UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION AND DIFFERENTIATION POTENTIAL OF RAT FULL-TERM AMNIOTIC FLUID STEM CELLS INTO THE DERIVATIVES OF THE THREE PRIMARY GERM LAYERS

HOO MUN FUN

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By

HOO MUN FUN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

CHARACTERISATION AND DIFFERENTIATION POTENTIAL OF RAT FULL-TERM AMNIOTIC FLUID STEM CELLS INTO THE DERIVATIVES OF THE THREE PRIMARY GERM LAYERS

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HOO MUN FUN

July 2015

Chairman : Norshariza Nordin, PhD
Faculty : Medicine and Health Sciences

Amniotic fluid (AF) is believed to contain highly potent stem cells which make them a reliable source for stem cells with wider differentiation spectrum. However, very few studies have established amniotic fluid stem cells (AFSCs) from full-term AF as most AFSCs were established from mid-term AF. Here, the study aimed to see if rat full-term AF harbours such stem cells with differentiation potential not only into derivatives of the three primary germ layers but also the functional ones. Isolation of amniotic fluid stem cells (AFSCs) from rat full-term AF was carried out using immuno-selection (miniMACS) against c-kit, a stem cell factor receptor that is expressed during embryogenesis. The requirement of leukaemia inhibitory factor (LIF) to maintain the differentiation potential of c-kit positive cells was investigated as part of the cell culture condition optimization. The cells were then characterized with population doubling time, panels of pluripotency and stemness markers and spontaneous differentiation capacity by assessing their ability to form good quality multicellular aggregates, embryoid bodies (EBs). The ability of the cells to undergo directed differentiation into the derivatives of the three primary germ layers, specifically the ectodermal (neurons and glial cells), mesodermal (cardiomyocytes, adipocytes and osteocytes) and endodermal (insulin secreting pancreatic β-cells) cells, as well as their functionality were then studied. Monolayer differentiation protocol was used for neural differentiation where the differentiated cells were analysed with specific markers for early (Pax6 and Nestin), post-mitotic (Class III β-tubulin) and mature neuronal markers (Calbindin, MAP2, GFAP, TH and Synaptophysin). For mesodermal differentiation, retinoic acid (RA), 5-Azacytidine C (5-Aza) and Vitamin C (Vc) treatments were applied prior to re-plating the EBs at high density. The cells were then subjected to O-Red oil

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(fat), Alizarin Red (bone), Alcian Blue (cartilage) staining and mature cardiac markers analysis (cardiac troponin, SERCA and GATA6). For endodermal differentiation, cells were sequentially treated with Activin A, Wnt3a, FGF7, Cyclopamine and retinoic acid before being analysed with pancreatic markers (brachyury, PDX1, CXCR4, NKX 6.1, HNF4A, MafA and insulin). Finally, functional analyses were carried out to examine the secretion of functional proteins, such as dopamine and insulin from the differentiated cells. In this study, the isolated rat full-term AFSCs not only possessed similar expression profiles as highly potent stem cells, but also exhibited a wide differentiation capability in generating not only the derivatives of the three primary germ lineages but also the functional ones. This strongly suggests AF of full-term pregnancy as a great potential source of stem cells with therapeutic value and opens the opportunity towards the establishment of full-term AFSCs in human counterpart as a potential candidate cells to treat various degenerative defects.

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PENCIRIAN DAN POTENSI PEMBEZAAN SEL-SEL STEM JANGKA PENUH CECAIR AMNIOTIK TIKUS KEPADA DERIVATIF DARI TIGA LAPISAN GERMA UTAMA

