UNIVERSITI PUTRA MALAYSIA

CHALLENGES IN OBTAINING GOOD METAPHASE SPREAD OF RAT FULL-TERM AMNIOTIC FLUID STEM CELL LINE (R3) FOR KARYOTYPING ANALYSIS

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ABSTRACT

Challenges in Obtaining Good Metaphase Spread of Rat Full-term Amniotic Fluid Stem Cell Line (R3) for karyotyping Analysis

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Introduction: Stem cells have been shown to be the promising cells for various therapeutic applications including for cell replacement therapy. Their ability to undergo self- renewability permits them to be preserved as stem cell lines for prolonged culture. Hence, making them as a reliable source for continuous supply of cells for therapy, namely as the cell source for transplantation. One such stem cell line is the newly in-house established rat full-term amniotic fluid stem cell (AFSC) line, known as R3. The line has been cultured up to 100 passages without losing its differentiation potential. However, its chromosomal stability has not been assessed before. Stable stem cell line is important before they could be used in transplantation. One of the ways is to check their chromosomal stability via karyotyping analysis. Nonetheless, it is not an easy process since the process relies on acquiring a good quality metaphase spread of the chromosomes, which is a tedious process as there is no standard karyotyping protocol attributed to AFSC line, albeit the protocol is well established for primary blood and amniotic fluid cells. **Objective:** In this study we aimed to evaluate the important variables in obtaining good metaphase spread of rat full-term AFSC line (R3) cultured at passage 37 (P37) and 38 (P38). **Hypothesis:** It is hypothesized that acquiring a good quality metaphase spread is dependent on different factors associated to karyotyping steps. **Methodology:** R3 was propagated to its respective passage number and karyotyping was performed to assess the effect of fixative drying time, the effect of adding colcemid after 34 hours instead of 18 hours and the effect of cell suspension dropping height on the quality of metaphase spread. **Result:** The good quality metaphase spread was not observed in this study despite of several attempts. However, we could observe the presence of clumped chromosomes and high number of chromosome spreads at a single area that might be due to the speed of fixative drying time. In addition, interphase-like cells were also observed that might be caused by the colcemid inefficiency. Structural disorganization of the chromosomes which could be attributed by a high dropping height of R3 cell suspension was also detected. **Conclusion:** Proper optimization for fixative drying time, colcemid incubation duration and suitable dropping height of cell suspension are essential elements in acquiring a good quality metaphase spread for full-term AFSC line. The specific optimized conditions could provide valuable parameters towards initial

checking of R3 stability. The stability of R3 upon prolonged culture is essential as this finding could be further translated to human counterpart as the prospective cells for transplantation from a source that is merely discarded, that is the human full-term amniotic fluid.

Keywords: Karyotyping, Full term gestation, Amniotic fluid stem cell, Metaphase spread

ABSTRAK

Cabaran dalam Memperoleh Penyebaran Metafasa Garisan Sel Stem Cecair Ketuban Tempoh Penuh daripada Tikus (R3) untuk Analisa Kariotip

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Latar Belakang: Sel stem telah terbukti menjadi sel yang menjanjikan pelbagai aplikasi terapi termasuk untuk terapi penggantian sel. Keupayaan mereka untuk menjalani pembaharuan diri membolehkan mereka dipelihara sebagai titisan sel stem untuk jangka masa berpanjangan. Oleh itu, menjadikannya sebagai sumber yang boleh dipercayai untuk bekalan sel yang berterusan untuk terapi, iaitu sebagai sumber sel untuk transplantasi. Salah satu titisan sel stem tersebut adalah sel stem cecair ketuban tempoh penuh tikus yang baru sahaja ditubuhkan dan dikenali sebagai R3. Titisan sel tersebut telah dikultur sehingga 100 laluan sel tanpa kehilangan potensi pembezaannya. Walau bagaimanapun, kestabilan kromosomnya belum pernah dinilai sebelum ini. Titisan sel stem yang stabil adalah penting sebelum dapat digunakan dalam rawatan transplantasi. Salah satu caranya adalah dengan memeriksa kestabilan kromosom melalui analisa kariotip. Walaupun begitu, ini bukan proses yang mudah kerana prosesnya bergantung pada pemerolehan penyebaran metafasa kromosom yang berkualiti baik, yang merupakan proses yang mencabar kerana tidak ada protokol kariotip standard untuk titisan sel stem cecair ketuban tempoh penuh walaupun protokolnya banyak terdapat untuk sel-sel primer seperti darah dan cecair amniotik. **Objektif:** Kajian ini bertujuan untuk menilai faktor penting dalam memperolehi penyebaran metafasa yang berkualiti tinggi dari titisan sel stem cecair ketuban tempoh penuh tikus (R3) yang dikultur pada 37 (P37) dan 38 (P38) laluan sel. **Hipotesis**: Dihipotesiskan bahawa pemerolehan penyebaran metafasa berkualiti baik bergantung pada faktor yang berkaitan dengan langkah-langkah semasa proses kariotip. **Metodologi:** R3 dibiakkan sehingga ke laluan sel yang dikehendaki dan analisa kariotip dilakukan dengan menilai kesan masa pengeringan fiksatif, kesan penambahan colcemid selepas 34 jam dan bukannya 18 jam, dan kesan menjatuhkan suspensi sel pada jarak yang tinggi terhadap kualiti penyebaran metafasa. **Hasil dan Perbincangan**: Penyebaran metafasa berkualiti baik tidak dapat diperolehi dalam kajian ini walaupun beberapa kali percubaan dilakukan. Walau bagaimanapun, kita dapat melihat adanya kromosom yang bergumpal dan jumlah penyebaran kromosom yang tinggi di satu kawasan yang mungkin disebabkan oleh kelajuan masa pengeringan fiksatif. Selain itu, sel-sel menyerupai sel interfasa juga diperhatikan yang

mungkin disebabkan oleh ketidakefisienan penyerapan colcemid oleh sel. Disorganisasi struktur kromosom yang mungkin disebabkan oleh kejatuhan suspensi sel R3 dari aras yang tinggi juga dikesan. **Kesimpulan:** Pengoptimuman yang tepat untuk masa pengeringan fiksatif, tempoh inkubasi colcemid dan aras ketinggian kejatuhan suspensi sel yang sesuai adalah elemen penting dalam memperolehi penyebaran metafasa yang berkualiti untuk titisan sel stem cecair ketuban tempoh penuh tikus. Pengoptimuman khusus dapat memberikan parameter yang berharga untuk pemeriksaan awal kestabilan R3. Kestabilan R3 pada kultur yang berpanjangan sangat penting kerana penemuan ini dapat diterjemahkan kepada sel manusia sebagai sel prospektif untuk terapi pemindahan sel dari sumber yang lazimnya hanya dibuang, iaitu cairan ketuban tempoh penuh manusia.

Kata kunci: Kariotip, Kehamilan penuh, Sel stem cecair ketuban, Penyebaran Metafasa,

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
AF	Amniotic Fluid
AFSCs	Amniotic Fluid Stem Cells
AFMSCs	Amniotic fluid mesenchymal stem cells
ARM	Artificial Rupture Membrane
AF-type	Amniotic Fluid-type
BSC	Biosafety Cabinet
COVID-19	Coronavirus Disease 2019
CGH	Comparative Genomic Hybridization
C-MYC	MYC Proto-oncogene
CD117	Cluster of differentiation 117
CD11b	Cluster of Differentiation Molecule 11B
CD 29	Cluster of Differentiation 29
CD34	Cluster of Differentiation 34
CD 44	Cluster of Differentiation 44
CD45	Cluster of Differentiation 45
CD 73	Cluster of Differentiation 73
CD 90	Cluster of Differentiation 90
CD 105	Cluster of Differentiation 105
CO_2	Carbon Dioxide
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ESCs	Embryonic Stem Cells
E-type	Epithelioid-type
FBS	Fetal Bovine Serum
FITC	Fluorescein-5- Thiocyanate
FACS	Fluorescence-Activated Cell Sorting
FISH	Fluorescence In Situ Hybridization
F-type	Fibroblast-type
GFAP	Glial Fibrillary Acidic Protein
H_2O	Water
HSCs	Hematopoietic Stem Cells
hAFSCs	Human Amniotic Fluid Stem Cells
hESCs	Human Embryonic Stem Cells
HLA-DR	Human Leukocyte Antigen-DR isotype
iPSCs	Induced Pluripotent Stem Cells
KCL	Potassium Chloride
Klf4	Kruppel-like factor 4
LIF	Leukemia Inhibitory Factor
MCO	Movement Control Order
M-FISH	Multicolor Fluorescence in -Situ Hybridization

MSCs	Mesenchymal Stem Cells
OCT-4	Octamer-Binding Transcription Factor 4
PHD	Doctor of Philosophy
PBS	Phosphate Buffer Saline
PSCs	Pluripotent Stem Cells
Pax 6	Paired Box Protein-6
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RPM	Rotation Per Minute
SKY	Spectral Karyotyping
Sox 2	Sex Determining Region Y-Box 2
SSEA-1	Stage-specific Embryonic Antigen-1
SSEA-4	Stage-specific Embryonic Antigen-4
Tert	Telomerase Reverse Transcriptase
TH	Tyrosine Hydroxylase

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Regenerative medicine is one the prominent area of medicine which holds great responsibility and become a hope for the community. It focuses on repairing and regeneration of cells, tissues or organs to replace their altered functions due to disease, loss of tissue, ageing or injury. In today's practise, end-stage diseases or massive tissue loss dependent on organ transplantation as a treatment option. Nevertheless, such options come along with some drawbacks like long waiting lists, less donor availability, low donor, and recipient tissue immunocompatibility which subjects recipient to immunosuppression throughout his life. To solve this, stem cell-based therapy appeared to be a promising alternative to cure various inherited and degenerative disorders through their special characteristics namely their ability to self-renew and differentiate into various cell types. These valuable characteristics serve as a platform for an adequate amount of therapeutically applicable cell types (Ramasamy, Velaithan, Yeow, & Sarkar, 2018). Specifically, their ability to undergo self-renewability permits them to be preserved as stem cell lines for prolonged culture. Hence, making them as a reliable source for continuous supply of cells for therapy, namely as the cell source for transplantation.

Of all different stem cell types available, human embryonic stem cells (hESCs) isolated from the inner cell mass of pre-implanted blastocyst hold the highest plasticity since they are pluripotent and able to differentiate into derivatives of the three primary germ layers; ectoderm, endoderm and mesoderm. However, their promising

pluripotency application in research and clinical trials hampered by the presence of ethical issue and lack of safety. According to King and Perin (2014), destruction of 5-day-old preimplantation embryo is necessary in order to harvest hESCs. Thus, hESCs research opponents claim that since the development of the embryo into a human being is possible, it has a noteworthy moral standing and thus it is unethical. In addition to this, hESCs characteristics of forming teratoma in vivo highlights its drawback concerning safety issue. On the other hand, adult stem cells are devoid of an ethical issue, possessing easy procurement method but have less proliferation and differentiation ability which pushed them aside from the ideal option for cell therapy since it requires abundant cells with greater differentiation potential.

To compensate for this issue, steps have been taken to explore an alternative source of stem cell with pluripotent or broadly multipotent characteristics, non-tumorigenic and devoid of ethical issues. One such stem cell line is the newly in-house established from both human and rat amniotic fluid stem cells (AFSCs) line from either amniotic fluid of mid-term or full-term gestation. They are small cell populations in amniotic fluid (AF) expressing Oct-4 with fulfilling criteria of an alternative source for stem cell which seems to gain huge interest among researchers (Ramasamy, Velaithan, Yeow, & Sarkar, 2018).

In this study, rat- full term amniotic fluid stem cell line (R3) has been used. It has been established by Mun Fun et.al (2015) from the amniotic fluid of 20^{th} day gestation of rat. The line has been cultured up to 100 passages without losing its differentiation potential. However, its chromosomal stability has not been assessed before. Stable stem cell line is important before they could be used in transplantation. One of the ways is to check their chromosomal stability via karyotyping analysis.

According to Hwang et.al (2013), a vital part of stem cell research and clinical application is the cytogenetic study especially karyotypic analysis for safety testing. Karyotyping analysis is fundamental in toxicity and tumorigenicity testing of the stem cell although some studies report chromosomes of stem cell are stable. However, there is evidence of chromosomal aberrations in prolonged or even early passages. Generally, karyotyping is a technique to determine numerical abnormalities or structural aberrations in chromosome (Catalina et al., 2007). Normal karyotype is a good indicator of chromosome stability (Borgonovo et.al, 2014). It is well-known for its application in routine analysis to diagnose genetic diseases, birth defects, and blood - related disorders from the primary cells harvested from samples like blood and amniotic fluid where they have standardized karyotyping protocol (LabtTestOnline,2020).

Nonetheless, it is not an easy process since the process relies on acquiring a good quality metaphase spread of the chromosomes, which is a tedious process as there is no standard karyotyping protocol attributed to AFSC line, albeit the protocol is well established for primary blood and amniotic fluid cells. Thus, in this study we aimed to evaluate the important variables in obtaining good metaphase spread of rat full-term AFSC line (R3). The specific optimized conditions could provide valuable parameters towards initial checking of R3 stability. The stability of R3 upon prolonged culture is essential as this finding could be further translated to human counterpart as the prospective cells for transplantation from a source that is merely discarded, that is the human full-term amniotic fluid.

1.2 Problem Statements

Stem cells derived from amniotic fluid show a compelling possible benefaction to the regenerative medicine field and have the noteworthy potential for application in research and therapy (Joo, Ko, Atala, Yoo, & Lee, 2012). Although cultivated stem cell has high potential to be used in cell therapy, ex vivo expansion could cause genetic instability which leads to tumorigenicity (Borgonovo, Vaz, Senegaglia, Rebelatto, & Brofman, 2014). Although karyotyping analysis could rule out cell line's stability, it is not easy to obtain a good quality metaphase spread for a stem cell line as there is no standard karyotyping protocol attributed to stem cell line, albeit the protocol is well established for primary blood and amniotic fluid cells. In this study we aimed to evaluate the important variables in obtaining good metaphase spread of rat full-term AFSC line (R3) cultured at passage 37 (P37) and 38 (P38). If the factors influence the good quality of metaphase spread of rat full-term AFSC line could be ruled out, this data could serve as a valuable prerequisite for R3 stability checking. Those findings could be translated for human counterpart as a promising cell for transplantation, a source that is merely discarded.

