A COMPARATIVE STUDY OF CONDITIONED MEDIUM DERIVED FROM HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS AND MOUSE EMBRYONIC FIBROBLASTS FOR CONVERTING PRIMED TO NAÏVE STATE OF RHESUS MONKEY EMBRYONIC STEM CELLS

RATREE MOORAWONG

A Thesis Submitted in Partial Fulfillment of the Requirements for the
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การศึกษาเปรียบเทียบการเปลี่ยนแปลงเซลล์ต้นกำเนิดตัวอ่อนลิงวอก สถานะไพรม์เป็นสถานะนาอีฟ ด้วยน้ำยาที่ผลิตจากเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อวาร์ตันเจลลี่มนุษย์ และเซลล์ไฟโบรบาสต์ของตัวอ่อนหนูเมาส์

นางสาวราตรี มูระวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เซลล์ต้นกำเนิดตัวอ่อนลิงวอก มีบทบาทสำคัญในการสร้างแบบจำลองในสัตว์ทางสาขาวิทยาศาสตร์การแพทย์ โดยเฉพาะการสร้างเนื้อเยื่อใหม่และการรักษาความผิดปกติทางพันธุกรรม ซึ่งเซลล์ต้นกำเนิดชนิดนี้แสดงสถานะไพรม์เท่านั้น ดังนั้นจึงมีข้อจำกัดของการเข้าไปเป็นส่วนหนึ่งในตัวอ่อน และทำให้มีข้อด้อยที่สำคัญต่อการวิจัยทางวิทยาศาสตร์การแพทย์ ซึ่งการประสบความสำเร็จในการรีโปรแกรมเซลล์สถานะไพรม์ให้เป็นสถานะนาอีฟถือเป็นจุดเปลี่ยนในการวิจัยด้านเซลล์ต้นกำเนิด เนื่องจากเซลล์สถานะนาอีฟมีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ประเภทใดก็ได้ การศึกษาครั้งนี้มีจุดมุ่งหมายเพื่อศึกษาประสิทธิภาพระบบน้ำยาเลี้ยงเซลล์ในการเปลี่ยนแปลงเซลล์ต้นกำเนิดตัวอ่อนลิงวอกจากสถานะไพรม์ให้เป็นสถานะนาอีฟในสภาวะการเลี้ยงเซลล์ที่ปราศจากเซลล์พี่เลี้ยง โดยใช้ระบบน้ำยาเลี้ยงสองชนิด ได้แก่ น้ำยาเลี้ยงเซลล์ที่ผลิตจากเซลล์ไฟโบรบาสต์ของตัวอ่อนหนูเมาส์สายพันธุ์ OF1 (OF1-MEFs-CM) และน้ำยาเลี้ยงเซลล์ที่ผลิตจากเซลล์ต้นกำเนิดมีเซ็นไคม์จากเนื้อเยื่อวาร์ตันเจลลี่ของสายสะดือมนุษย์ (hWJ-MSCs-CM) ผลการวิจัยพบว่าการเปลี่ยนแปลงเซลล์ต้นกำเนิดตัวอ่อนลิงวอก ในน้ำยาทั้งสองชนิดแสดงให้เห็นถึงการเพิ่มระดับการแสดงออกของยีนพลูริโพเทนต์ ได้แก่ *SOX2* และยังมีการสูญเสียการแสดงออกของยีนที่เกี่ยวข้องกับสถานะไพรม์ ได้แก่ *TBXT* และ *OTX2* ในการเปรียบเทียบการแสดงออกของยีนที่จำเพาะต่อเซลล์สถานะนาอีฟ พบว่าในวันที่ 9 ของการเปลี่ยนแปลงเซลล์ต้นกำเนิดตัวอ่อนลิงวอกในน้ำยาเลี้ยงเซลล์ที่ผลิตจาก hWJ-MSCs-CM มีระดับการแสดงออกของยีนที่สูงขึ้นอย่างมีนัยสำคัญ ได้แก่ *KLF4, KLF17, ESRRB, TFAP2C, DPPA2* และ *DPPA5* ในทางตรงกันข้าม การเปลี่ยนแปลงสถานะเซลล์โดยใช้น้ำยาเลี้ยงเซลล์ที่ผลิตจาก OF1-MEFs-CM มีระดับการแสดงออกของยีน *DPPA5* ที่สูงขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับการเหนี่ยวนำในน้ำยา hWJ-MSCs-CM ในวันที่ 9 และวันที่ 21 ผลการทดลองนี้บ่งชี้ได้ว่าน้ำยาเลี้ยงเซลล์ที่ผลิตจาก hWJ-MSCs-CM มีประสิทธิภาพการเหนี่ยวนำเซลล์ต้นกำเนิดตัวอ่อนลิงวอกระยะไพรม์เป็นระยะนาอีฟได้ดี ซึ่งจะเป็นประโยชน์ต่อการนำไปประยุกต์ใช้เพื่อเหนี่ยวนำเซลล์พลูริโพเทนต์ชนิดอื่น