Oleh

HOO MUN FUN

Julai 2015

Pengerusi : Norshariza Nordin, PhD
Fakulti : Perubatan dan Sains Kesihatan

Cecair amniotik (AF) dipercayai mengandungi sel-sel stem yang berpotensi tinggi yang sering digunakan sebagai sumber sel stem dengan spektrum pembezaan yang luas. Namun, tidak banyak kajian yang dijalankan pada sel stem cecair amnionik (AFSCs) dari AF jangka penuh dan kebanyakan data adalah dari AF jangka pertengahan. Di sini, kajian ini bertujuan untuk melihat jika AF jangka penuh tikus mengandungi sel-sel stem dengan potensi pembezaan kepada derivatif daripada tiga lapisan germa berfungsi. Sel-sel stem cecair amnionik (AFSCs) telah diisolasikan daripada AF jangka penuh tikus dengan menggunakan immunon pilihan (miniMACS) terhadap C-kit, faktor reseptor sel stem yang didapati dalam proses embriogenesis. Keperluan faktor inhibitori leukemia (LIF) dalam mengekalkan potensi perbezaan sel-sel c-kit positif telah disiasat sebagai sebahagian daripada pengoptimuman keadaan kultul. Sel-sel c-kit positif kemudian dicirikan dengan masa pengandaan populasi, panel penanda-penanda pluripotensi dan kapasiti pembezaan secara spontan melalui pembentukan agregat multisellular, iaitu badan embrioid (EBs). Kemudian, keupayaan sel-sel c-kit positif untuk dibezakan kepada derivatif daripada tiga lapisan germa utama, seperti ectodermal (sel-sel saraf dan glia), mesodermal (sel jantung, sel lemak, sel tulang dan sel kondrosit) dan endodermal (β-sel pankreas), dianalisa serta fungsi sel-sel selepas pembezaan dinilai. Protokol monolayer differentiation (MD), telah digunakan untuk membezakan sel-sel c-kit positif kepada sel-sel saraf dan glia, di mana sel-sel yang telah diperbezakan akan dinilai dengan penanda-penada awal sel-sel saraf (Pax6 dan Nestin), penanda tamat mitosis (Kelas III β-tubulin) dan matang (Calbindin, MAP2, GFAP, TH dan Synaptophysin). Untuk pembezaan mesodermal, rawatan menggunakan asid retinoik (RA), 5-Azacytidine C(5-Aza) dan asid askorbik

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(Vc) telah diberikan kepada EBs hari kedua, sebelum pengkulturan kepadatan tinggi dalam pinggan kultur pada hari keempat. Sel-sel kemudian diajukan kepada analisa dengan O-Red Oil (sel lemak), Alizarin red (sel tulang), Alcian Blue (sel kondrosit) dan dinilai dengan penanda sel jantung matang (Cardiac troponin, Serca dan GATA6). Manakala, untuk pembezaan endodermal, sel-sel c-kit positif dirawat dengan Activin A, Wnt3a , FGF7, Cyclopamine dan asid retinoic secara bersiri sebelum dianalisa dengan penanda pankreas (brachyury , PDX1, CXCR4, NKX 6.1, HNF4A, MafA dan insulin). Akhir sekali, analisa fungsian telah dijalankan untuk mengkaji rembesan protein berfungsi seperti dopamin dan insulin pada sel-sel selepas pembezaan. Hasil kajian menunjukkan bahawa AFSCs tikus yang dipencil pada penghamilan jangka penuh, bukan sahaja memiliki profil-profil penanda sel stem berpotensi tinggi, tetapi juga mempunyai keupayaan perbezaan yang luas. Mereka berupaya membeza kepada derivatif-derivatif daripada tiga germa utama yang berfungsi. Ini jelas menunjukkan bahawa AF pada penghamilan jangka penuh boleh dijadikan sumber sel-sel stem berpotensi tinggi yang mempunyai nilai terapeutik. Dengan penemuan ini, AFSCs jangka penuh dipercayai boleh dicapai dalam manusia dan berpotensi tinggi sebagai calon sel stem untuk merawat pelbagai kecacatan degeneratif.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Norshariza Binti Nordin, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Syahrilnizam Bin Abdullah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Rajesh A/L Ramasamy, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Cheah Pike See, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Dr. Norshariza Binti Nordin