1.3 Hypothesis

It is hypothesized that acquiring a good quality metaphase spread is dependent on different factors associated to karyotyping steps.

1.4 Objectives

General objective: To evaluate the important variables in obtaining good metaphase spread

Specific objectives:

1. To revive and propagate good quality rat full-term amniotic fluid stem cells (R3).
2. To observe the effect of fixative drying time on the quality of metaphase spread.
3. To monitor the effect of adding colcemid to the cells after 34 hours in the culture on the quality of metaphase spread.
4. To assess the effect of cell suspension dropping height on the quality of metaphase spread.

CHAPTER 2

LIERATURE REVIEW

2.1 Introduction to Stem cell

2.1.1 Definition and characteristic

Germ cells, somatic cells and stem cells are three basic cell types which make up the human body. Somatic cells consist of a mass of cells which make up the human adult where each of it in its differentiated state possesses its own genomic copy except for red blood cells which are without nuclei. Germ cells are cells that produce gametes like eggs and sperm (Bongso & Richards, 2004). Stem cells are undifferentiated cells that exist in different stages of life namely embryonic, foetal, and adult stages and become differentiated cells which serve as tissue and organs' building blocks. The ability of extensive proliferation (self-renewal), arising from a single cell (clonality) and differentiation capability into different types of cells (potency) mark the main characteristics of stem cells (Kolios & Moodley, 2012). In terms of stem cell division, symmetric or asymmetric are two modes of stem cell division where two daughter cells which are identical to mother cell are produced when stem cell undergoes symmetric division whereas one daughter cell that is identical to the mother cell and another more potential restricted daughter cell are produced in asymmetric division (Chen, Ye, & Ying, 2015) (Figure 2.1). According to Bongso and Richard (2004), the asymmetric mode of stem cell division is a significant physiological mechanism for the maintenance of the body's cellular composition of tissue and organ, known as homeostasis.

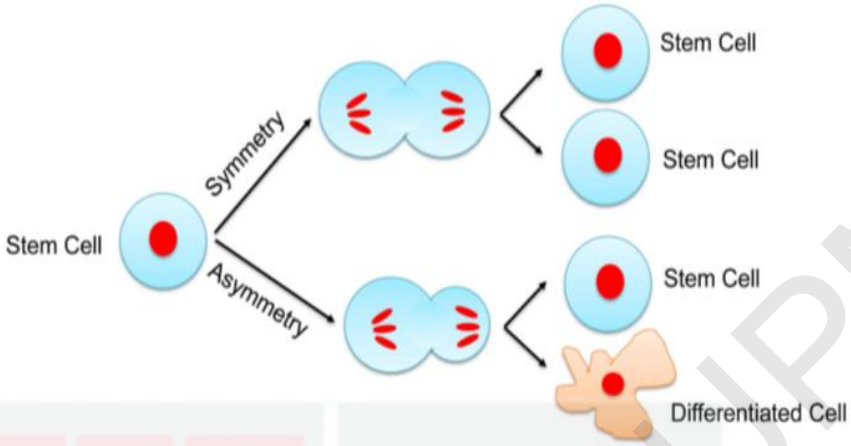


Figure 2.1 Symmetric and asymmetric stem cell division. Image adapted from (Chen, Ye, & Ying, 2015)

2.1.2 Classification

Stem cell can be classified based on their potency or differentiation potential and origin (Kolios & Moodley, 2012).

2.1.3.1 Differentiation potential

Different types of stem cell have different degree of differentiation potential or potency.

2.1.3.1.1 Totipotent Cells

Totipotent cells are also known as omnipotent cells which can be found in early development and they are the most undifferentiated cells. The cells of the first two divisions of a fertilized oocyte are known as totipotent cells since they form embryo and placenta by differentiating into both embryonic and extraembryonic tissues (Kolios & Moodley, 2012).

2.1.3.1.2 Pluripotent Cells

Pluripotent cells are capable of differentiation into derivatives of the three primary germ layers; ectoderm, mesoderm, and endoderm from where all tissues and organs evolve (De Miguel, Fuentes- Julian & Alcaina, 2010). Embryonic stem cells (ESCs) which are derived from the inner cell mass of the blastocyst and induced pluripotent stem cells (iPSCs) from reprogrammed somatic cells are the examples of pluripotent stem cells (Kolios & Moodley, 2012). Unlike totipotent cells, pluripotent stem cells cannot make the extraembryonic tissues including the placenta.

2.1.3.1.3 Multipotent stem cell

This group of stem cells can produce a different type of cells which is limited to one germinal layer like mesenchymal stem cells or just a particular cell line like hematopoietic cell line (Rajabzadeh, Fathi & Farahzadi, 2019). For instance, mesenchymal stem cells can specialize in mesoderm- derived tissue like muscle, cartilage, bone and adipose tissue (Kolios & Moodley, 2012).

2.1.3.1.4 Oligopotent stem cell

This group of cells are capable of self- renewal and formation of two or more lineages within a particular tissue. One of the common examples of oligopotent stem cell is hematopoietic stem cells as they are capable of differentiation into myeloid and lymphoid lineages. Besides, studies have shown that bronchoalveolar duct junction cells in the lung could be the source of bronchiolar epithelium and alveolar epithelium (Kolios & Moodley, 2012).

2.1.3.1.5 Unipotent stem cell

This group of stem cells could produce cells with only single lineage differentiation. The differentiation potential is very limited as compared to other types

of stem cells in adult tissues (Rajabzadeh, Fathi & Farahzadi, 2019). For instance, muscle stem cells develop into mature muscle cells and not others whereas epidermal stem cells only give rise to keratinocytes.

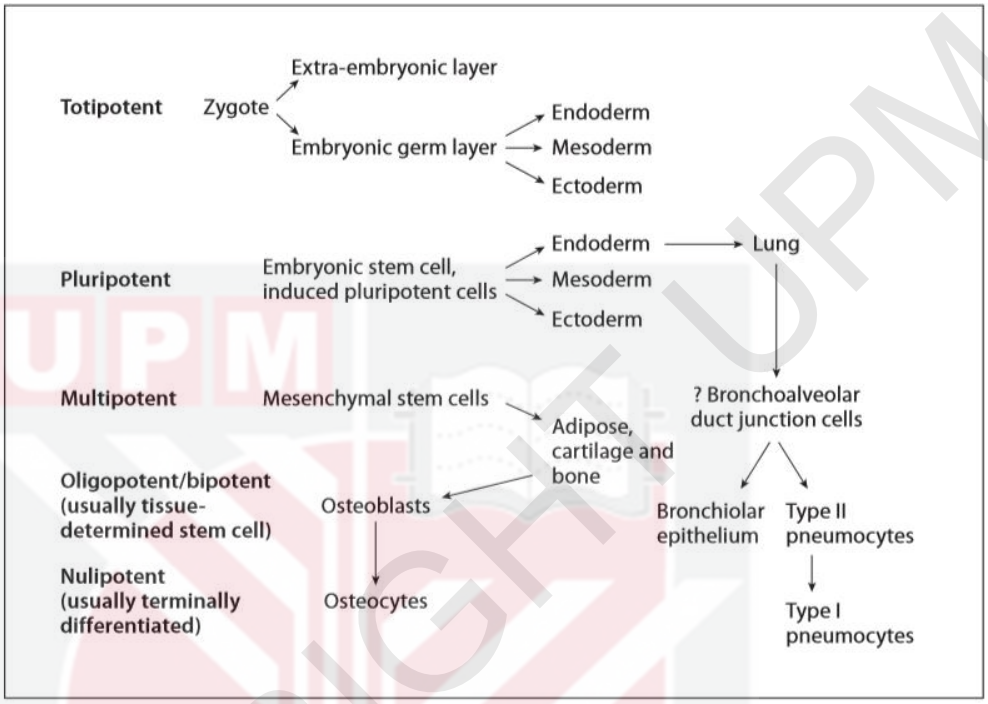


Figure 2.2 The hierarchy of stem cells. Image adapted from (Kolios & Moodley, 2012)

2.1.3.2 Origin

2.1.3.2.1 Embryonic stem cell

This type of stem cell is classified as pluripotent since they are derived from the inner cell mass of the early blastocyst, the preimplantation embryo of post-fertilization. They can differentiate into the tissue of the three primary germ layers and can also remain undifferentiated for a prolonged duration in culture. The blastocyst consists of the inner cell mass which leads to the formation of the embryo and the outer cell mass (trophoblast) which leads to the formation of the extra-embryonic tissues including the placenta. To develop ESCs lines, cells from the inner cell mass are harvested and transferred to a culture medium under a very specific condition. Besides, ESCs are

recognized by the presence of transcription factors such as Nanog and Oct 4 which sustain the cells in an undifferentiated state, making them able to self- renew (Kolios & Moodley, 2012). However, these cells are utilized less in research because of ethical barriers to embryo sampling and culture (Rajabzadeh, Fathi & Farahzadi, 2019). For instance, the opposer of human embryonic stem cell research defend that since the embryo has the potential to grow into a human being, it has a remarkable moral standing, thus its demolition is unethical (King & Perrin, 2014).

2.1.3.2.2 Adult stem cells

Since they are found after an embryo develops into a foetus, they are called as adult by people and no longer termed as embryonic stem cell. This group of stem cells are procured from adult tissue. MSCs and stem cell procured from placental tissue like human amnion epithelial cells are examples of adult stem cells (Kolios & Moodley, 2012). Besides, they have been identified in many other tissues including blood, intestine, skin, muscle, brain, and heart. Their differentiation capacity is limited and classified as multipotent stem cell where they only divide to self-renew and to grow into multiple specialised cell types exist in a specific tissue or organ. For example, hematopoietic stem cells (HSCs) are stem cells which differentiates into all blood cells (Birbrair & Frenette, 2016) while mammary stem cells supply cells for mammary gland growth during puberty and gestation and has a vital role in breast carcinogenesis(Liu, Dontu, &; Wicha, 2005).Apart from that, the good side of this stem cell is, there is no rejection issues or ethical controversies since they are autologous cells (Kolios & Moodley, 2012).

2.1.3.2.3 Tissue- resident stem cell

This stem cells are also known as adult tissue-specific/ progenitor stem cells (Noguchi et al., 2019). These cells in foetus or adult tissues are partially differentiated cells which divide and produce differentiated cells and usually referred as "committed" to differentiate in a specific cellular development pathway (National Institute of Health, 2001). Unlike, adult stem cells which both self-renew and differentiate into specialized cells, progenitor cells are only capable to differentiate and regarded to be more differentiated than adult stem cells. In an adult, the capability of renewal and repair of some tissues and organs upon injury is highly dependent on tissue-resident stem cells which produce terminally differentiated, tissue-specific cells. They inhabit in a 'stem cell niche, a microenvironment which regulates the self- renewal and differentiation of these cells. Their origin is from ontogenesis as reported by previous studies and they sustain in a quiescent state till activation caused by local stimuli leads to their proliferation, differentiation, or migration (Kolios & Moodley, 2012). Commonly known examples of this class of stem cells are, epidermal stem cell which regularly renew skin's outer layer upon worn out , and gut's epithelial stem cell constantly replacing the gut lining.(Nature Reports Stem Cells, 2007).

2.1.3.2.2 Induced Pluripotent stem cells

This group of stem cells are generated from adult somatic cells which are genetically reprogrammed to an 'ESC-like state' through gene transduction technique with certain transcription factors. For instance, Takahashi and Yamanaka (2006) had once generated induced pluripotent stem cells(iPSCs) from adult somatic cell such as adult fibroblasts by inserting four factors, Oct3/4, Sox2, c-Myc, and Klf4, in ES cell

culture conditions. They found that these cells akin to ESCs in many ways, for instance, morphology, growth properties and expression of ES cell gene markers.

2.2 Amniotic fluid

2.2.1 Function of amniotic fluid (AF)

Amniotic fluid (AF) is a yellowish clear fluid that encircles foetus that resides in the amnion sac of all mammals throughout the pregnancy. The major function of AF is to safeguard and cushion the foetus from abdominal trauma of the mother. Besides, by cushioning the foetus, it also prevents the compression between foetus and uterus. It also provides protection from infection through its antibacterial properties, function as a source of fluid, nutrients, and growth factors to aid normal foetus development. Apart from this, AF also serves as a lubricating agent for foetal movements and prevents heat loss to retain constant temperature (Hamid et al., 2017).

2.2.2 Development and changes of amniotic fluid throughout gestation

AF is known as an isotonic solution since it contains protein, carbohydrate, lipids, phospholipid, urea, and electrolytes. The constituent and volume change as gestation continues. Human pregnancy is divided into three stages; first trimester (week 1-13), second or mid-trimester (week 14- 27), and third trimester (begin at week 28 to full-term at week 38-42). Maternal-amnion membrane transport and surface of embryo contributes to the formation of AF in early pregnancy. In mid-trimester, urine excretion and secretion from foetal lungs contribute to AF. During the third trimester, foetal urine and lung liquid are the main sources of AF and lesser from the discharge of foetal oral-nasal cavities (Hamid et al., 2017).

2.2.3 Content of amniotic fluid

Water, chemical substance, and cells are the main compositions of AF. (Loukogeorgakis & De Coppi, 2016). It possesses different types of differentiated and undifferentiated cells. The cells are morphologically and biochemically heterogeneous. It was anticipated that the heterogeneity of cells is due to direct foetal and fluid contact via the flow of various fluid between foetus and amnion. Developing foetus itself which shed its skin and amnion membrane, in addition to the alimentary, respiratory, and urogenital tracts were found to be the source for these cells (Hamid et.al,2017). The cells count in AF affected by gestational age where it increases with respect to gestational age except for the presence of any pathological condition affecting the count (Loukogeorgakis & De Coppi, 2016). In terms of morphology and growth characteristics, viable adherent cells of AF are divided into three groups: amniotic fluid (AF)-type; epitheloid (E)- type; and fibroblastic(F) type cells (Ramasamy, Velaithan, Yeow, & Sarkar, 2018).

