelial cells in three-dimensional tube-like structures. EGFL6 is also secreted by osteoblasts during bone development (Chim *et al.*, 2011).

NPY (Neuropeptide Y) have roles in cell proliferation and angiogenesis. NPY induces proliferation of neonatal and adult cardiomyocytes and vascular smooth muscle (Wang *et al.*, 2010; Zukowska-Grojec *et al.*, 1993). NPY is also expressed in MSC (Igura *et al.*, 2011; Wang *et al.*, 2010) and Downregulation of its receptor in MSC was reported as the reason for low proliferation of MSC isolated from old donors (Igura *et*

al., 2011). In addition, NPY was reported to increase MSC migration and induce these cells to form capillary-like tubes (Wang *et al.*, 2010). MSC also differentiate into cardiomyocytes and endothelial cells when under NPY influence (Wang *et al.*, 2010). ESC were also responsive to NPY. This molecule was reported to be capable to maintain ESC in the undifferentiated state for more than 4 months without feeder layer and exposed to medium without serum(Son & Cho, 2012).

VIP (Vasoactive Intestinal Peptide) was previously reported as expressed by MSC-like populations isolated from heart (Pelekanos *et al.*, 2012). However, there are no other studies about VIP expression by cardiac cells. VIP is an interesting target due to their angiogenic effects (Yang *et al.*, 2009). VIP induces proliferation of endothelial cells and also promotes angiogenesis, both mediated by induction of VEGF expression (Yang *et al.*, 2013; Yang *et al.*, 2009). VIP was also reported as having effects over immune system cells (reviewed in Smalley *et al.* (2009).

TBX20 (T-box transcription factor) has important roles in heart morphogenesis. Mice embryos lacking TBX20 expression presented severe defective cardiac morphogenesis, decrease in cell proliferation in myocardium and died before birth (Cai *et al.*, 2005; Stennard *et al.*, 2005). During heart development, Tbx20 have roles in chamber formation and it is related to ventricular function in adult heart (Stennard *et al.*, 2005) Tbx20 ablation in adult hearts resulted in dilated hearts with several alterations of cardiac function, such as loss of systolic function, conduction delay and arrhythmia (Shen *et al.*, 2011). Besides, among downstream targets of Tbx20 are genes with important roles in cardiac development and function.

GATA-4 and GATA-6 are transcription factors expressed in myocardium (Charron *et al.*, 1999) and have redundant roles in the onset of cardiac myocyte differentiation during embryogenesis (Zhao et al., 2008). GATA-4/6 were reported to interact physically and co-localize in cardiomyocytes(Charron *et al.*, 1999). Their importance in heart development is evidenced by the absence of beating cells and downregulation of proteins whose expression is characteristic of cardiac myocyte differentiation in GATA-4/6 null embryoid bodies (Zhao *et al.*, 2008). Moreover, embryos lacking both transcritption factors presented acardia(Zhao *et al.*, 2008)

4. Conclusions

We conclude that MSC can be isolated and cultured *in vitro* from bone marrow and skeletal muscle from *Gallus gallus* fetuses. These cells showed the classical characteristics already described for MSC: they present fibroblat-like morphology, plastic adherence, express the molecular markers CD73, CD90 and CD105, and differentiate into osteoblasts and adipocyte. These cells, therefore, can be called MSC.

In addition, we were able to isolate a particular group o cells from the CM. Although CM isolated cells present many MSC characteristics, they are not able to differentiate into adipocytes. The transcriptome analysis revealed that these cells express genes associated with the cardiac tissue, what indicates that CM cells are more compromised than MSC. Based on the CM isolated cells behavior, it is possible that these cells are EPDCs. However, a deeper characterization of CM cells is necessary to better conclude about its identity.

3.3. References

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5. DISCUSSÃO GERAL

5.1. *Gallus gallus* como modelo para estudo de células-tronco mesenquimais

O frango doméstico é empregado como modelo biológico há, pelo menos, dois mil anos (Stern, 2005) para estudos de desenvolvimento. No entanto, no campo de pesquisa das células-tronco mesenquimais, o uso deste organismo é bastante limitado. Apesar da existência de possíveis CTM distribuídas em vários compartimentos corporais deste organismo (Young *et al*., 1995), os estudos com células-tronco isoladas de frango limitam-se à caracterização das células isoladas de medula óssea, pulmão e tecido adiposo (Gong *et al*., 2011; Khatri *et al*., 2010; Khatri *et al*., 2009). Estudos mais complexos da biologia de CTM utilizando células de frango são praticamente inexistentes.