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Syahrilnizam Bin Abdullah

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Rajesh A/L Ramasamy

Signature: _____
Name of Member of Supervisory Committee: Dr. Cheah Pike See

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LIST OF ABBREVIATIONS

5-Aza	5-azacytidine
AECs	Amniotic fluid epithelial cells
AF	Amniotic fluid
AFCs	Amniotic fluid cells
AF-MSCs	Amniotic fluid mesenchymal stem cells
AFSCs	Amniotic fluid stem cells
AF-type	Amniotic fluid type
ANP	Atrial natriuretic peptide
Ap2	Adipocytes protein 2
Asc-2-P	Ascorbic acid 2-phosphate
ASCl1	Achaete–scute homologue 1
bFGF	Basic fibroblast growth factors
BM-MSCs	Bone marrow mesenchymal stem cells
BMPs	Bone morphogenic proteins
BRn2	Brain specific homeobox and POU domain 2
BSP	Bone sialoprotein
C/EBPa	CAAT/enhancer-binding proteins
cAMP	Cyclic adenosine monophosphate
CDC25A	Cell division cycle 25 homolog A
CDK6	Cyclin dependent kinase 6
CeBPβ	CCAAT/Enhancer-Binding Protein Beta-2 Isoform
ChAT	Choline acetyltransferase
c-kit	Type III tyrosine kinase receptor of the stem cell factor, also known as CD117

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CLC	B-cell stimulating factor-3
CMs	Cardiomyocytes
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
Col11	Collectin sub-family member 11
CT-1	Cardiotropin-1
cTnI	Cardiac troponin I
c-TnT	Cardiac troponin-T
CXCR4	Chemokine receptor type 4
D	Day
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter
DE	Definitive endoderm
DHBA	3,4-dihydroxyhenzene benzylamine
DIA	Differentiation inhibiting activity
DKK-1	Dickkopf
DM	Diabetes mellitus
DMEM	*Dulbecco's Modified Eagle's medium*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTZ staining	Dithizone staining
E	Embryonic day
EBM	Embryoid body medium (without LIF)

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EBs	Embryoid bodies
ECD	Electrochemical detector
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immuno assay
END-2	Visceral endoderm-like cell line
ERK	Extracellular signal-regulated kinases
ESCMs	Embryonic stem cell derived cardiomyocytes
ESCs	Embryonic stem cells
ESM	Embryonic stem cell medium (with LIF)
E-type	Epithelioid- type
FABP4	Fatty acid binding protein 4
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSCs	Fetal stem cells
F-type	Fibroblastic-type
GAGs	Glycosaminoglycan
GATA4	GATA binding protein 4
GFAP	Glial fibrillary acidic protein
GIRK2	Protein-regulated inward-rectifier potassium channel 2
GJA1	Gap junction alpha-1 protein
GLP	Glucagon-like peptide
GLUT4	glucose transporter type-4

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GMEM	Glasgow Minimum Essential medium
GP130	Glycoprotein 130
HAFFT	Human amniotic fluid fibroblastoid-type cells
HD	Hanging drop
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESCs	Human embryonic stem cells
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
HSCs	Hematopoietic stem cells
IBMX	Isobutylmethylxanthin
ICC	Immunocytochemistry
ICM	Inner cell mass
IGF	Insulin growth factor
IL-6	Interleukin-6
ILV	Indolactam V
INS-1	Insulin-1
iPSCs	Induced pluripotent stem cells
ITS	Insulin, transferrin, selenious acid
JAK	Januse Kinase
K_2HPO_4	Dipotassium Phosphate
KAAD-CYC	3-Keto-N-(aminoethyl-aminocaproyl-dihydro-cinnamoyl) cyclopamine
KCL	Potassium Chloride
Kdm5b	Lysine (K)-specific demethylase 5B
KGF	Keratinocyte growth factor
Klf4	Kruppel-like factor 4