2.3 Amniotic fluid derived stem cells

2.3.1 History

Immunochemical analysis to detect the presence of dystrophin protein in patients with Duchenne's muscular dystrophy could not be done in amniocytes from prenatal diagnosis due to the absence of dystrophin in these cells. To overcome this, scientist induced myogenesis in amniocyte cultures by incorporating rhabdomyosarcoma's cell line supernatant where rhabdomyosarcomas are known to produce myogenic factors. Later, the initial idea of stem cell presence in amniotic fluid was born based on the

expression of skeletal muscle protein, dystrophin in amniotic fluid-derived cells when exposed to the rhabdomyosarcoma cell lines' supernatant while in culture (Streubel et al, 1996). Besides, Prusa et al. (2003) were the earliest to report that that Octamer Transcription Factor-4 (Oct-4) is expressed in cells from the mid-term amniotic fluid at both transcriptional and protein levels. Eventually, the presence of mesenchymal stem cell surface marker profile and the capability of differentiation into osteocytes, adipocytes and fibroblasts was proven (Loukogeorgakis & De Coppi, 2017). Furthermore, more evidence indicating stem cells present in amniotic fluid was demonstrated. Some groups have used CD117 (c-Kit; type III tyrosine kinase receptor for stem cell factor with significant roles in gametogenesis, melanogenesis, and hematopoiesis) for isolation of undifferentiated population from amniotic fluid (Loukogeorgakis & De Coppi, 2017). De Coppi et al. (2007) had isolated c-Kit (CD117) positive cells from mid-term amniotic fluid obtained from amniocentesis of human specimens while Gao et al. (2016) had isolated c-kit (CD117) positive cells from full-term amniotic fluid obtained from caesarean delivery. Amniotic fluid mesenchymal stem cells (AFMSCs) and the c-Kit+ amniotic fluid stem cells (AFSCs) are the two prominent stem cell population within the amniotic fluid. Among these two, due to the expression of pluripotency markers and greater differentiation potential, AFSC appeared to be a good candidate for therapeutic applications (Ramasamy, Velaithan, Yeow, & Sarkar, 2018)

2.3.2 Isolation and establishment

2.3.2.1 One stage culture

AF procured from second-trimester amniocentesis is centrifuged. Hemocytometer is used to count the number of cells and mixed with an equal volume of culture medium, generally, Dulbecco's Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS) and the cells are able to attach to a plastic culture plate at 10^4 cells/ cm2 and incubated overnight at 37°C under 5% CO2. After 3∼5 days, the culture medium is changed to get rid of non-adherent cells and this continued twice weekly. For 4∼5 days, the primary cells are cultured until confluency had been reached and termed as a passage "0". The cell population is of heterogeneous morphology and domination of fibroblastic like cells occur upon few passaging. Confluency usually attained in 4 to 6 days and leftover cells are cryopreserved in

cryopreservation media (10% DMSO, 90% FBS), frozen at - 80°C for 24 h, and the next day, stored in liquid nitrogen (Gholizadeh-ghalehaziz, Farahzadi, Fathi, & Pashaiasl, 2015).

2.3.2.2 Two-stage culture

Tsai et.al utilized non- adhering AF cells of primary amniocytes culture for isolation and establishment of two-stage culture protocol of AFSC. The non-adhering AF cells collected from supernatant of AFCs which cultured in serum-free changes medium (first stage) are plated for AFSC culturing after foetal chromosome analysis (second stage) completed (Gholizadeh-ghalehaziz, Farahzadi, Fathi, & Pashaiasl, 2015).

2.3.2.3 Immunoselection

This method is based on surface antigen c-Kit (CD117), the receptor for stem cell factor 8. Ditadi was the earliest researcher to demonstrate that c-Kit population cells extracted from AF possess hematopoietic potential (Gholizadeh-ghalehaziz, Farahzadi, Fathi, & Pashaiasl, 2015). De Coppi et.al (2007) proved that about 1% of cells in the culture of human amniocentesis samples procured for prenatal genetic diagnosis by using magnetic microspheres, shows the surface antigen c-Kit. They proved that those cells which they termed AFSC could be expanded in culture in the absence of feeder layer with doubling time about 36 h and maintained for more than 250 population doublings with conserved long telomeres and a normal karyotype without being tumorigenic. Besides, by implementing magnetic associated cell sorting, Arnhold et.al have extracted 3.2±1.03% of CD117 positive cells of the whole cell population and showed that they are capable of differentiation into adipogenic, osteogenic, myogenic and neurogenic lineage (cited in De Coppi et.al,2007). However, Loukogeorgakis & De Coppi (2017) demonstrated that CD117 + human AFSC with high purity could be only possible through FACS sorting since abundant cellular debris and less CD117+ cells do not permit efficient enrichment by MACS.

2.3.3 Characterization of full-term Amniotic Fluid Stem Cell (AFSC)

Full characterization has been done by De Coppi et.al for mid-term human AFSC. Mid-term AFSC has been categorised as broadly multipotent or potentially pluripotent stem cells (Hamid et.al.2017). This position is due to their expression of stemness and pluripotency-associated markers and incapability of tumor formation upon transplantation (Mun Fun et.al, 2015). Based on growth characteristics and morphology, viable adherent cells of AF divided into three groups: amniotic fluid

(AF)-type; epitheloid (E)- type; and fibroblastic type cells (Ramasamy, Velaithan, Yeow, & Sarkar, 2018). AFSCs found to be nontumorigenic and its injection to immunodeficient mice have shown no formation of teratoma in vivo (Loukogeorgakis & De Coppi, 2017). However, most studies investigate the capability of tumor formation from AFSCs derived from mid-term AF and very few groups studied this on full-term AFSCs. Both mid-term and full-term AFSCs isolation from AF are not associated with ethical issues as present in the study of hESCs (Joo, Ko, Atala, Yoo, & Lee, 2012). Hamid (2019) have reported RNA level expression of pluripotency-associated markers: Oct-4, Nanog, Sox2, Klf4, c-Myc, and early embryonic and primitive stem cell marker, SSEA-4 in AFSC derived from AF of full-term gestation shows highly primitive AF cells when compared to adult stem cells. Apart from that, it was found that not more than 95% human full-term AF cells fulfil the criteria to be addressed as MSCs as they are negative for the expression of MSCs-related markers such as CD 73, CD90, and CD105, however, they are negative for MSC negative markers such as CD34, CD45, CD11b and HLA-DR although previous studies of full-term AFSC showed a high level of MSC markers expression. It was suggested that this discrepancy might be due to the isolation protocol used. For example, third-trimester human AFSC isolated using an attachment to plastic/ CD117 selection protocol was found to express MSC markers such as CD29, CD44, CD73, CD90, and CD105 (Loukogeorgakis & De Coppi, 2017) while two-stage cultivation protocol was used by the aforementioned study by Hamid et.al (2017). CD73, a classical marker to detect MSCs population and found to involve in cell proliferation, differentiation and immune tolerance highly expressed in full-term human AFSCs. Surface antigen c-Kit (CD117) expression was found negative. Moreover, further characterization of human full-term AFSCs proved the formation of embryoid body (EB) with a smooth boundary

through the suspension method. Differentiation capability of AFSCs into osteogenic, adipogenic, chondrogenic and neurogenic lineage were also visible from different staining methods and the presence of matured markers (Hamid et.al, 2017).

2.3.4 Full term vs Mid-term AFSC

Both full-term and mid-term AFSCs could be obtained from AF of different species such as human, cow, canine and rodents. The main difference between them is the period where the AF is obtained for AFSCs isolation. For example, procurement of mid-term AF usually involves amniocentesis, an invasive procedure associated with complications due to amnion sac infection from the needle, sac's leakage and in some cases, miscarriages. In contrast, procurement of full-term AF is devoid of an invasive issue since it is obtained from merely discarded AF during normal delivery through Artificial Rupture of Membrane (ARM) procedure or caesarean section (Hamid et.al,2017). Besides, in terms of samples availability, lack of sample accessibility is one of the limitations of mid-term AF while full-term AF provides an ample amount of AF samples with higher volumes. Looking at their similarities, mid and full-term AFSCs of human and animal have been reported to have similar pluripotency-associated markers, stem cell surface markers, differentiation potential, and devoid of ethical issue related to their isolation (Mun Fun et.al,2015).

2.4 Rat full term Amniotic Fluid Stem Cell (R3)

2.4.1 Isolation protocol

Samples of AF were procured from 20th day of gestation (full-term) time-mated Sprague Dawley rats. The primary culture was propagated before achieving an adequate number of cells for isolation. To isolate the c-Kit+ amniotic fluid stem cells

(AFSCs), Mun Fun et.al have implemented immune-selection of C-kit positive cells protocol (Mun fun et.al, 2015). This isolated c-kit+ AFSC was then named as R3.

2.4.2Characterization

2.4.2.1Molecular characterization

Molecular characterization for R3 has been performed for pluripotent -associated markers and cell-surface markers associated with various stem cell types by Mun fun et.al. It was found that a vital transcription factor for maintenance of pluripotent stem cells, Oct 4 expressed in 70-90 % of the cells as proved by both immunocytochemistry (ICC) and fluorescence-activated cell sorting (FACS) analyses. Besides. its presence also confirmed by RT-PCR. Next, a vital transcription factor which regulates pluripotency, Nanog, was found to be expressed by 35- 70% of the cells. Moreover, expression of other markers such as SSEA-1 (embryonic stem cell surface marker), Sox2 (pluripotency marker) and Tert (stemness- related gene) which code for telomerase was also detected. Apart from this, other stem cells associated with cell-surface markers were also detected via FACS analysis. This includes general stem cell markers CD90-1 and CD29 where 90-99 % and 80-99% of the cells express them respectively, mesenchymal stem markers CD105 expressed highly by 51-95% of the cells, and CD44 expressed moderately by 40-60 % of the cells. Nevertheless, endothelial and hematopoietic lineage associated markers (CD34, CD11b and CD45) were not recognized (Mun fun et.al,2015).

2.4.2.2 Functional characterization

Functional characterization was performed to investigate the differentiation capability of C-kit+ AFSCs in vitro and eventually to proof the presence of highly potent stem cells among C-kit positive cells. These criteria were assessed by looking at their capability to form embryoid bodies (EBs), spontaneous differentiation and neural lineage differentiation. Formation of EBs was done by using hanging drop and single-cell suspension methods and examined based on boundary smoothness, area size and diameter. Both methods yielded EBs with good quality where it has smooth boundaries and experienced cavitation process. These results parallel with standard pluripotent stem cells like mouse embryonic stem cells. Apart from this, lineage-specific markers for derivatives of three primary germ layers' presence were assessed to evaluate the ability of C-kit+ AFSCs to differentiate spontaneously. These markers include class III beta-tubulin (ectoderm), brachyury (mesoderm) and albumin (endoderm). Class III beta-tubulin and brachyury expressed after 5 days and albumin expressed after 20 days in culture. Additionally, the capability of spontaneous differentiation into functional adipocytes, osteocytes and chondrocytes were proven by positively stained EBs with O Red Oil, Alizarin Red and Alcian Blue after 10 days being in culture. Characterization further proceeded on differentiation capability of AFSC into neural lineages upon exposure to serum-free monolayer differentiation (MD) where successful differentiation into neuroectodermal precursors express crucial markers for neuroectodermal cells, nestin and Pax 6 were observed Expression of post-mitotic neuronal marker class III beta-tubulin, glial marker, GFAP (glial fibrillary acidic protein) and matured neuronal marker such as TH (tyrosine hydroxylase) were also detected. All those expressions highlight the differentiation capability of C-kit positive cells into glial and specific subtypes of a neuron (Mun fun et.al,2015).

2.5 Genomic Stability & Cytogenetic Analysis

2.5.1 Definition

Cytogenetic studies include chromosome counting, in situ hybridization, banding techniques and karyotype and ideogram construction (Rodríguez-Domínguezet.al, 2017). According to Polipalli, Karra, Jindal, and Puppala (2016) cytogenetic analysis is an analysis which yield a genomic picture of chromosomes of an individual by orderly pair and arrange them and at the same time unveil changes in chromosome numbers (aneuploids) and fine structural changes, for instance, chromosomal deletions, duplications, translocations and inversions in the autosomes or sex chromosomes.

2.5.2 Importance of cytogenetic analysis of cell line

Xie et.al (2011) have stated that before therapeutic applications could be achieved, the potential adverse effects of long -term cell culture of human embryonic stem cells must be examined. A similar concept applies to any other stem cell lines which hold a greater prospect in terms of therapeutic application in future. Besides, whenever prolonged cultured of stem cell is concerned, cryopreservation is also one of the major concerns. This is because vital point for stem cell application in clinical veterinary and human medicine is the likelihood of long-term storage since cryopreservation serve a pool of cells for basic research and test of feasibility (reviewed in Kulikova et al., 2019). Although cultivated stem cells have portrayed its capability of utilization in different areas of cell therapy, pooling of genetic and epigenetic alterations which highlights the genetic instability that could lead to

tumorigenicity could have resulted from the ex vivo expansion of stem cells (Borgonovo, Vaz, Senegaglia, Rebelatto, & Brofman, 2014). Besides, numerical chromosome alteration happens in every dividing cell because of errors in the mitotic event and chromosome aberrations are the principal mechanism by which cancer cells develop due to abnormal expression of many genes resulted from chromosomal changes (Meisner & Johnson, 2008). At the same time, according to Hwang et. al (2013), there are reports on chromosome abnormalities in prolonged or even in early passages although many other studies reported that stem cells' chromosome is stable. Aforementioned statements from literature, gave us an alarming call to track the chromosome stability of stem cell lines. Studies suggested that the basis of stem cell line establishment is the introduction of the cytogenetic programme to determine the chromosome stability (Catalina et al., 2007). Cytogenetic or chromosome analysis is extensively used for the identification of chromosome instability (Howe, Umrigar, & Tsien, 2014). Also, stem cells' cytogenetic study is a vital part of stem cell research and stem cells clinical application at which safety and proof of principle for desired therapeutic effect need to be proven in preclinical studies of stem cells. Cytogenetic studies particularly karyotyping analysis for safety testing, is fundamental in the test of toxicity and tumorigenicity of stem cell (Hwang et. al,2013). However, the successfulness of complex molecular cytogenetic studies relies on a good quality of metaphase spread. Unfortunately, inconstancy of optimum chromosome spreading prevail as an utmost obstacle in cytogenetic studies (Deng, Tsao, Lucas, Leung, & Cheung, 2003). There are two types of cytogenetic analysis: classical or conventional and molecular cytogenetic analysis.