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา ราตรี มูระวงษ์
ลายมือชื่ออาจารย์ที่ปรึกษา

RATREE MOORAWONG: A COMPARATIVE STUDY OF CONDITIONED MEDIUM DERIVED FROM HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS AND MOUSE EMBRYONIC FIBROBLASTS FOR CONVERTING PRIMED TO NAÏVE STATE OF RHESUS MONKEY EMBRYONIC STEM CELLS.
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Keyword: Rhesus Monkey/Embryonic Stem Cells/Conditioned Medium/Primed State/ NAÏVE State

Rhesus monkey embryonic stem cells (rhESCs) have a significant role used as animal model in medical sciences, especially, tissue regeneration and treatment of genetic disorders. In general, rhESCs are only at the primed state, thus posing limitations on engraftment in host embryos which is significant disadvantages for medical research. The successful reprogramming of primed state cells into the naïve state is considered a breakthrough in stem cell research, as naïve cells have the ability to differentiate into any cell types. The aim of this study was to evaluate the ability of culture media to convert primed rhESCs to a naïve state using two different conditioned media under feeder-free conditions. The first conditioned medium was derived from mouse embryonic fibroblasts of OF1 strain (OF1-MEFs-CM) and the second from human umbilical cord Wharton's jelly mesenchymal stem cells (hWJ-MSCs-CM). The results revealed that the conversion of rhESCs in both conditioned media strongly expressed gene of the core pluripotent markers *SOX2*, while resulting in the loss of genes expression of primed markers including *TBXT* and *OTX2*. The comparison of genes expression of naïve markers found that rhESCs grow in hWJ-MSCs-CM conversion at day 9 exhibited significantly higher levels of gene expression including *KLF4, KLF17, ESRRB, TFAP2C, DPPA2* and *DPPA5*. Conversely, the conversion using rhESCs grow in OF1-MEFs-CM exhibited significantly higher expression of *DPPA5* compared to cells grow in hWJ-MSCs-CM on both day 9 and day 21. These results suggest that the efficiency of each conditioned medium differs in the expression of naïve markers at different stages. The results of this experiment indicate that the hWJ-MSCs-CM efficiently reprograms primed rhESCs into the naïve state. This could be beneficial for application in reprogramming other types of pluripotent stem cells.

School of Biotechnology
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Student's Signature Ratree Moorawong
Advisor's Signature

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LIST OF ABBREVIATIONS

bFGF	=	Basic fibroblast growth factors
DAPI	=	4′,6-Diamidino-2-Phenylindole, dihydrochloride
DMEM	=	Dulbecco's modified Eagle medium
DMEM-F12	=	Dulbecco's modified Eagle medium/Nutrient mixture F-12
DPPA2	=	Developmental pluripotency associated 2
DPPA5	=	Developmental pluripotency associated 5
ESRRB	=	Estrogen-related receptor beta
FBS	=	Fetal bovine serum
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
H3K14ac	=	Histone H3 lysine 14 acetylation
H3K9me3	=	Histone H3 lysine 9 trimethylation
hWJ-MSCs	=	Human Wharton’s jelly-derived mesenchymal stem cells
KDM4A	=	Histone lysine demethylase 4A
KLF17	=	Krueppel-like factor 17
KLF4	=	Kruppel-like factor 4
KO-DMEM	=	Knockout Dulbecco's Modified Eagle's Medium
KOSR	=	Knockout serum replacement
LIF	=	Leukemia inhibitory factor
MMC	=	Mitomycin C
NANOG	=	Nanog homeobox
NEAA	=	non-essential amino acids
OCT4	=	Octamer-binding transcription factor 4
OF1-MEFs	=	Mouse embryonic fibroblasts strain OF1
OTX2	=	Orthodenticle homeobox 2
PSG	=	Penicillin streptomycin glutamine
RT-qPCR	=	Real-time quantitative polymerase chain reaction
SOX2	=	SRY-box transcription factor 2
TBXT	=	T-box transcription factor T
TFAP2C	=	Transcription factor AP-2 gamma