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LIF	Leukemia inhibitory factor
LIFr	Leukemia inhibitory factor receptor
Lin28	Protein Lin-28 homolog A
LRRK-2	Leucine-rich repeat kinase 2
MACS	Magnetic-activated cell sorting (MACS)
MafA	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A
MafB	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
MAP	Mitogen-activated protein
MAP2	Microtubule-associated protein 2
MAPCs	Multipotent adult progenitor cells
MD	Monolayer differentiation
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cells
Mest	Mesoderm specific transcripts gene
$MgSO_4$	Magnesium Sulphate
MIAMI cells	Marrow-isolated adult multi-lineage inducible cell
MLC-2a	Myosin light chain-2a
MLC-2v	Myosin light chain-2v
MMP13	Matrix metallopeptidase 13
MSCs	Mesenchymal stem cells
myT1l	Myelin transcription factor 1 like
NA	Norepinephrine
NaCl	Sodium Chloride
Nanog	Homeobox transcription factor nanog

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NCAM	Neural cell adhesion molecule
NeuroD	Neurogenic helix loop helix protein
NF	Neurofilamant
NGN3	Neurogenin 3
Nkx2.5	Homeobox protein nkx2.5
Nkx6.1	Homeobox protein nkx6.1
NPCs	Neural precursor cells
NSCs	Neural stem cells
Oct4	Octamer-binding transcription factor 4
OPC	Oligodendrocytes precursor cells
OSM	Oncostatin M
OSX	Osterix
P	Passage
PBS	Phosphate buffer saline
PCL	Poly-ε-caprolactone
PD	Parkinson's disease
PDX1	Duodenal homoeobox-1
PE	Phycoerythrin
PEC1	hESCs derived pancreatic progenitor cells
PI	Propidium iodide
PI3K signaling	Phosphatidylinositol 3-kinase signaling
PLLA	Poly-L-lactic acid
PPAR	Peroxisome proliferation-activated receptors
RA	Retinoic acid
rCMs	Rat cardiomyocytes

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RET	trk, trkB, and RET
Rex1	Known as Zfp-42, zinc-finger protein-42
RGD	Arginine, glycine, aspartic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RPMI	Roswell park memorial institute
Runx2	Runt-related transcription factor 2
SCF	Stem cell factor
SDIA	Stromal cell-derived inducing activity
SERCA	Sacroendoplasmic reticulum calcium transport ATPase
Shh	Sonic hedgehog
SOS	Sodium octyl sulphate
Sox1	Sex determining region Y-box 1
Sox2	Sex determining region Y-box 2
SSEA1	Stage specific embryonic antigen1
SSEA3	Stage specific embryonic antigen3
SSEA4	Stage specific embryonic antigen4
STAP	Stimulus triggered acquisition of pluripotency cells
STAT	Signal transducer and activator of transcription
Tbx3	T-box transcription factor
Tert	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-beta
TH	Tyrosine hydroxylase
trk	Tropomyosin receptor kinase

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trkB	Tropomyosin receptor kinase B
Vc	Vitamin C
VEGF	Vascular endothelial growth factor
VM	Ventral midbrain
VMAT	Vesicular monoamines transporters
VSELs	Very small embryonic-like cells
W	Weeks
WHO	World Health Organization
α-MHC	α- myosin heavy chain
β-MHC	β-myosin heavy chain

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CHAPTER 1

INTRODUCTION

Stem cells are characterized by two unique properties; the self renewability and their differentiation potential, which make them a great promising candidate for regenerative medicine. Since the discovery of stem cell biology, different types of stem cells have been isolated and characterized, ranging from the unipotent spermatogonial stem cells to pluripotent embryonic stem cells (ESCs). Among all, only certain types of stem cells, such as ESCs and mesenchymal stem cells (MSCs), have been used in clinical trials in the last decade; while, hematopoietic stem cells (HSCs), that have been applied to replace cancer patients' normal growing blood cells during chemotherapy (Stanevsky et al. 2009), remained to be the only successful stem cell for clinical use.