2.5.3 Types of cytogenetic analysis

2.5.3.1 Conventional cytogenetic analysis (Karyotyping)

Karyotyping is a technique to determine numerical abnormalities or structural aberrations in chromosomes (Catalina et al., 2007). Normal karyotype is a good indicator of chromosome stability (Borgonovo et.al, 2014). It is well-known for its application in routine analysis to diagnose genetic diseases, birth defects, and blood - related disorders from the primary cells harvested from samples like blood and amniotic fluid where they have standardized karyotyping protocol established(LabtTestOnline,2020).Moreover, karyotyping is a traditional way of evaluating a cell line's stability and safety (Xie et.al, 2011). Conventional cytogenetic analysis(karyotyping) involves blocking of the cells at metaphase, harvesting of cells, swelling, and fixing, staining and microscopical observation. It is highly informative and gold standard in aiding genetic evaluation of cell lines which eventually enables the identification of both numerical and structural chromosomal abnormalities (Borgonovo, Vaz, Senegaglia, Rebelatto, & Brofman, 2014).For instance, previously, Kulikova et al. (2019) have conducted karyotyping analysis to determine chromosomal aberration (aneuploidy) in fresh, slow- frozen or vitrified amniotic fluid mesenchymal stem cells (AFMSCs) of rabbit to identify any genetic instability arise due to cryopreservation by counting the number of chromosomes. The number of chromosomes remained similar in almost all the cells regardless of cryopreservation technique.

2.5.3.2 Molecular cytogenetic analysis

Molecular cytogenetic plays a vital role in the analysis of genomic integrity of cells. Fluorescence in situ hybridization (FISH) is extensively established molecular cytogenetic techniques that are commonly used in diagnostic to detect specific DNA sequences present in chromosome and nuclei of cancer cells (Das & Tan, 2013). There are many different types of FISH based techniques such as spectral karyotyping (SKY), Multicolor FISH (M-FISH), and Comparative Genomic Hybridization (CGH).

2.5.3.2.1 Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) technique involves hybridization of the directly labelled probe with a fluorochrome (rhodamine or fluorscein −5-thiocyanate) or indirectly labelled with haptens (biotin or digoxigenin) to distinctive DNA sequences along the chromosomes (Catalina et al., 2007; Das&Tan,2013). FISH can be conducted on metaphase or interphase of cells. This technique enables complex and cryptic chromosomal abnormalities such as gene rearrangement, amplification, and deletions at a single-cell level without prior information of chromosomal loci involved. (Catalina et al., 2007). There are three kinds of FISH DNA probes: (i) whole chromosome painting probes to analyse structural rearrangements by identifying the complete sequence of a given chromosome, (ii) centromeric probes, created to detect repetitive probes which target repeated tandem sequences of α-satellite DNA in the centromere of a chromosome and (iii) locus-specific probe to detect particular gene or chromosome region possessing copy number alterations and structural rearrangement (Das& Tan,2013).

2.5.3.2.2 Spectral Karyotyping (SKY) and Multicolor FISH (M-FISH)

Spectral karyotyping (SKY) technique utilizes fluorescent dye which binds to a distinctive part of chromosomes. Different chromosomal pairs have distinctive spectral characteristics since a series of specific probes each with dyes of varying amounts being used. The outcome of the analysis is in a digital image than film in full colour. Since homologous chromosomes are in the same colour, the pairing of chromosomes is easy and crossovers aberrations are easily identifiable. Translocations that is undetectable by traditional banding analysis is dateable upon using this technique (Catalina,2007). Besides, the common term for multi-colour FISH assays is M-FISH but the technologies behind the way on how the fluorochrome information is produced have to lead to two different M-FISH systems; spectral karyotyping (SKY) and M-FISH. Both have identical experimental procedure; Probes for all 24 human chromosomes are labelled by abiding labelling scheme and hybridized to metaphase spread for the duration of 24 to 48 hours, continued with post- hybridization washes and, if needed, antibody detection. Both SKY and M-FISH differs on their respective imaging technique at where SKY detects the differentiation of chromosomes depending on their spectral properties, while M-FISH detects the differentiation of chromosomes depending on presence or absence of fluorochrome when visualization done using specific filters (Pinkel & Albertson, 2006).

2.5.3.2.3 Comparative genomic hybridization (CGH)

Comparative genomic hybridization (CGH) provides whole genome overview and enable the recognition of DNA copy number changes. Without preliminary knowledge of the genomic region of interest, this method enables a genome screening of chromosomal imbalances. Target DNA from any stem cell is compared with a reference control DNA from commercially available standard DNA to identify potential anomalies in the DNA. Green fluorophore (fluorescein-5- thiocyanate;FITC) and red fluorophore (tetramethylrhodamine isothiocyanate; TRITC) used to label target DNA and control DNA respectively. These are mixed in equal amounts, hybridized to normal metaphase preparations and computer software is used to analyse the green: red ratio yielded by the two samples to identify gains and losses of material. Peaks and valleys highlight possible gains while losses of DNA confirmed if ratio exceeded below 0.75 and beyond 1.25. The normal amount of genetic material will appear as yellow signal due to equal hybridization target green DNA and red control DNA. Genetic material loss indicated by a red signal while gain indicated by the green signal. This ratio profile will be calculated along all chromosome in normal metaphase to enable the identification over or under-presentation of target genome's chromosomal region compared to control which at the end generate chromosome imbalances data are created (Catalina et al., 2007)

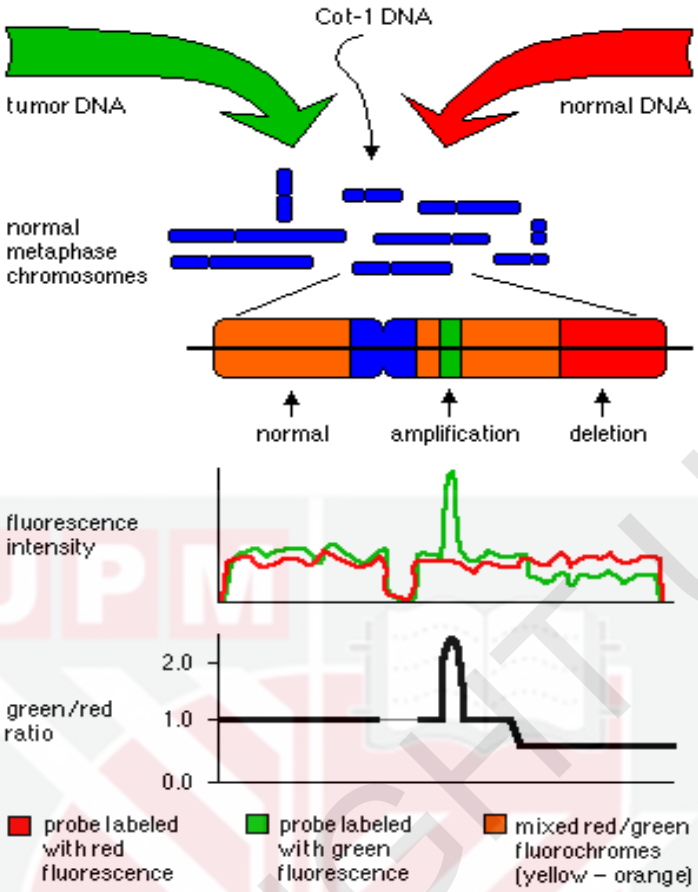


Figure 2.3 Simplified idea of Comparative genomic hybridization (CGH). Obtained from Comparative genomic hybridization (CGH)- WikiLectures.

2.6 Factors influencing a good quality of metaphase spread in karyotyping

As mentioned previously, the successfulness of complex molecular cytogenetic studies relies on a good quality of metaphase spread. Three basic method to acquire metaphase spread includes squashing, smearing and dropping of the cells (Rodríguez-Domínguezet.al, 2017). Unfortunately, the inconstancy of optimum chromosome spreading prevails as an utmost obstacle in cytogenetic studies (Deng, Tsao, Lucas, Leung, & Cheung, 2003). According to Meisner & Johnson (2008) “slide making is more of an art than a science, and each laboratory must develop its own approach”. There are various factors found to influence the quality of a good metaphase spread.

One of it is, dropping height of the cell suspension where the dropping of cells from a high position was reported to enhance the spreading of chromosomes (Hliscs, Mühlig, & Claussen, 1997). Besides, Spurbeck, Zinsmeister, Meyer, and Jalal (1996) have reported that accurate control of drying rate (a time where metaphase cells dry) holds a critical role in attaining a good metaphase spread. They have proposed that achieving optimum chromosome spreading is possible by implementing a various combination of relative humidity and temperature while drying the culture with the use of an environmental control unit. Apart from that, Kwasny et al. (2014) have stated that the rate of fixative evaporation determines the spreading of metaphase chromosomes. Achieving a single cell suspension was also reported to be a crucial step in getting a good metaphase spread. Moreover, the incubation time of cells in hypotonic solution mentioned to be vital also because longer timing leads to much earlier cell membrane bursting and cause loss of chromosomes while shorter timing cause trouble in obtaining chromosome spreads because disruption of cell membrane might not occur (Campos, Sartore, Abdalla, & Rehen, 2009). Other reported factors were the role of humidity alone which influence the quality of metaphase spread. According to Deng, Tsao, Lucas, Leung, and Cheung (2003), fixative absorbs water from the air when it spreads over the slides and eventually leads to dynamic rehydration of cells. They stated that dramatic release of free energy happens upon the mixture of fixative with water where they anticipate it to contribute for chromosome spreading and cytoplasm relaxation. Thus, they proposed that insufficient rehydration due to little ambient humidity or if chromosome spreading occurs after dramatic dynamic rehydration, optimal spreading of chromosome might not happen. Finally, the quality of spreads was also reported to be dependent on personnel skills and quality of glass slides (Kwasny et.al, 2014).

2.7Advantages and disadvantages of conventional cytogenetic (karyotyping)

First, karyotyping is commonly addressed as the gold standard for genetic testing. It is not expensive and the equipment for preparation could be easily obtained. However, the evaluation could be done on only dividing cells and no frozen tissue could be utilized. The need of metaphases and troublesome in analysis and interpretation of data are also disadvantages of this technique. Apart from this, less automation in the processing of sample and time required to analyse each division made this technique as a time -consuming method (Catalina et al., 2007).

2.8 Application of AFSCs in Regenerative Medicine

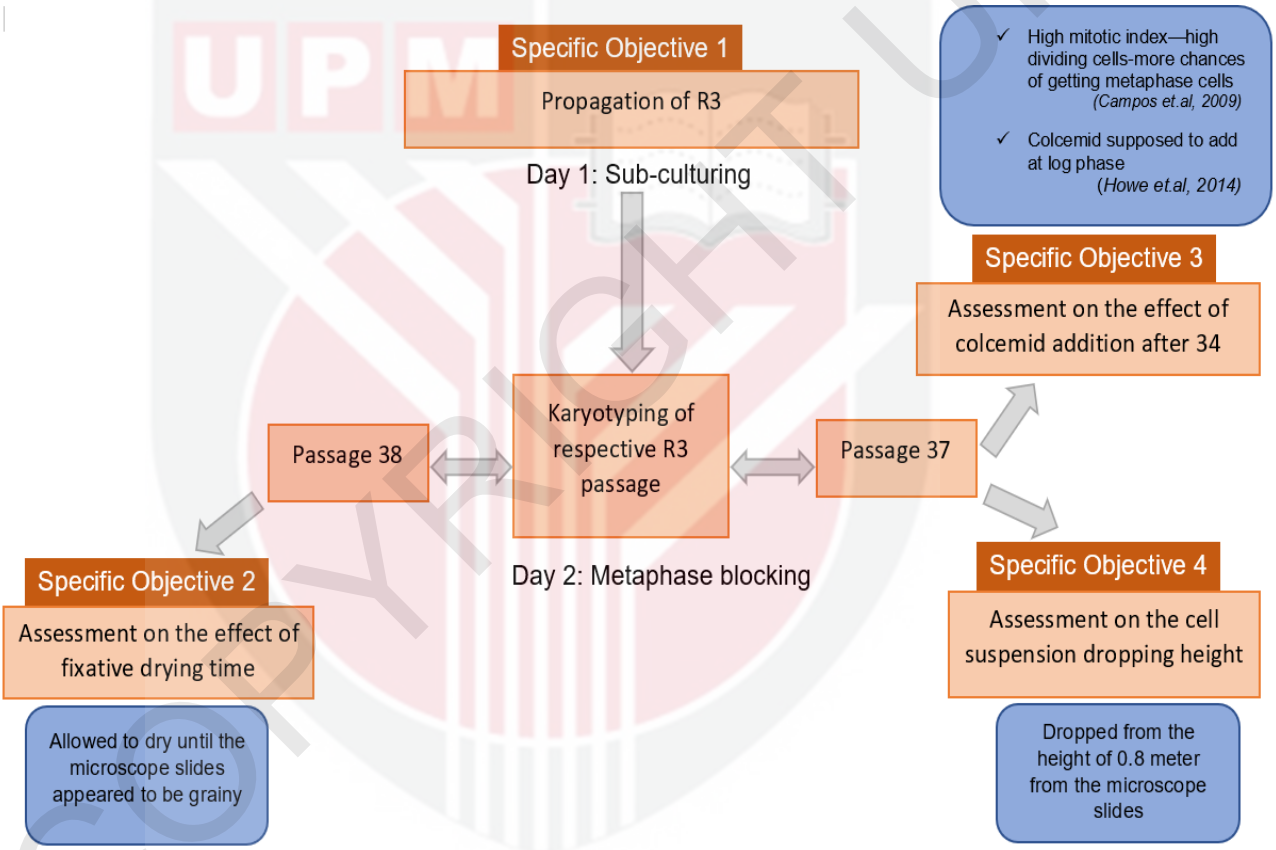
Regenerative medicine has made an appearance as a prominent field which focuses on the repair, replacement, or regeneration of cells, tissues, and organs. In cell-based therapies, stem cells are used for the regeneration process (Srivastava et al., 2018). Many types of stem cells serve as a promising candidate for regenerative but each of it possessing its own limitation. Although hESCs are pluripotent stem cells with a wide spectrum of differentiation potential, their clinical translation has been hampered by ethical concerns, harmful risk of immunogenicity and tumorigenicity. Production of iPSCs seems to overcome these issues (ethical issue and tumorigenicity) but low efficiency of iPSCs protocols and the threat of insertional mutagenesis/tumorigenesis in using viral vectors hampered their usage in the clinic (Loukogeorgakis & De Coppi, 2017). AFSCs could be a solution for these problems because it has been suggested by Hamid et.al (2017) that it has high differentiation potential, rapid propagation and lack

of immunogenic properties which make as a promising tool for tissue engineering and regeneration. Srivastava et. Al (2018) has reported many exciting applications of AFSCs in a various system such as cardiovascular, gastrointestinal, musculoskeletal, nervous, respiratory, skin, and urinary systems using animal models.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Research Design



3.2 Rat full-term amniotic fluid stem cell line (R3)

R3 is a rat full-term AFSCs which was isolated using immune-selection of C-kit positive cells protocol from the samples of AF procured from 20th day of gestation (full-term) time-mated Sprague Dawley rats and propagated as a cell line by Associate Professor Dr. Norshariza Nordin's research group. The isolated c-kit+ AFSC was then named as R3.