CHAPTER I
INTRODUCTION

1.1 Background and significance

Pluripotent stem cells (PSCs) are cells that have the capacity to self-renew by dividing and to develop into the three primary germ cell layers of the early embryo. The PSCs including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). Ordinarily, ESCs derived from the inner cell mass (ICM) of blastocyst stage embryos. They are widely used in scientific research because they have the ability to differentiate to be various tissues in the body (Biehl & Russell, 2009). ESCs were first produced from the ICM of mouse blastocyst (Evans & Kaufman, 1981). Consequently, numerous ESCs have been reported in various animals, including humans (Thomson et al., 1998), non-human primate (NHPs) (Thomson et al., 1995), bovine (Mitalipova et al., 2001), rats (Buehr et al., 2008) and pig (Chen et al., 1999).

In rodents, ESCs have the ability to transition between two distinct states: the naïve pluripotent state and the primed pluripotent state (Nichols et al., 2009). Naïve and primed states exhibit dramatic differences, including transcription profile, epigenetic profile, cell cycle regulation, and energy production, which subsequently influence their characteristics and functions (Weinberger et al., 2016). A primary criterion for discerning between the naïve and primed states of pluripotency lies in the status of X chromosome activity: female ESCs in the naïve state display two active X chromosomes, while female ESCs in the primed state have experienced random inactivation of one or both X chromosomes, a pattern consistent with the cell's developmental maturity. Until recently, human PSCs predominantly existed in a primed-like state, dependent on bFGF and activin A for inhibition of differentiation. However, a few naïve ESCs have been derived from human blastocysts. (Guo et al., 2016). Achieving this involves using cocktails of growth factors and small molecules (chemical inhibitors of PKC, MEK, and GSK3b), which often yield cell lines reputed to be unstable. A more reliable approach entails treating PSCs, which are in the primed state, with resetting factors to restore naïve-type characteristics.

In NHPs, ESCs in the primed state have been treated with resetting factors to instate naïve-type characteristics. Of note, Fang and colleagues pioneered the conversion of primates to naivety in the monkey using 4i/L/b medium, supplemented

with growth factors LIF and bFGF, as well as inhibitors PD0325901 (MEK), CHIR99021 (GS3b), SB203580 (p38MAPK), and SP600125 (JNK) (Fang et al., 2014). Many reports have demonstrated successful conversion of primed PSCs to a naïve state in humans (Chan et al., 2013; Gafni et al., 2013; G. Guo et al., 2017), but there has been relatively less study on this conversion in rhesus monkeys. Recently, rhesus monkey embryonic stem cells (rhESCs) are highly valuable tools for regenerative medicine owing to their similarity to human ESCs, which renders them particularly relevant for studying human development and disease progression (Vallier et al., 2005). Usually, NHPs ESCs have been established on mouse embryonic fibroblasts (MEFs) feeder cells or in matrix-coated dishes in feeder-conditioned medium supplemented with bFGF and KOSR, which are known to support the self-renewal in the primed state of pluripotency. Subsequently, resetting factors are used in conditioned medium (CM) to induce naïve-type characteristics in this primed state. Since, the number of published papers on CM production and characterization has exponentially increased over the past 20 years, CM can be considered a promising pharmaceutical product in regenerative medicine (Kuo et al., 2021). Generally, CM is mostly produced from MEFs and human Wharton's Jelly mesenchymal stem cells (hWJ-MSCs).

In particular, mesenchymal stem cells (MSCs) are present in various types of tissues. They are pluripotent and can differentiate into many kinds of mesodermal cells, such as adipocytes, chondrocytes, and osteocytes (Abbasi-Malati et al., 2018). There have been many attempts to use MSCs for the treatment of many types of diseases, based on their ability to suppress immunocyte proliferation and to control inflammation, because of their secretion of various cytokines and growth factors (Chamberlain et al., 2007). However, cell-free therapy based on CM derived from human Wharton's jelly mesenchymal stem cells (hWJ-MSCs) has gained attention in the field of protective and regenerative medicine owing to the many advantages of hWJ-MSCs. These advantages include non-invasive collection, no ethical issues, low tumorigenicity, and suitability for allogeneic transplantation due to their reduced immunogenicity, which lowers the risk of rejection by the recipient’s immune system. (Acuto et al., 2023). Moreover, to facilitate their clinical use and ensure safety, protocols for converting primed ESCs to naïve state must comply with good manufacturing practice guidelines and avoid xenogeneic products.