O intuito de isolar CTM de fetos de frango é o de propor *G. gallus* como alternativa de modelo para o estudo de CTM. Como passo inicial, a caracterização de CTM obtidas de medula óssea e músculo esquelético, bem como o isolamento de possíveis progenitores cardíacos (EPDCs), disponibiliza três modelos celulares para estudo. O isolamento e caracterização molecular de células de outros compartimentos fetais expandirão o potencial do modelo no campo das CTM, também servindo de confirmação e complementação as pesquisas de Young *et al*. (1995).

Uma vez estabelecidos estes modelos para estudo de CTM, lacunas de conhecimento presentes no campo de CTM poderão ser preenchidas utilizando-se células isoladas de fetos de frango. Entre as áreas com conhecimento escasso sobre os microRNAs expressos em CTM. Há apenas quatro estudos de levantamento do *pool* de miRNAs característicos de CTM, e estes concentram-se apenas nas células isoladas da medula óssea e do tecido adiposo (Bae *et al*., 2009; Gao *et al*., 2011; Greco & Rameshwar, 2007; Kao *et al*., 2009). Estudos da função dos miRNAs identificados no estado tronco também é incipiente, enfocando apenas nas moléculas com papel na indução da diferenciação (Gao *et al*., 2011). Outro campo também pouco explorado é o papel de miRNA na senescência de CTM. Apenas sabe-se que hsamir-371, hsamir-369-5P, hsa-mir-29c, hsa-mir-499 and hsa-mir-217 apresentam aumento de expressão

quando comparam-se células recém isoladas e células senescentes (Wagner *et al*., 2008).

Além disso, a análise de transcriptoma realizada com as células obtidas da medula óssea e do músculo cardíaco de fetos de frango revelou genes cuja expressão não fora reportada em CTM previamente. A confirmação de que se tratam de moléculas características deste tipo celular, via análise de CTM isoladas de outros compartimentos, e a análise do seu papel nestas células também são perspectivas que se abrem com este trabalho.

5.2. Características de células-tronco mesenquimais isoladas de fetos de *Gallus gallus*

A medula óssea é o tecido de onde comumente as CTM são isoladas para a maioria dos estudos envolvendo este tipo celular. No entanto, há evidências da existência de células similares às CTM da medula distribuídas em outros locais no organismo. Young *et al*. (1995) obtiveram células capazes de diferenciarem-se em tecido ósseo, adiposo, cartilaginoso e muscular de 26 diferentes compartimentos de fetos de frango com 11 dias. Células isoladas do espaço perivascular de uma série de compartimentos corporais demonstraram capacidade de diferenciarem-se em células musculares, adipócitos, osteoblastos e condrócitos, bem como expressão de marcadores de superfície celular característicos de CTM (Crisan *et al*., 2008). Esses resultados estão de acordo com a hipóteses de que as CTM estão distribuídas em todo o organismo, ocupando um nicho perivascular (da Silva Meirelles *et al*., 2006). Em outras palavras, as CTM estariam associadas à membrana basal de vasos sanguíneos, funcionando como uma reserva de células indiferenciadas que atuariam no reparo e regeneração de lesões do tecido/órgão onde se encontram (da Silva Meirelles *et al*., 2008; da Silva Meirelles *et al*., 2006).

Dentro deste contexto, conseguiu-se isolar células com características de CTM não só da medula óssea, mas também da musculatura esquelética de *G. gallus*. Planejava-se também isolar CTM de músculo cardíaco, uma vez que já há relatos da presença de CTM neste nicho (Pelekanos *et al*., 2012). No entanto, as células obtidas do tecido cardíaco apresentaram fenótipo mais próximo ao observado em EPDCs (ver item 4.2).