The clinical applications of both ESCs and induced pluripotent stem cells (iPSCs) are still limited. Although ESCs entered clinical trials in 2010, they remain controversial to some, as their generation involves the destruction of embryos. Another serious concern is the safety issues of ESCs, as they can form tumours upon transplantation (Kielman et al. 2002; Ishikawa et al. 2003). There are also issues with clinical applications of iPSCs. Most iPSCs studies are restricted to laboratories as the generation of iPSCs can be expensive, labour intensive and inefficient due to poor pluripotency conversion (Wolfrum et al. 2010). Similar to ESCs, iPSCs also are associated with mutagenesis and tumorigenesis (Gore et al. 2011; Hussein et al. 2011; Lister et al. 2011). Meanwhile, MSCs have lower differentiation capacity, which are restricted to mesodermal lineages, thus has limited their potential use in clinical applications (Malatesta et al. 2008; Ding et al. 2011). Their limited life-span (lesser than 10 passages) in culture is another drawback for their use in clinical applications (Taléns-Visconti et al. 2006a; Yang et al. 2011; Sancho-Martinez et al. 2012). Hence, it is still an immense task before mesenchymal stem cells could be widely applied in clinical settings primarily due to their limited differentiation capacity and their shorter lifespan (Rosenbaum et al. 2008; Trounson et al. 2011; X. He et al. 2012).

Clearly, the discovery of stem cells that possess similar differentiation potential as ESCs, but safer would be highly desirable. A number of new stem cells types that are categorized as potentially pluripotent stem cells have been reported; including very small embryonic like cells (VSELs), multipotent adult progenitor cells (MAPCs) and marrow isolated adult multi-lineage inducing cells (MIAMIs) (reviewed in Gao et al. 2013). Generally, these cells have greater differentiation potential than multipotent cells, where they can differentiate into the cell derivatives of the three primary germ layers. They also exhibit, to some degree, markers that are expressed in the

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standard pluripotent stem cells, the ESCs (D'Ippolito et al. 2004; Kucia et al. 2007; Ratajczak et al. 2008; Zuba-Surma et al. 2009; Ratajczak et al. 2011). Regardless of the bright side, many challenges still need to be tackled before their usage in clinical application could be applied, as these cells are newly discovered where the isolation, characterization and long-term maintenance can be technically challenging (D'Ippolito et al. 2004; Ratajczak et al. 2008; Zuba-Surma et al. 2009; Gao et al. 2013).

Stem cells have been isolated from various body compartments and cavities, such as the cardiac cavity, dental pulp or to a greater extend the extra-embryonic compartment (Hilmi et al. 2008; Dobreva et al. 2010; Makino and Fukuda 2011). Triggered by the idea that amniotic fluid is harbouring a rich source of foetal and maternal cells, scientists have isolated various types of stem cells from amniotic fluid; such as amniotic fluid mesenchymal stem cells (AF-MSCs) and amniotic fluid epithelial stem cells (AECs) (Fauza 2004; Abdulrazzak et al. 2010). However, the majority of studies were targeted on the isolation of multipotent stem cells with a simple one-stage or two stage protocols. This had resulted in the isolation of multipotent stem cells with low passage number and limited differentiation potential (Prusa et al. 2004; Antonucci et al. 2009; Mauro et al. 2010; Da Sacco et al. 2010; Klemmt et al. 2011; Yadav et al. 2011; Janz et al. 2012; Jezierski et al. 2012; Hartmann et al. 2013; Pratheesh et al. 2013).

Interestingly, the additional step of magnetic activated cell sorting (MACS) against c-kit (stem cells receptor), a type of broad multipotent stem cells, termed amniotic fluid stem cells (AFSCs), were first successfully isolated from amniotic fluid collected from mid-pregnancy in 2007 (De Coppi et al. 2007). These AFSCs express Oct4, indicating their pluripotency status (De Coppi et al. 2007; Perin et al. 2008; Gekas et al. 2010; Bollini et al. 2011; Bai et al. 2012; Maraldi et al. 2014). Additionally, *Tert*, which is responsible for indefinite replicative capacity, is also expressed in these cells (Mosquera et al. 1999; Kim et al. 2007a). The doubling time for AFSCs is 36 hours, and they can be cultured for more than 250 populations (De Coppi et al. 2007; Phermthai et al. 2010a). AFSCs may have good prospects in cell therapy, because they possess similar developmental potential as ESCs, they are not ethically controversial and they are likely to be safer in clinical settings as they do not form tumours upon transplantation (De Coppi et al. 2007; Pozzobon et al. 2010; Chen et al. 2011). Their discovery not only gives an alternative source for stem cells, but also marks a potentially significant advancement in regenerative medicine (Siddiqui and Atala 2004; Da Sacco et al. 2010).