Therefore, the aim of this study was to compare the efficiency of converting primed rhESCs to naïve state using two different CM which derived from MEFs and hWJ-MSCs, under feeder-free conditions. Specifically, we sought to evaluate the

potential of culture system and assess their stemness to understand the benefits of using these CM to converting primed to the naïve state for medical applications.

1.2 Research objectives

1.2.1 To generate the CMs from OF1-MEFs and hWJ-MSCs for use in the base media during the converting primed to the naïve state of rhESCs.

1.2.2 To evaluate the efficiency of the CMs between OF1-MEFs and hWJ-MSCs for converting primed rhESCs to the naïve state.

1.3 Scope of the study

Primed rhESCs have been converted and maintained in ALGöX medium containing Activin A, LIF, the PKC inhibitor (Gö6983), and the tankyrase inhibitor (XAV939) using different culture medium produced from OF1-MEFs-CM (Gold standard method) and hWJ-MSCs-CM under feeder-free conditions. In the conversion process, primed rhESCs were first cultured in ALGöX with ascorbic acid (VALGöX) for 9 days, then switched to ALGöX for 12 days. Subsequently, the cells were evaluated the characteristics of naïve-like cells using immunocytochemistry (ICC) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) techniques. The illustration of rhESCs conversion process is shown in Figure 1.1

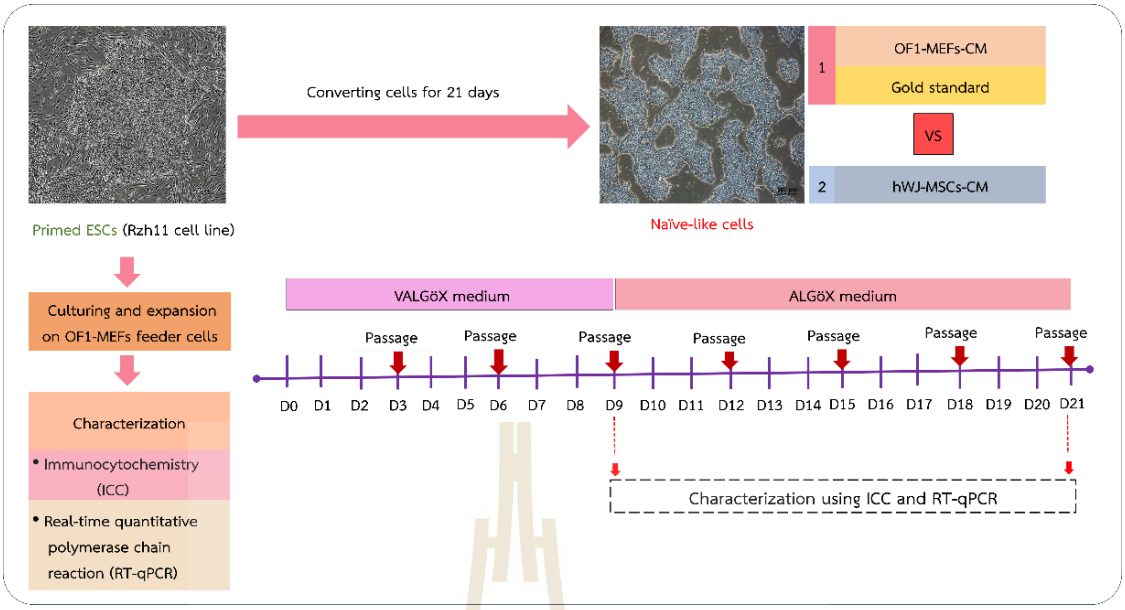

Figure 1.1 Schematic diagram of converting primed rhESCs to the naïve state using CMs derived from OF1-MEFs and hWJ-MSCs in feeder-free conditions.

1.4 Benefits of this research

Typically, the naïve state of ESCs possesses the ability to differentiate into any cell type, making them highly promising for cellular therapy in regenerative medicine. However, to facilitate their clinical use and ensure safety, protocols for converting primed ESCs to naïve state must comply with good manufacturing practice guidelines and avoid xenogeneic products. Therefore, the benefits of this study encompass identifying the efficiency of CMs (OF1-MEFs-CM and hWJ-MSCs-CM) in converting primed rhESCs to the naïve state. Specifically, we aimed to evaluate the potential of culture system and assess their stemness to understand the advantages of using these CMs to induce primed rhESCs to the naïve state for medical applications.