O isolamento e a caracterização das CTM são feitos via adesão das células à superfície do recipiente de cultura e pela avaliação da expressão de proteínas de superfície celular associada ao estudo do potencial de diferenciação, respectivamente (Dominici *et al*., 2006). Considerando uma cultura homogênea de CTM seria a condição ideal para a sua expansão para posterior uso em terapia celular, marcadores para o isolamento de populações puras de CTM se tornam importantes. Em 2006, estabeleceu-se a expressão de CD73, CD90 e CD105 como marcadores positivos e CD14, CD34, CD45, ou CD11b, CD79a ou CD19 e HLA classe II como marcadores negativos de CTM (Dominici *et al*., 2006). No entanto, o levantamento bibliográfico das CTM isoladas e caracterizadas até o momento expandiu o conjunto de marcadores positivos para CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166 e HLA-ABC. Em adição, CD14, CD31, CD34, CD45, CD62E, CD62L, CD62P e HLA-DR parecem não ser detectados na superfície celular de CTM (Calloni *et al*., 2013).

O conjunto de marcadores comumente utilizado para caracterizar as CTM é bastante variável entre estudos e entre células isoladas de diferentes locais (Calloni *et al*., 2013), geralmente atendo-se à observação a presença de CD73, CD90 e CD105 em adição à não detecção de CD34. Estudos mais abrangentes de superfície celular permitiriam estabelecer mais precisamente os marcadores que são universais às CTM e também os que podem caracterizá-las quanto ao local de origem. Até o momento, análises em larga escala enfocando proteínas de membrana de CTM foram procedidas apenas com células oriundas da medula óssea (Delorme *et al*., 2008; Jeong *et al*., 2007; Niehage *et al*., 2011). Além disso, há pouco conhecimento sobre como os marcadores de superfície celular já estabelecidos para CTM comportam-se durante o processo de diferenciação. Neste trabalho, observou-se que, de maneira geral, há uma diminuição da expressão de CD73, CD90 e CD105 durante o processo de ostegênse e adipogênese de CTM de medula óssea e de músculo esquelético. O acompanhamento do comportamento de um pool maior de marcadores durante a diferenciação seria interessante no sentido de abrir a possibilidade de identificar os tipos celulares intermediários entre a CTM e a célula terminalmente diferenciada dela originada.

Ainda dentro do contexto de marcadores de estado tronco, a expressão de marcadores de CTE em CTM não é consenso na literatura. As células isoladas neste estudo não apresentam expressão de Oct-4, Sox-2, Nanog e telomerase, em contraste

ao observado por outros autores em células do mesmo organismo (Khatri *et al*., 2009). Por isso, um dos gargalos desta questão está em definir se as CTM em geral expressam de fato esses marcadores ou se a expressão destes é um artefato do seu cultivo in vitro. Além disso, em caso da presença de marcadores de CTE ser uma característica também das CTM, pouco se sabe sobre o papel destas moléculas neste tipo de célula-tronco e estudos de função, que ainda são bastante escassos, se fazem necessários.

As linhagens celulares para as quais as CTM são capazes de se diferenciarem já estão bem estabelecidas. Em conformidade com a literatura, as células isoladas de fetos de frango foram capazes de diferenciarem-se em osteoblastos (células isoladas da medula óssea e dos músculos esquelético e cardíaco) e em pré-adipócitos (células isoladas de medula óssea e músculo esquelético). O padrão de expressão dos marcadores osteogênicos (fosfatase alcalina, ostenectina, osteopontina e osteocalcina) durante a diferenciação osteogênica já se encontra relativamente estabelecido (Frank *et al*., 2002; Liu *et al*., 2003; Liu *et al*., 1994; Malaval *et al*., 1999; Shur *et al*., 2001). No entanto, estes estudos foram procedidos a partir da diferenciação de osteoblastos (Shur et al., 2001) ou de osteoprogenitores não caracterizados (Liu *et al*., 2003; Liu *et al*., 1994; Malaval *et al*., 1999). O estudo com CTM de *G. gallus* está entre os pioneiros no acompanhamento, via PCR em tempo real, da expressão de um conjunto abrangente de marcadores oestogênicos antes e durante o processo diferenciação, juntamente com estudo de Frank *et al*. (2002). Além disso, pela primeira vez a expressão de BMP-4 é avaliada em uma CTM. A expansão da análise quantitativa para outros genes sabidamente envolvidos no processo de osteogênese, tais como Runx2, Osterix, osteoprotegerina e BSP, nas células isoladas de frango enriqueceria o conhecimento molecular da diferenciação osteogênica destas células.