Most previous studies have focused to isolate stem cells from mid-term AF collected *via* amniocentesis, including from human, dog, pig, horse and buffalo (De Coppi et al. 2007; Chen et al. 2011; Filioli Uranio et al. 2011; Yadav et al. 2011; K Dev et al. 2012; Kapil Dev et al. 2012; Pratheesh et al.

2013). Nonetheless, there are concerns about the potential risks posed by the mid-term AF acquisition through amniocentesis, such as complications associated with infection of the amnion sac from the needle, leakage of the sac and most seriously, miscarriage (Leschot et al. 1985; Kong et al. 2006). Although the advancement of technology has greatly reduced the risk, but exploring the possibility of isolating AFSCs from full-term amniotic fluid, can increase the accessibility of getting the AF samples, since they are large in volume (estimately 600ml) and the full-term AF are generally meant to be discarded (Abramovich 1970). Thus, AF from full-term pregnancy could be an alternative source to isolate AFSCs.

Only a small number of studies reported the isolation of stem cells from full-term pregnancy, namely from human (You et al. 2008; You et al. 2009), horse (Iacono et al. 2012), cow (Rossi et al. 2014) and dog (Fernandes et al. 2012; Choi et al. 2013). According to previous literature, the isolation step done was rather simple, which the primary AF cells were cultured in a standard culturing medium in serum supplemented with certain growth factors through one- or two-stage culture methods. However, these isolated AF-MSCs or AFSCs exhibited mesenchymal-like morphology limited differentiation potential (You et al. 2008; You et al. 2009; Fernandes et al. 2012; Iacono et al. 2012; Choi et al. 2013; Rossi et al. 2014).

Different from the previous studies on stem cells derived from full-term AF studies, the additional step of magnetic activated cell sorting (MACS) against c-kit (stem cells receptor) was applied in this study, in order to isolate a purer stem cell population from full-term AF. Then, the isolated c-kit positive cells were optimized with the requirement of Leukaemia Inhibitory Factor (LIF) in culture. These isolated full-term AFSCs are believed to have higher differentiation potential and stemness than those isolated from single- and two-staged protocols as previously described. Moreover, LIF supplemented culture is believed to be essential to maintain the differentiation potential of the cells. Finally, these cells are hoped to behave similarly as those isolated from mid-term AF through immuno-selection against c-kit.

Research questions:

1. Does rat full-term AF harbour highly potent stem cells, similar to those isolated from mid-term AF?
2. Do the isolated stem cells possess a wide differentiation spectrum to differentiate into derivatives from the three primary germ layers, specifically into the functional ones?
3. Besides, does the isolated full term AFSC require LIF in culture, similar to ESCs, in order to maintain them in undifferentiated state?

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Hypothesis:

Rat full-term amniotic fluid harbours highly potent stem cells with the ability to differentiate into functional derivatives of the three primary germ layers.

General Objective and the specific aims

In this study, the general objective is to enrich broadly multipotent AFSCs lines which were isolated in the lab previously using magnetic activated cell sorting (MACS) against c-kit (stem cells receptor), and to explore the differentiation potential of the isolated AFSCs into derivatives of the three primary germ layers. The specific objectives are:

1. To evaluate the effects of c-kit and LIF for AFSCs culture.
2. To molecularly and functionally characterize the established AFSCs.
3. To explore the ectodermal differentiation potential of AFSCs and to evaluate their functional neurogenic capabilities.
4. To examine the mesodermal differentiation potential of the AFSCs into cardiomyocytes, osteocytes, chondrocytes and adipocytes.
5. To unravel the endodermal differentiation capacity of AFSCs and to assess their functional pancreatic potential.

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