3.3 Cell Culture of rat full-term AFSCs (R3)

3.3.1 Preparation of vital solutions and medium

3.3.1.1 Preparation of ES medium

Protocol for the preparation of ES medium was adapted from Mun fun et.al (2015). The preparation begun by preparing stock solution containing 18.1 ml of 2.3% sodium bicarbonate (Gibco), 2.9 ml of 1mM L-glutamine (Gibco), 2.9 ml 0f 0.5mM sodium pyruvate (Gibco), 1ml of 0.1mM β-mercaptoethanol (Gibco) and 5.9 ml of MEM. After that, complete media was prepared for regular usage by mixing 42.5 ml of stock solution, 7.5ml of 15% foetal bovine serum (FBS) (Gibco), and 50ul of 10ng/ml of rat LIF (EMD Millipore).

3.3.1.2 Preparation of Phosphate-buffered saline (PBS)

PBS with the concentration of 1X was prepared from the stock with the concentration of 10X in 400 ml. For this, 40 ml of PBS from the stock was added to 360ml of sterile distilled water making up the final concentration into 1X.

3.3.1.3 Preparation of gelatine

Gelatine of 0.1 % was prepared by using Porcine Gelatine (Sigma). 0.1 gm gelatine powder was measured using weighing scale (Sartorius) and added into 100ml of PBS. At the end, the gelatine solution was autoclaved to sterilize it.

3.2.2 Thawing of rat full-term AFSC line (R3)

The frozen R3 with desired passage number in cryovial was removed from the -80°C freezer and transferred into 37°C water bath quickly. Thawing was done quickly by swirling the vial gently in the 37°C water bath until small quantity of ice remain in the vial. The vial was wiped with 70% alcohol before transferred into Class II Biosafety Cabinet (BSC). The thawed cells were suspended in 1 ml of ES medium, an established medium for R3 and transferred to a 15ml centrifuge tube. After that, centrifugation was performed at 1000 rpm for 5 minutes. While centrifugation occurs, T25 flask was coated with 0.1 % gelatine. To do this, 2ml of 0.1% gelatine was transferred to the flask and left for about 1 minute. After 1 minute, the gelatine was discarded. Once the centrifugation stops, without disturbing the cell pellet, the supernatant was removed carefully. The cell pellet was suspended in 1 ml of ES medium before transferred into T25 cell culture flask. An additional 4ml of ES medium

was added to the flask making the total media volume 5ml. The cells were incubated at 37°C with 5% humidified CO_2.

3.2.3 Propagation of rat full-term AFSC line (R3)

R3 were cultured in T25 cell culture flask and incubated at 37°C with 5% humidified CO_2. It was provided with 5 ml of ES medium. Every day, the cells were inspected for its morphology and colour change of the media.

3.2.4 Subculture and cell counting of rat full-term AFSC line (R3)

Once cells reached 70-80% confluency, the cells were sub-cultured by washing with 1x phosphate buffer saline (PBS) twice (Gibco), trypsinised with 1 ml of 0.25% trypsin-EDTA (Gibco) and incubated in incubator to enable the detachment of R3 from the surface of the cell culture flask. One millilitre of foetal bovine serum (FBS)(Gibco) was added to deactivate the trypsin to prevent any damage to the cell. The cell suspension was transferred to 15 ml tube. Centrifugation was performed for 5 minutes at 1000rpm. After that, the supernatant was discarded and the pellet was resuspended with 1 ml ES medium. After this step, the cells were counted using haemocytometer After this step, the cells were counted using haemocytometer. To do this, 10 µl of trypan blue (Sigma) was mixed with 10 µl of the cell suspension and by using the haemocytometer, the cells was counted. The cells were classified as viable if it is unstained whereas blue stained cells were classified as dead cells since only the dead

cells absorb the blue dye. The total number of viable cells was calculated using the formula as below.

$$\text{Viable cells} = \text{(Total cell counted on the grid)}/4 \times 10\text{^}4 \times \text{volume of cell suspension} \times \text{dilution factor}$$

After cell counting step, the ratio shown below was used to estimate the volume of cell suspension that need to be transferred to desired pre-coated T25 flask with 0.1% gelatine.

Volume of cell suspension:

Number of viable cells: 1ml

750 000 cells in 25 cm^2: Volume of cell suspension that need to be transferred

After calculation, the estimated volume of cell suspension was transferred to the flask and ES media was added making up the total media volume as 5ml. The flask was labelled with respective passage number, experimenter and supervisor name, date and then incubated in 37 $^{\circ}C$, 5% CO_2 incubator until confluency phase achieved.

3.2.5 Cryopreservation of rat full-term AFSC line (R3)

To detach the cells, R3 were washed with 1x PBS(Gibco) twice before being treated with 1 ml of 0.25% trypsin-EDTA (Gibco) for 3 minutes. After the cells detached, 1 mL of FBS (Gibco) was added to deactivate the action of trypsin. Then,

the cell suspension was centrifuged at 1000 rpm for 5 minutes. After centrifugation, the supernatant was removed and the pellet was resuspended in 1 ml of ES medium. One hundred microlitre of dimethyl sulfoxide (DMSO) was transferred to the cell suspension and aliquoted into labelled cryovials. The cryovials was then placed in -80°C freezer.

3.4 Original karyotyping protocol of rat full-term AFSC line (R3)

The protocol for karyotyping was adapted from Punovouri et.al (2018) from her thesis entitled "The role of the E- to N-cadherin switch in the neural differentiation of embryonic stem cells".

3.4.1 Preparation of vital solutions

3.4.1.1 Potassium chloride (KCL hypotonic solution)

For karyotyping procedure, 0.75 M of KCL (Scharlau) was required. It was prepared in 10 ml of distilled water. To do this, 0.056 gm of KCL was weighed using weighing scale (Sartorius) and dissolved in 10 ml of distilled water. This solution was prepared freshly for each karyotyping procedure.

3.4.1.2 Carnoy's fixative (Methanol: Glacial acetic acid)

Carnoy's fixative of ratio 3 parts of methanol and 1 part of glacial acetic acid was used. This fixative was prepared to the final volume of 20ml. To do this, 15 ml of methanol (J.T. Baker) and 5ml of glacial acetic acid (Friedemann Schmidt) was

measured using measuring cylinder and mixed in a 50 ml tube. This solution was prepared freshly for each karyotyping procedure.

3.4.1.3 Giemsa stain

Giemsa stain (R&M Chemical) of ratio 1 part of Giemsa and 50 part of PBS (Gibco) was used. This stain was prepared to the final volume of 200 ml. To do this, 4ml of Giemsa was dissolved in 196 ml of PBS.

3.4.2 Metaphase blocking

One day before karyotyping procedure, the cells were passaged at 4-5pm in 5ml of ES media and the time of splitting was noted (the time was noted once the flask placed in the incubator. This would be considered as day 1 of the experiment. On day 2, which is 18 hours after splitting, 10 ul of colcemid (Gibco) with the concentration of 100ng/mL was added to the culture media. More metaphases expected to be yielded from longer incubation in colcemid but the chromosomes will gradually become smaller and fatter, making them more difficult to count. The whole procedure was be performed in the Biosafety Cabinet Level II.

3.4.3 Cell harvesting

Two hours and thirty minutes after blocking the cell division at metaphase, the cells were harvested for karyotyping. Firstly, the culture medium was discarded from the flask to a labelled 15 ml tube. Then, 15 ml of PBS(Gibco) was added to the flask

to wash the cells and the PBS was discarded into a waste beaker. Five hundred microlitre of trypsin (Gibco) was added into the flask and incubated at 37°C for 5 minutes or until the cell being lift. After that, by using media in the falcon, the trypsin was quenched and pipetted up and down to get as near a single-cell suspension as possible. The suspension was transferred back to the tube.

3.4.4 Cell swelling and fixing

The cell was spinned at 800 rpm for 8 minutes. The supernatant was discarded into a waste beaker while leaving 1 ml of media. The tube was tapped gently to resuspend the pellet in residual media. Then, 9 ml of freshly prepared 0.075M KCL was added slowly. To do this, 4ml of solution was added by the tube wall by while the tube being placed on a vortex set at mild speed. Then, remaining 5ml of solution was added and kept in water bath at 37°C for 25 minutes. After 25 minutes, 3 drops of cold fixative were added by using Pasteur pipette to the tube wall while the tube being placed on a vortex set at mild speed. Then, it was spinned at 800 rpm for 8 minutes without break and upon completion of spinning, the supernatant was discarded. The pellet was resuspended in the residual media by gently tapping the tube. Then, 3 Pasteur pipettes full of fixative was added slowly by following similar technique mentioned previously, spinned at 800 rpm for 8 minutes and the supernatant was discarded. The same step was repeated with 2 Pasteur pipette and 1 Pasteur pipette full in fixative. Upon completion of last centrifugation step, the supernatant was discarded and 4 ml of fixatives was added to the pellet and stored at 4°C for the continuation of karyotyping procedure the next day.

3.4.5 Staining and microscopical imaging

The cells in the fridge was revived. The cells were taken out from the fridge and washed 3 times with freshly made fixative by following similar procedure mentioned in previous step. Then, clean glass slides were soaked in 70% ethanol and wiped using soft tissue. The slides were humidified by breathing on it. Three drops (40-50uL) of the cell suspension was dropped on the slide by using Pasteur pipette from an appropriate height. The slides were allowed to air dry until the microscope slides appeared to be grainy. The cells were expected to break apart from hitting the slide. At the same time, dropping from height is said to improve the cell membrane breaking. Later, Giemsa was prepared in a staining chamber. The slides were stained in the chamber for 8-10 minutes. The Giemsa solution can be used again but it should be stored separately and mixed before each new staining since the solution tends to set over time and yield debris on the slides. After the staining, the slides were rinsed using distilled water and complete drying of the blot was allowed. After drying, two to three drops of mounting medium (Sigma) was added to a coverslip and the coverslip was dropped onto the sample. The slides were placed on the lab bench and airdried for 1 hour. Finally, microscopic imaging was performed at different magnification to capture the metaphase spread of R3.

3.5 Assessment on the effect of fixative drying time on the quality of metaphase spread of R3

Passage 38 of R3 was used to assess this factor. There was no optimization made to the original protocol to assess this factor. The microscope slides with the cell suspension of R3 dropped on it were allowed to air dry naturally on the lab bench without any specific time limit but until it appeared to be grainy.

3.6 Assessment on the effect of adding colcemid after 34 hours to the culture on the quality of metaphase spread of R3

Passage 37 was used to assess this factor. The only manipulation made to the original protocol was, the addition of colcemid to the culture after 34 hours instead of 18 hours.

3.7 Assessment on the effect of cell suspension dropping height on the quality of metaphase spread of R3

Passage 37 which was used to assess the factor in 3.6, was also used to assess these factors. Initially, there were two heights planned to be used as dropping height of R3 cell suspension. One is 0.8 meter which is from a higher height from the surface of microscope slide and the other one is 0.15 meter which is quite closer to the microscope slide. Unfortunately, due to sample insufficiency, the experiment was just ended up by just implementing 0.8 meter as a height for dropping the cell suspension of R3.

CHAPTER FOUR

RESULTS

4.1 Propagation of good quality rat full-term amniotic fluid stem cell line (R3)

4.1.1 Morphology of rat full-term amniotic fluid stem cell line (R3)

R3 was grown in embryonic stem cell (ES) medium, an established medium for R3 by Mun Fun et.al (2015). The cells attain its confluency in 2 days' time. The morphology of R3 was captured using Olympus CKX41 inverted microscope. They remained homogenous throughout the culture when observed at 4X magnification (A). Besides, at the magnification of 10X, they resemble cuboidal-shaped morphology(B). Moreover, at the magnification of 20X, it was very clear that the cells possess high nucleus to cytoplasmic ration due to the presence of multiple nucleoli in the nucleus (C).