Quanto a diferenciação adipogênica, apesar da observação de depósitos lipídicos intracelulares, existe a possibilidade de as células isoladas de medula óssea e músculo esquelético não terem atingido o estágio de adipócito. Esta hipótese é levantada baseando-se na expressão ausente ou baixa de marcadores adipogênicos como PPAR-γ, FABP-4 e adiponectina, juntamente com a expressão de DLK1, marcador de pré-adipócitos, em todos os momentos analisados. A avaliação de outros genes expressos durante o processo de geração de adipócitos, como C/EBP-β, C/EBP-δ e lipoproteína lipase, marcadores iniciais da transição pré-adipócito/adipócito, e

adipsina, angiotensiongênio II, leptina e ACBP, marcadores tardios (Niemela *et al*., 2007), poderia auxiliar na confirmação da hipótese de diferenciação parcial.

Para a completa caracterização das CTM isoladas de frango, estudos adicionais visando verificar o potencial das células obtidas neste trabalho de diferenciarem-se em condroblastos, myoblastos, células beta-pancreáticas e neurônios também seriam interessantes para a sua completa caracterização. As características observadas nas CTM de frango estão de acordo com as observadas em uma série de outros organismos modelo. O levantamento bibliográfico das características das CTM de diferentes espécies revelou que, independentemente do organismo e do local de isolamento, a capacidade de aderir ao plástico, a morfologia similar a de fibroblastos, o padrão de marcadores de superfície e a capacidade de diferenciar-se em adipócitos, condrócitos e osteoblastos parecem ser características universais deste tipo celular (Calloni *et al*., 2013; manuscrito em preparação).

5.3 EPDCs (*Epicardium-derived cells*)

O epicárdio forma-se através da migração de células pró-epicárdicas para a superfície do tubo cardíaco em formação, as quais aderem ao miocárdio e migram lateralmente até o completo envolvimento do órgão (Lie-Venema *et al*., 2007). Após o total encobrimento do coração, um grupo de células do epicárdio sofre transição epitelial-mesenquimal, gerando as EPDCs (do inglês *epicardium-derived cells*) (Lie-Venema *et al*., 2007). As EPDCs migram através do miocárdio, contribuindo para o desenvolvimento dos vasos coronarianos, das válvulas atrioventriculares, da arquitetura do miocárdio e do sistema de condução periférico (Lie-Venema *et al*., 2007). Dado o seu potencial de diferenciação, EPDCs já foram propostas como células-tronco cardíacas (Wessels & Perez-Pomares, 2004).

Um estudo que caracterizou EPDCs humanas revelou que as mesmas apresentam morfologia similar à de fibroblastos, expressam as proteínas de superfície CD44, CD46, CD90, CD105 e HLA-ABC e são possíveis indução a diferenciação em osteoblastos e células de musculatura lisa, porém não são capazes de gerar adipócitos (van Tuyn *et al*., 2007). Além disso, essas células expressam uma série de genes

característicos de células de músculo liso e cardíaco, dentre elas GATA-4 (van Tuyn *et al*., 2007).

As EPDCs apresentam potencial de emprego em terapia celular. Quando administradas em corações de camundongos infartados, observa-se uma melhora da função cardíaca, juntamente com um aumento da vascularização do local do dano e aumento da sobrevivência dos indivíduos infartados (Winter *et al*., 2007). Além disso, os efeitos benéficos das EPDCS perduram por um longo tempo (Winter *et al*., 2007). A aplicação de EPDCs associadas a precursores de cardiomiócitos em corações infartados também revelou uma melhora da função cardíaca, resultado da interação sinérgica dos dois tipos celulares, possivelmente por ação parácrina (Winter *et al*., 2009).