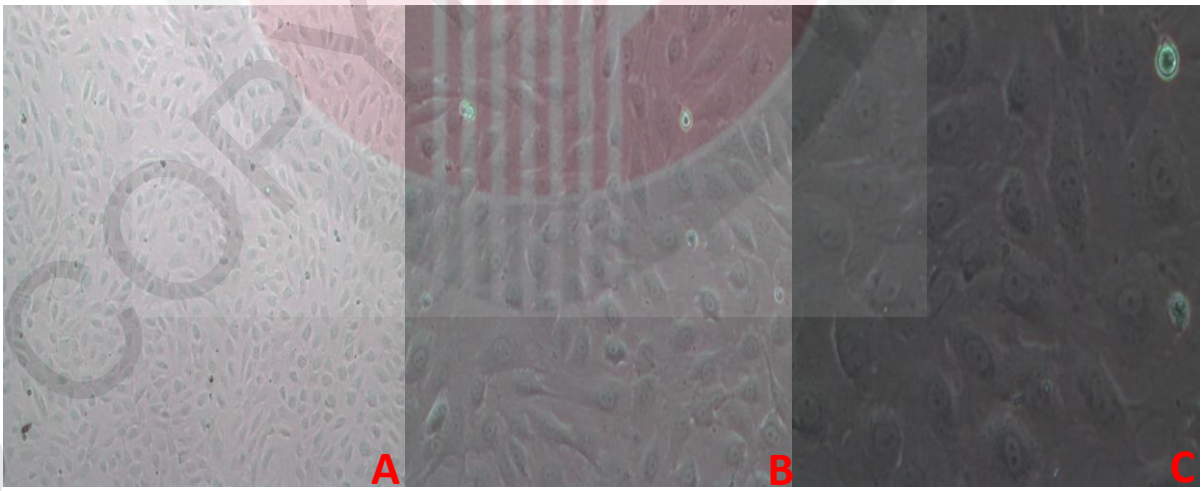


Figure 4.1.1 (A-C): Morphology of R3 cells at Passage 38; A) At magnification of 4X, B)10X and C)20X of inverted microscope

4.1.2 Immunocytochemistry staining (ICC) of R3 for pluripotency-associated markers

Molecular characterization of R3 was performed to identify the expression of pluripotency- associated markers such as Oct-4, Nanog and Sox2 by postgraduate senior, Miss Nur Izzati Mansor who are doing her Doctor of Philosophy (PHD) study involving R3 cell line. As depicted by the figure 4.1.2 below, the markers fluorescent in green colour while the cells' respective nucleus which was counterstained with DAPI fluorescent in blue colour. The observation was made under fluorescent inverted microscope (Olympus) in a dark room. As the images two types of images was merged using Image J software, it indicates the presence of pluripotency- associated marker in the stem cell line culture of R3.

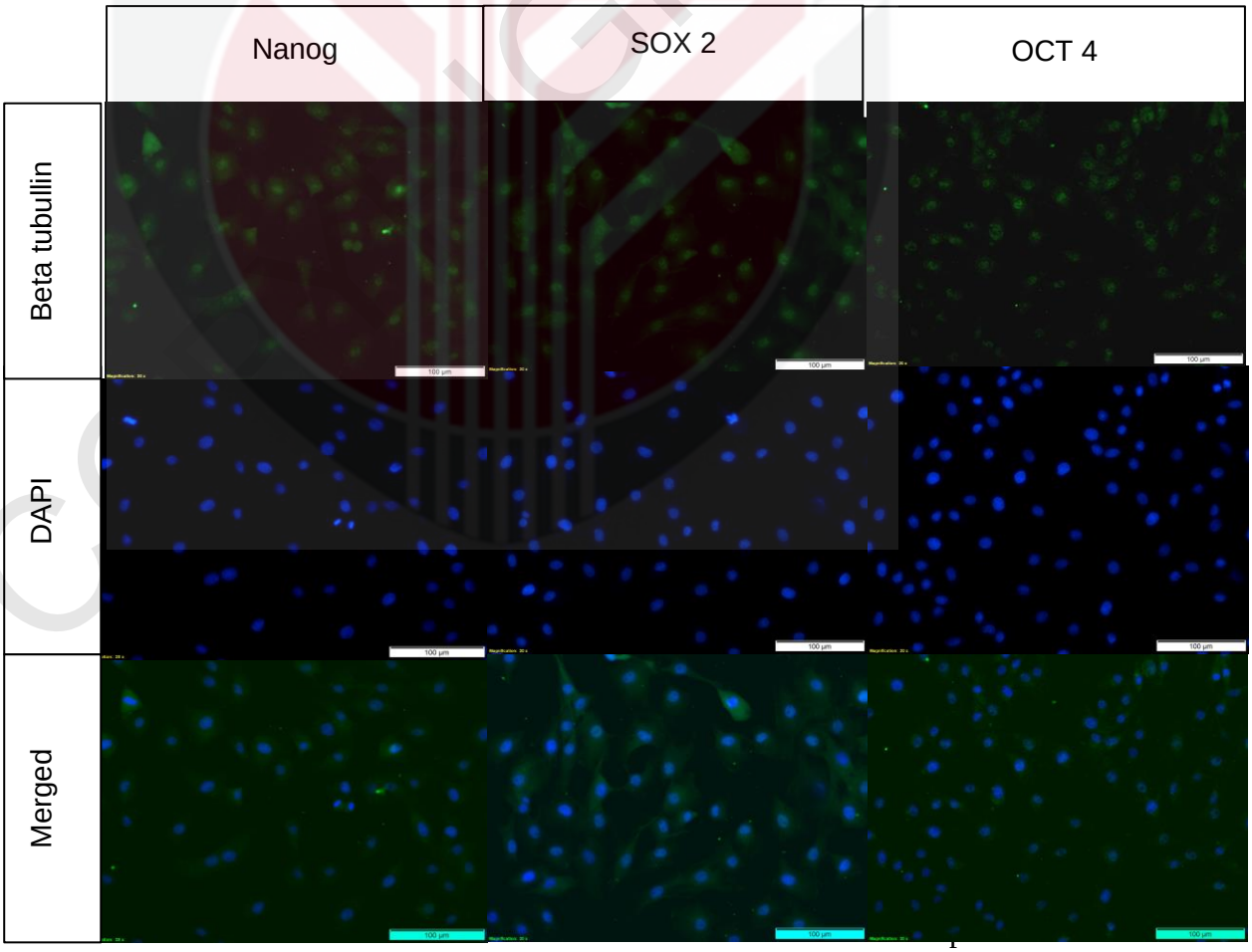


Figure 4. 1.2: Immunofluorescent staining of R3 for pluripotency markers Nanog, SOX 2 and OCT 4. The nuclei were counterstained with DAPI. Result adapted from (Unpublished data from Nur 'Izzati Mansor, 2020)

4.1.3 Flow cytometry analysis of R3 for pluripotency-associated markers

Molecular characterization was further confirmed by flow cytometry analysis for pluripotency-associated markers such as OCT4, Nanog and SOX 2 by postgraduate senior, Miss Nur Izzati Mansor who are doing her Doctor of Philosophy (PHD) study involving R3 cell line. As depicted by figure 4.1.3 below, the percentage of cells with the presence of Oct-4, Nanog and Sox2 were 52.2 %, 35.1 % and 99.2% respectively. Thus, these further indicates the presence of pluripotency- associated marker in the stem cell line culture of R3.

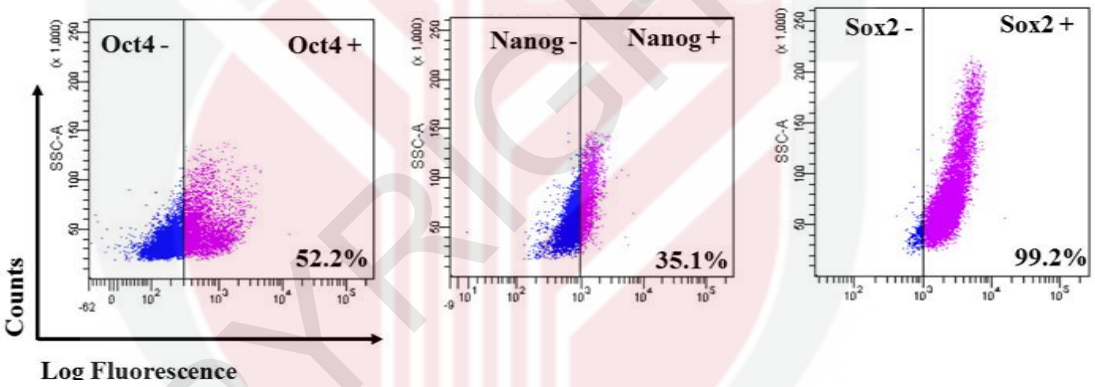


Figure 4.1.3: Flow cytometric analysis of pluripotent transcription markers (Oct4, Nanog and Sox2 for detection of pluripotency state of R3 cells. Result adapted from (Unpublished data from Nur 'Izzati Mansor, 2020)

4.2 Assessment on the effect of fixative drying time on the quality metaphase spread of R3

As for specific objective 2, assessment on the effect of fixative drying on the quality of metaphase spread of R3 was done. Passage 38 of R3 was cultured and karyotyped to obtain metaphase spread. No optimization was made to the original karyotyping protocol adapted from (Punovuori et.al,2018). The microscope slides with the cell suspension of R3 dropped unto it was allowed to air-dry until it appeared to be grainy without any specific time limit. Unfortunately, the results turned out to be the

unexpected one as depicted by the figures below. Figure 4.2.1, shows the presence of clumped chromosomes, encircled with in red. The images were taken using smart phone camera at the magnification of 40X using fluorescent inverted microscope (Olympus) in a dark room Besides, figure 4.2.2 shows too much metaphase spread in the same area. The images were also taken using smart phone camera at the magnification of 40X using Nikon Eclipse TS100. These results will be compared to the previous studies and discussed in discussion chapter.

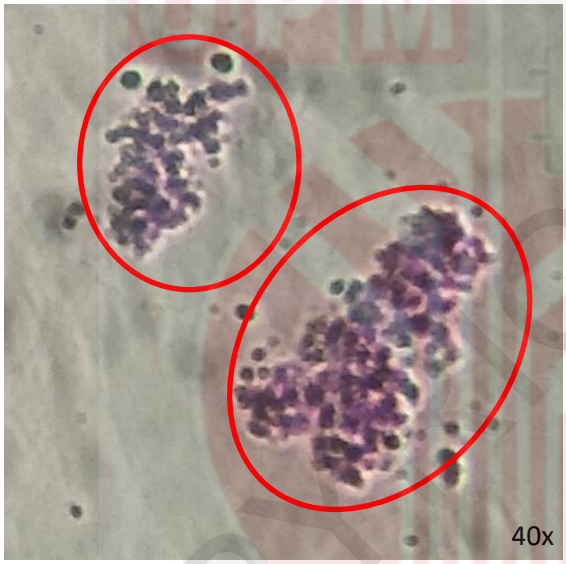


Figure 4.2.1: Presence clumped chromosomes

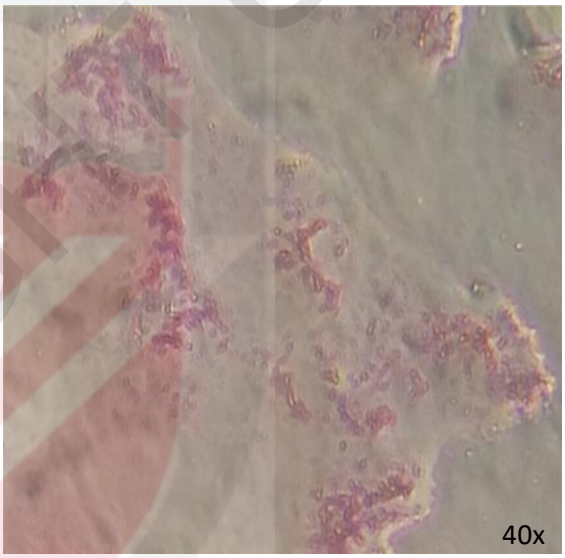


Figure 4.2.2: Too much metaphase spread of same area

4.3 Assessment on the effect of colcemid addition after 34 hours on the quality of metaphase spread of R3

As for specific objective 3, assessment on the effect of colcemid addition after 34 hours on the quality of metaphase spread of R3 was done. Passage 37 of R3 was cultured and karyotyped to obtain metaphase spread. So, the optimization made was the addition of colcemid after 34 hours to R3 culture instead of 18 hours as suggested by the original protocol adapted from (Punovuori ,2018). Despite of the optimization made, there were absence of dividing cells population which is being arrested at metaphase. Instead, a lot of interphase-like cells been having been observed. Figures 4.6 and 4.7 below show the interphase-like cells which could be in different stages of interphase. The images were taken at 100X magnification using Leica Optical Microscope Model DM2500. These results will be compared to the previous studies and discussed in discussion chapter.

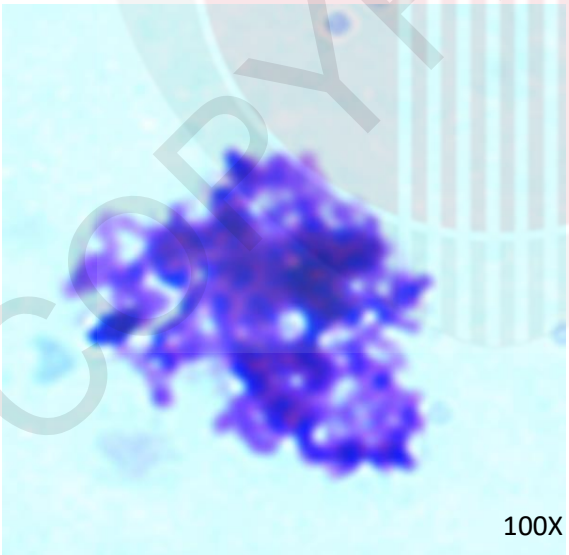


Figure 4.3.1: Presence of interphase-like cells (colcemid addition after 34 hours)

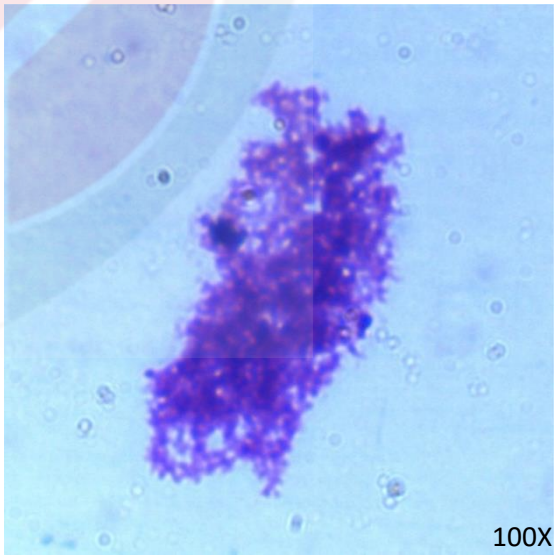


Figure 4.3.2: Presence of interphase-like cells (colcemid addition after 34 hours)

4.4 Assessment on the effect of cell suspension dropping height on the quality of metaphase spread of R3

As for specific objective 4, assessment on the effect of cell suspension dropping height on the quality of metaphase spread of R3 was done. The same Passage 37 of R3 which was cultured and karyotyped to obtain metaphase spread to assess the specific objective 3, was used for the purpose of this objective too. The cell suspension of R3 was dropped from the height 0.8 meter from the microscope slide. Figure 4.4.1 below shows the structurally- disorganized like chromosome dropped from the height of 0.8 meter. The images were taken at 100X magnification using Leica Optical Microscope Model DM2500. These results will be compared to the previous studies and discussed in discussion chapter.