A similaridade de características com as células estudadas por van Tuyn *et al*. (2007) levantou a possibilidade de que as células isoladas de tecido cardíaco neste trabalho seriam EPDCs. Além disso, os dados de expressão obtidos na análise de transcriptoma corroboram essa hipótese. EPDCs parecem induzir angiogênse em miocárdios enfartados (Winter *et al*., 2007) e as células obtidas de coração de *G. gallus* expressam uma série de fatores envolvidos com o processo de angiogênese (EDN1, TBX20, PRKCB1, GATA-4, GATA-6, AQP1 e LAMA5). As EPDCs também são capazes de diferenciarem-se em células de músculo liso da parede de vasos sanguíneos (Gittenberger-de Groot *et al*., 2010). Em concordância com esta característica, as células isoladas de coração neste trabalho também expressam genes associados a diferenciação de células de músculo liso (EDN1, IGFBP5, GATA-4, GATA-6, EPAS1 and FHL1).

Considerando o potencial das EPDCs e a necessidade de conhecer melhor a sua biologia, as células isoladas de coração de *G. gallus* apresentam-se como um modelo atraente para o estudo de EPDCs. No entanto, apesar de haverem fortes indícios de se tratarem EPDCs, estudos adicionais são necessários para a confirmação da identidade destas células.

6. CONCLUSÕES GERAIS

Considerando os resultados obtidos durante o desenvolvimento deste trabalho, conclui-se que:

- é possível isolar células-tronco mesenquimais da medula óssea e do músculo esquelético de fetos de frango com 18-19 dias de incubação;

- apesar do grande volume de pesquisas visando a identificação de marcadores de células-tronco, ainda são necessários estudos para melhor caracterizar molecularmente as CTE, CTM e CTH;

- com base em um levantamento da literatura, propõe-se a atualização da lista de marcadores positivos característicos de CTM para CD13, CD29, CD44, CD49e, CD54, CD71, CD73, CD90, CD105, CD106, CD166 e HLA-ABC, e de marcadores negativos para CD14, CD31, CD34, CD45, CD62E, CD62 L, CD62P e HLA-DR;

- independentemente do organismo ou do compartimento de isolamento, as CTM apresentam um conjunto de características aparentemente universal para estas células: aderência ao plástico, morfologia similar à de fibroblasto, capacidade de diferenciação em, pelo menos, adopócitos, condrócitos e osteoblastos e a expressão de, pelo menos, CD73, CD90 e CD105;

- as células isoladas de músculo cardíaco apresentam morfologia e marcadores de superfície característicos de células-tronco mesenquimais, porém quanto ao potencial de diferenciação testado, são incapazes de gerarem adipócitos. Análises moleculares e do potencial de diferenciação para outras linhagens além das testadas são necessários para elucidar a identidade destas células.

- as CTMs isoladas de medula óssea apresentam super-expressão de genes associados a morfogênse óssea, ao transporte de ácidos graxos e de ligação a ácidos nucleicos.

Estudos adicionais se fazem necessários para verificar o papel dos mesmos na biologia desta células;

- as células isoladas de músculo cardíco apresentam super-expressão de genes relacionados a morfogênse cardiaca, a angiogênese, a diferenciação de células de musculatura lisa e a coagulação sanguínea, o que corrobora com a hipótese de que não se tratam de CTMs, mas sim de células da linhagem cardíaca, possivelmente EPDCs.

7. PERSPECTIVAS

A conclusão deste trabalho deixa como perspectivas:

- o isolamento e caracterização de de células-tronco mesenquimais de outros compartimentos corporais de fetos de *G. gallus*;

- a verificação do potencial de diferenciação para condroblastos, neurônios, mioblastos e células beta-pancreáticas das CTM obtidas de medula óssea e músculo esquelético;

- a confirmação da identidade das células isoladas de músculo cardíaco como EPDCs;

- a investigação do papel dos genes observados como super-expressos em CTM de medula óssea nesse tipo celular, uma vez que a maior parte deles tem sua função analizada em CTM;

- os estudos de proteômica de superfície de células isoladas de outros compartimentos corporais que não a medula óssea de *G. gallus*;

- a identificação de miRNAs expressos em CTM antes da indução da diferenciação e estudo do seu papel para a manutenção do estado tronco;

- o estudo do papel de miRNAs no processo de senescência de CTM.

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