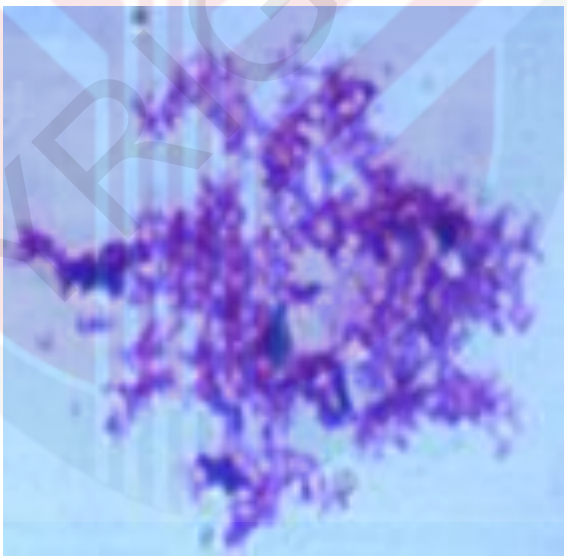


Figure 4.4.1: Presence of structurally disorganized-like chromosome dropped from the height of 0.8 meter

CHAPTER FIVE

DISCUSSION

Stem cells are well-known for their unique properties such as self-renewability and differentiation capability. Self-renewability properties of stem cells make them to be preserved as a stem cell line and increases chance of continuous supply for therapeutic application and eventually marks them as a reliable source for therapeutic application such as cell replacement therapy. For this reason, stem cells which are safe and devoid of ethical issue are really demanding. One such cells is the newly established amniotic fluid stem cell (AFSC) line. Human and murine AFSCs have been found to be safe as they do not form tumour in vivo upon transplantation (Loukogeorgakis & De Coppi, 2017) and do not have any ethical issues as present in embryonic stem cells, which are known to have the widest differentiation potential. However, most of these cells were isolated from mid-term amniotic fluid through a risky procedure, amniocentesis. Alternatively, stem cells isolated from full-term amniotic fluid could be used. However, their genomic stability status is still lacking. Generally, a cell line which holds a therapeutic value should be proven to be stable by not forming tumour in vivo upon prolonged culture before its clinical application.

One of the aspects to prove the stability of amniotic fluid stem cell (AFSC) line is by performing cytogenetic studies such as karyotyping. Karyotyping is reported to be a gold standard for cell line's genetic evaluation for the identification of numerical and structural chromosomes abnormalities (Borgonovo, Vaz, Senegaglia,

Rebelatto, & Brofman, 2014). However, karyotyping is highly dependent on a good metaphase spread of chromosomes for further analysis. Moreover, there is no standard karyotyping protocol solely attributed to stem cell line, albeit the protocol is well established for primary blood and amniotic fluid cells. Thus, in this study we aimed to evaluate the important variables in obtaining good metaphase spread of rat full-term amniotic fluid stem cell line (R3). These findings could serve as a valuable factor for R3 stability checking in the future. In future, although the karyotyping findings would be derived from the rat stem cell line, it could be translated for human counterpart as a promising cell for transplantation, a source that is merely discarded which is human full-term amniotic fluid.

Hence, to achieve this objective, firstly we have propagated a good quality of R3 with the supplementation of ES medium, an established growth medium for R3 culture proposed by Mun Fun et.al (2015). Upon its confluency, which is in about 48 hours, it was sub-cultured to its respective passage number and on the next day karyotyping was continued by following protocol adapted from Punovuori et al. (2018). There are six major steps involved in karyotyping which includes metaphase blocking, cell harvesting, cell swelling, cell fixing, staining and finally microscopical evaluation. The metaphase arresting agent used in this experiment was colcemid. Cultures were arrested at metaphase because that is the mitotic phase where chromosomes are highly condensed and distinguishable. Colcemid inhibits the polymerization of tubulin and hinders the mitotic spindle fibre formation (Lagos & Jimnez, 2012). Thus, the chromosomes could not move to either poles of cells to proceed through the mitosis and divide into two daughter cells. Besides, the cells were harvested to get as near single cell suspension as possible in order to acquire a good metaphase spread by titrating the cell suspension for a few times slowly. Later, the

cells were induced to cell by adding hypotonic solution, potassium chloride. Hypotonic solution usually expands the cellular volumes and helps to disentangle the chromosomes (Campos et.al, 2009). After that, the cells were fixed using Carnoy's fixative. Fixatives fix the tissue wither respective components at a specific stage thereupon particular divisional stages are blocked. In chromosomal studies, fixatives preserve structural integrity of nucleic acid and protein of the chromosomes (Arora, 2016). Coming back to the methodology of this experiment, except for assessment on fixative drying time, the original protocol was optimized to monitor the effect of different factors on the quality of metaphase spread of rat full- term amniotic fluid stem cell line (R3).

5.1 Propagation of good quality of rat full-term amniotic fluid stem cell line (R3)

A good quality of R3 could be defined by its morphology. Firstly, as shown by figure 4.1.1, at magnification of 4X, it appeared to be homogenous and at 10X shown to have cuboidal shape. These observations perfectly match the morphological description of R3 by Mun Fun et.al (2015) where it was mentioned that c-kit positive cells isolated from rat full-term AF (AFSC) were more homogenous than c-kit negative cells and most of the cells displayed cuboidal-shaped morphology. Besides, at 20X, it has high nucleus to cytoplasmic ratio which indicates that there was abundance of nucleoli. Stepinski (2018) have proposed that the rate of cell growth and proliferation are highly dependent on the rate at which nucleolus produce ribosomes and in turn ribosomes regulate protein biosynthesis. Therefore, from here we can say that highly nucleoli indicate high proliferation rate of our R3.

Besides, a good quality of R3 could also be defined by the qualitative and quantitative analysis for main stem cell- specific markers such as Oct-4, Nanog

and Sox2. Figure 4.1.2 shows the immunofluorescent staining for the aforementioned markers. The markers were stained in green colour by beta-tubulin antibodies while their respective nucleus was stained in blue by 4′,6-diamidino-2-phenylindole (DAPI) staining. This qualitative analysis is further supported by the quantitative analysis for the similar markers through flow cytometry analysis as shown by figure 4.1.3. Prusa et al. (2003) were the earliest to report that that Octamer Transcription Factor-4 (Oct-4) is expressed in cells from the mid-term amniotic fluid at both transcriptional and protein levels. At the same time, previously it has been reported that ESCs are recognized by the presence of transcription factors such as Nanog and Oct 4 which sustain the cells in an undifferentiated state, making them able to self- renew (Kolios & Moodley, 2012). Zhang (2014) has reported that Sex determining region Y-box 2 (Sox2) is a vital transcription regulator where they form complex with Oct-4 and Nanog where they collectively control the gene expression in pluripotent stem cells (PSCs) and maintain its pluripotency.

Therefore, the presence of stem cell-specific markers such Oct-4, Nanog and Sox-2 in my culture confirms the presence of stem cell in my propagated culture and eventually proves the stemness of R3.

5.2 Assessment on the effect of fixative drying time on the quality metaphase spread of R3

Other than fixation of cell as it is for karyotyping, fixative plays a huge role in the drying process of microscope slides once the cell suspension (cells inside the fixatives) were dropped unto the microscope slides and allowed to air-dry naturally. According to Spurbeck et. al (1996), an optimum metaphase spread is

highly relying on fixative drying rate which is influenced by both humidity and temperature. Relative humidity is the moisture percentage present in the air compared to its potential saturation at that temperature. Air's capability to hold water increases as the temperature increases. It was also suggested that fast fixative evaporation occurs in dry air whilst slow fixative evaporation occurs in moist air at which both situations are not ideal for since it would end up producing a poor metaphase spreading. Generally, the fixatives layer becomes thinner as it evaporates and this presses the cells from the top, enlarges the cellular area and presses the metaphase chromosome within upper and lower membranes and, spread out the chromosomes and finally collapse the cell membrane (Arsham et.al, 2017). Result from the figure 4.2.1, presence of the clumped chromosomes could be attributed to speed of fixative drying time as suggested by Spurbeck et.al (1996).

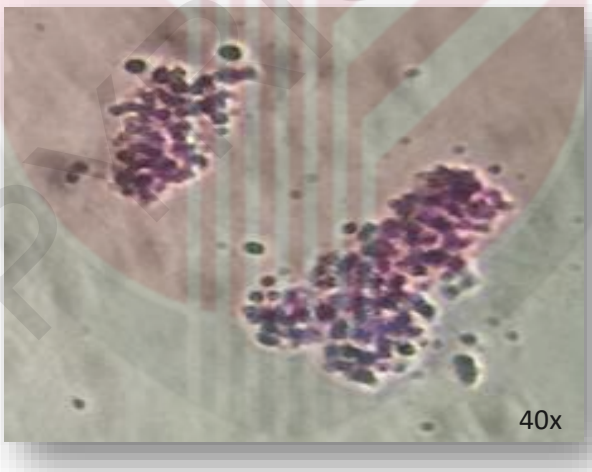


Figure 4.3.1

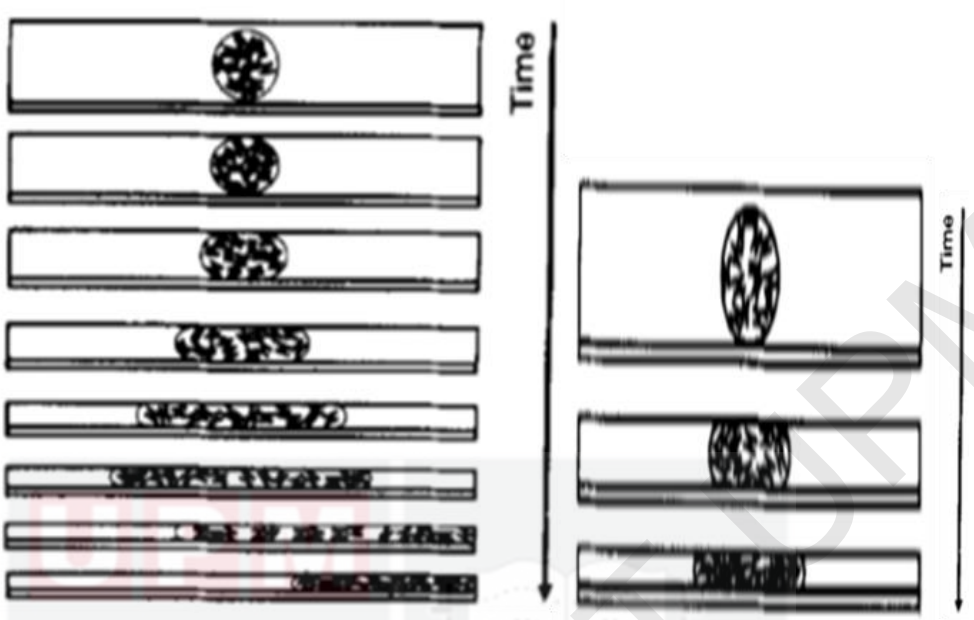


Figure 5.2.1: Metaphase spread as a function of the duration of drying time, obtained from Spurbeck et. al. 1996)

Spurbeck et.al (1996) proposed that speed of fixative trying which is either too slow or too fast as indicated by the time scales in figure 5.2.1 could results in clumpy metaphase chromosomes. If the fixative dries too slow, the cells might have enlarged to its optimum diameter but the fixative did not dry. One of the fates that might happened to the cells were, the cell membrane did not rupture and fixative's current moves the metaphase cells and while moving, the chromosomes inside the cells roll and clump together. Whilst, if the fixative dries too fast, the cells might have dried before it has the chance to reach its optimum diameter. Therefore, results in clumpy chromosomes.

Besides, result from figure 4.2.2 shows too much of metaphase spread at a single area. This result could be comparable to similar phenomena shown by human pluripotent stem cells in the figure below from previous study by Peterson et.al (2011).

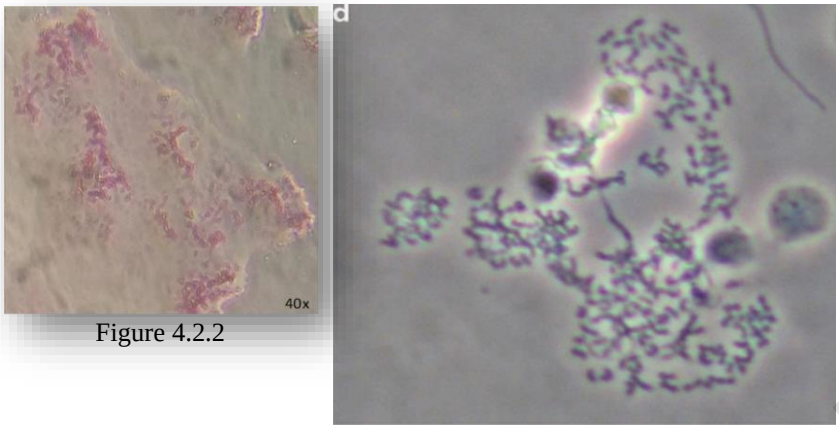


Figure 4.2.2

Figure 5.2.2: Metaphase chromosome spread morphology of human pluripotent stem cell. Too many chromosome spreads in the same area, obtained from Peterson, Chun, & Loring (2011)

This phenomenon could be attributed to slow speed of fixative drying time as suggested by Arsham et.al (2017). As mentioned earlier, the fixatives layer becomes thinner as it evaporates and this presses the cells from the top, enlarges the cellular area and presses the metaphase chromosome within upper and lower membranes , spread out the chromosomes and finally collapse the cell membrane, But in the situation where slow fixative drying occurs, the cell membrane become weakened which in turn create holes and eventually cause the chromosomes to be spilled out and yield a poorly spread metaphase chromosomes.

5.3 Assessment on the effect of colcemid addition after 34 hours on the quality of metaphase spread of R3

The idea of colcemid addition after 34 hours instead of 18 hours as suggested by original protocol is actually to have more population of dividing cells being arrested at metaphase as suggested by previous researcher. Campos et.al (2009) has suggested that it is good to have a culture with high mitotic index with many dividing cells in order to increase the chances of getting more metaphase cells for karyotyping analysis. Besides, according to Howe et.al (2014), the colcemid supposed

to be added to the culture when it is in the logarithmic or exponential phase which characterized by the cell doubling time. The calculated population doubling time for our R3 line is about 35 hours where we anticipate that at 35 hour, the cell population has already doubled (undergone cell division through cytokinesis to produce daughter cells) and thus the colcemid should be added the moment when the cells are still in the mitotic phase (at about 34 hours) which only lasts for about one hour. Despite of the manipulation made, we observed the presence of interphase-like cells as shown in figure 4.3.1 and 4.3.2 in the result section.

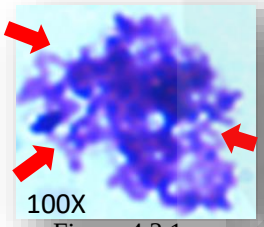


Figure 4.3.1

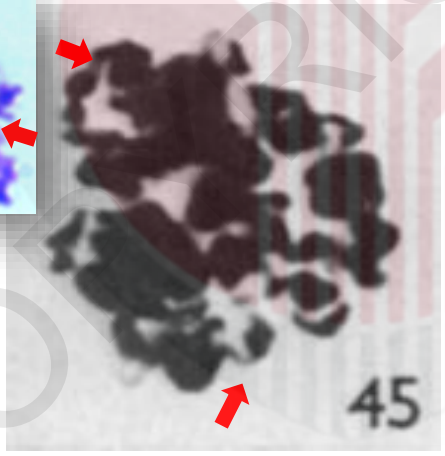


Figure 5.3.1: Condensing chromosomes of Chinese hamster cells in Mid- to late G, nucleus in which chromosomes have contracted into distinct thickened strands with uneven diameters (Stack, Brown d., & Dewey, 1977)

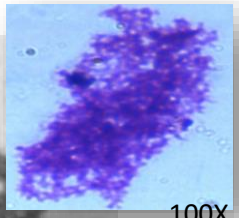


Figure 4.3.2

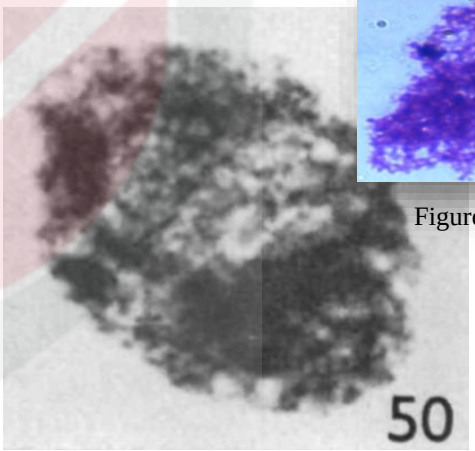


Figure 5.3.2:S phase chromosomes of Chinese hamster cells (CHO line) with generally diffuse chromatin with some clumping evident, obtained from (Stack, Brown& Dewey, 1977)

As shown above, figure 4.3.1 is comparable to figure 5.3.1 from previous research on mammalian cell where they visualized the interphase nuclei of cells. The study indicates that it could be a mid-to-late G phase nucleus with some chromosome

condensation of uneven diameters as indicated by the red arrows. Besides, figure 4.3.2 is comparable to figure 5.3.2 from previous research also where they studied the morphology of interphase nuclei of mammalian cell. The study suggests that it could be an S phase chromosome with some diffused chromatin and clumping evident.

This phenomenon could be attributed to the colcemid treatment inefficiency as suggested by Bickmore et.al (2001) or long cell population doubling time as suggested by MacLeod et.al (2007). Bickmore et.al (2001) proposed that cells which are derived from the mouse often evade the colcemid blockade and continue the cell cycle where they eventually enter interphase when incubated with colcemid for a prolonged time. This seems to be true in our case since R3 cells were derived from the rat amniotic fluid, a species which is closely related to mice. Perhaps, it could be not the timepoint of colcemid addition is the one really matters to acquire a good quality of metaphase spread but the incubation period of cells with colcemid. In contrast, MacLeod et.al (2007) proposed that long cell population doubling time could be the reason for insufficient metaphases where he suggests prolonged incubation with colcemid overnight could solve this issue.

5.4 Assessment on the effect of cell suspension dropping height on the quality of metaphase spread of R3

The idea of dropping the cell suspension from higher height popped out from the suggestion by previous studies that dropping the cell suspension from a higher position was reported to enhance the spreading of chromosomes (Hliscs, Mühlig, & Claussen, 1997).

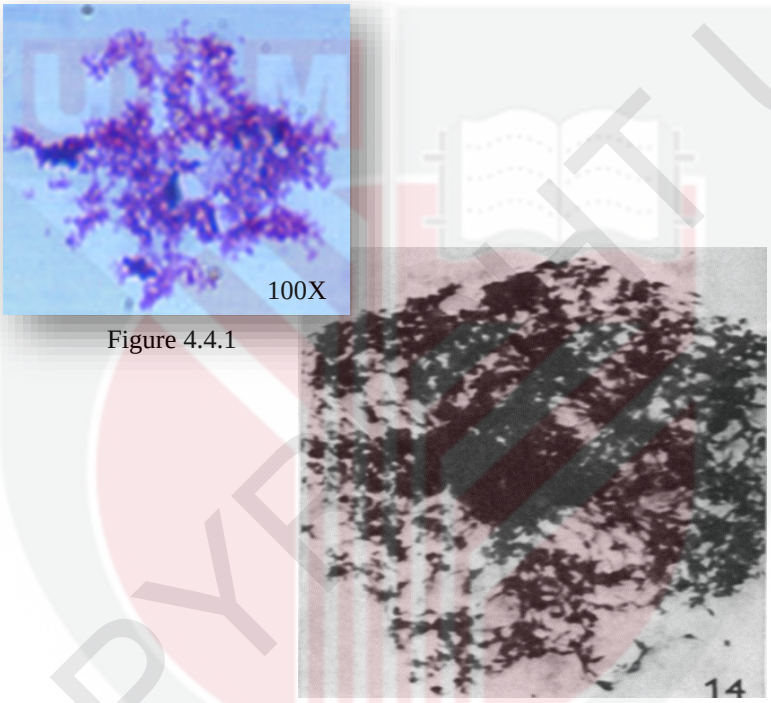


Figure 4.4.1

Figure 5.4.1:S phase chromosomes of Allium cepa root tips with long strands of finely dispersed chromatin due to intense squashing, obtained from (Stack, Brown d., & Dewey, 1977)

Unfortunately, as shown by figure 4.4.1, a structurally disorganized-like chromosomes were observed when dropped from the height of 0.8 meter. This phenomenon could be attributed to high impact force exerted towards the chromosomes while trying to obtain a good metaphase spread as suggested by Stack et.al (1977). This observation could be compared to previous study which visualize the interphase nuclei morphology of a plant cell as shown in figure 5.4.1. The figure shows an S phase chromosome with

some chromatin finely dispersed due to intense squashing. Now, what is vital to notice here is that, not the phase of which the chromosomes are in but the effect of implementation of different method in getting the metaphase spread towards the structural integrity of the chromosomes. Therefore, we anticipate that the force generated from either intense squashing or dropping from a higher height could leave an effect on the structure of chromosomes which could eventually leads to the formation of structurally disorganized-like cells in our experiment.

CHAPTER SIX

CONCLUSION, LIMITATIONS AND FUTURE RECOMMENDATIONS

6.1 Conclusion

In summary, in this this study we could be able to propagate a good quality of rat full-term amniotic fluid stem cell line (R3) which is defined by a good homogeneity, high nucleus to cytoplasmic ratio and which express stem cell-specific markers (pluripotency-associated markers) such as oct-4, Nanog and sox2. However, a good quality metaphase spread could not be obtained in this study despite of several attempts. Nevertheless, we could be able to demonstrate few challenges faced in obtaining a good metaphase spread that could be attributed to various factors associated to karyotyping steps. The presence of clumped chromosomes and high number of chromosome spreads at a single area could be attributed to the speed of fixative drying time. In addition, presence of interphase-like cells could be caused by the colcemid treatment inefficiency or long population doubling time of cells. Structural disorganization of the chromosomes could be attributed to a high dropping height of R3 cell suspension. Proper optimization for fixative drying time, colcemid incubation duration and suitable dropping height of cell suspension are vital in acquiring a good quality metaphase spread for full-term AFSC line. These specific optimizations could serve as valuable factors for R3 stability checking through karyotyping. This finding in turn, although would be derived from a rat AFSC line, it could be still translated for human counterpart as a promising cells transplantation, a source that is merely discarded which is human full-term amniotic fluid.

6.2 Limitation

Our study could be able to demonstrate the vital factors for obtaining a good quality metaphase spread of full-term AFSC. However, several challenges and limitations were also encountered. The main limitation was lack of personal experience and skills in using microscope to get a best plane of view of metaphase spread on the microscope slides. Due to this, there is high tendency to overlook certain important observation on the microscope slide of metaphase spread which supposed to be addressed. Besides, lack of experience in interpreting the observation of metaphase spread on the microscope slide is another limitation. As a result, this could have led misinterpretation of karyotyping result to other totally unrelated or irrelevant subject matters. Finally, time constraint is another major limitation to this study due to implementation of Movement Control Order (MCO) in Malaysia due COVID-19 pandemic. This limitation hampers the replication of experiment which need to be done for at least three time for each factor assessed in this study in order to make the result more scientifically sound and accurate.

6.3 Future Recommendation

Firstly, addressing the issue of speed of fixative drying time which is either too slow or too fast, as for future recommendation, proper optimization should be made on this factor as standard relative humidity for slide-preparation is highly dependent on the temperature differences, hygrometer accuracy, technique and type of specimen (Ashram et.al, 2017). Literatures provides various suggestion to overcome this issue. Ashram et.al (2017) have suggested that fixative drying from the range of

30 to 45 seconds is an appropriate duration. Besides, he also proposed that it is vital to slow down the fixative drying in dry climates as the fixatives dries too fast in low humidity. To do this, he suggested to use humidifier or placing the slides on wet paper towels or above steam in a sink in order to dry the slides. It is also good to breathe on the slides to provide humidity. In contrast, he also proposed to speed up the fixative drying time in humid climate as the fixatives dries too slow in high humidity. To do this, he proposed to use different composition of Carnoys's fixative which is 6:1 methanol-acetic acid instead of 3:1 ratio. He also reports that laboratories in east and Midwest utilize dehumidifiers for slide preparation to cope with high humidity. Apart from that, Lundsten et.al proposed temperature of 20 °C and humidity with optimum range of 40% to 50%. He also proposed that it's better to uniquely designed "climate-room" or slide making cabinet which is commercially available to ease the process of temperature and humidity control in a small area than in a large room for slide preparation (cited in Arsham et.al, 2017). Spurbeck et.al (1996) have used a modified Thermotron environmental control unit to adjust for humidity of 50% and temperature of 25 °C as an optimum setpoints. Looking at all these different recommendations from the previous study ranging from simplest to the complex one, taking into account prospect and budget allocated for a respective project, future researchers should be able to optimize the protocol accordingly.

Besides, addressing the issue of colcemid treatment inefficiency, as for future recommendation, it would be better if the researchers could be able to optimize the colcemid incubation period with the cells by reducing the duration of incubation since prolonged incubation with colcemid was the one might have led the cells to escape the blockade in this experiment. In contrast, to compensate for the issue of long population doubling time of the cells, prolonged incubation of culture with

colcemid might will help. Although, the justification and solution in addressing these problems seems to be contradict, there is no harm in executing trial and error approach to optimize the karyotyping procedure for stem cell line as karyotyping steps are very technical and requires ample of time for troubleshooting.

Apart from that, addressing the issue of high dropping height of R3 cell suspension, as for future recommendation it is better to follow the range of dropping height provided by literatures instead of personally amending the protocol which would not work all the time. Kuo et.al (2016) have implemented dropping height of 0.5 meter in order to obtain a well-spread meiotic pachytene chromosome from pollinia of Phalaenopsis orchids while Yao et.al (2019) have used dropping height of 35 cm to get well-spread metaphase chromosomes of maize inbred lines. In contrast, it was reported that cell suspension dropping height is not relevant as high heights only helps in even cells distribution on microscope slide. Moreover, a good chromosome spreads could be still obtained despite of lower or higher dropping height from the microscope slide (cited in Rodríguez-Domínguez et.al, 2017). From here it is clear that if one would like to optimize cell suspension dropping height, as for starting, they could try the range of height from 35 to 50 cm or even try to drop it from the lowest and highest point possible in order to rule out the real influence of cell suspension dropping height. From there, height which is not suitable could be excluded from the list.

Finally, each factor assessed in this study should be repeated many times to assess and validate its influence on a good quality of metaphase spread since data from this study is very limited. It is all about sufficient time, ability to troubleshoot

and proper optimization when it comes to acquiring a good quality metaphase spread as karyotyping procedure is very technical and different cell line response differently. It is also highly recommended to use a proper microscope with good quality of metaphase spread view in order to capture a clear and presentable image since microscope is an important equipment in cytogenetic studies.

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