CHAPTER II
LITERATURE REVIEW

2.1 Basic of stem cells

Stem cells are indeed defined as precursor cells with the ability to proliferate and differentiate into various cell types. They are characterized by their capacity for self-renewal and their potential to differentiate into specialized cell types. Generally, stem cells can be classified into two types including classified according to their origin (Table 2.1) and classified according to their potency (Table 2.2).

2.1.1 Classification of stem cells according to their origin

2.1.1.1 Embryonic stem cells (ESCs) are the cells derived from the inner cell mass (ICM) of embryos at blastocyst stage (Zakrzewski et al., 2019). They are taken from the very early stages of embryo development, typically 4-5 days after fertilization (Lerou, 2011; Ying et al., 2003). They can be maintained in culture as undifferentiated cell lines because they are capable of self-renewal and can be stimulated to differentiate into any cell lineage (Klimanskaya et al., 2006). They can differentiate into the endoderm, mesoderm, and ectoderm of embryonic germ layers, as well as any type of somatic and germline cells. Therefore, they hold great potential in tissue regeneration therapy (Zhang & Huang, 2010).

2.1.1.2 Adult stem cells (ASCs) are the cells obtained from bone marrow, peripheral blood, and umbilical cord blood. These cells can only produce a limited number of cell types. They have limited potential differentiate when compare with ESCs because of the state of development of these cells (Robinson, 2001). These cells are important in tissue repair and regeneration medicine because they can replace cells that die due to injury or disease (Hansen & Inselman, 2011).

2.1.1.3 Induced Pluripotent Stem Cells (iPSCs) are stem cells that can be directly generated from adult cells such as, skin cells, through reprogramming using specific transcription factors. This process returns the cells to a state resembling embryonic stem cells, enabling them to differentiate into various cell types (Takahashi & Yamanaka, 2006).

Table 2.1 A summary table classifying stem cells according to their origin.

Cell types	Type of stem cells	Origin	Self-renewal	Applications
ESCs	Pluripotent	ICM of blastocyst	High self-renewal capacity	Basic research, regenerative medicine, drug testing
iPSCs	Pluripotent	Somatic cells	High self-renewal capacity	Regenerative medicine, disease modeling, drug testing
ASCs	Multipotent	Bone marrow, brain, skin	Limited self-renewal capacity	Tissue repair, regeneration, treating specific diseases (e.g., leukemia)

2.1.2 Classification of stem cells according to potency

Pluripotency refers to a single cell's capacity to generate all cell types found in mature organisms. In mammalian development, pluripotency is confined to cells of the embryo or epiblast, which serve as the foundational tissue for the entire embryo. *In vivo*, pluripotent cells are transient and exist only for a brief period during mammalian embryo development, lacking long-term self-renewal capability. However, when provided with appropriate culture conditions, cells originating from the embryo or epiblast can gain indefinite self-renewal capacity while preserving their pluripotent traits *in vitro*, giving rise to pluripotent ESC lines. Usually, there are four main types of stem cells depend on the potency character including, totipotent, pluripotent, multipotent, and unipotent stem cells.

2.1.2.1 Totipotent stem cells are a class of stem cells with the highest potential. These cells can generate and form an entire embryo, including extra-embryonic cells (placenta), which can differentiate into almost all cell types (Ghazimoradi, Khalafizadeh, & Babashah, 2022).

2.1.2.2 Pluripotent stem cells are a class of stem cells with high potential. These cells can give rise to all cell types of the body except the placenta and can differentiate into derivatives of the three germ layers formed in the early stages of gastrulation. (Ralston & Rossant, 2010).

2.1.2.3 Multipotent stem cells are one of the main cell types capable of differentiating into specialized cells with specific functions. These cells can differentiate into various types such as bone, cartilage, fat, muscle, and other tissues, and they are isolated from the placenta, adipose tissue, bone marrow, neural tissues and etc. (Trounson & McDonald, 2015). These cells can self-renew and have therapeutic potential for the treatment of a range of diseases because they do not have the ethical issues associated with pluripotent and totipotent stem cells. (Mirzaei et al., 2018).

2.1.2.4 Unipotent stem cells are stem cells that have the property of self-renewal, distinguishing them from non-stem cells. These cells can produce only one cell type and differentiate to become a specific type of tissue.

Table 2.2 A summary table classifying stem cells according to their potency.

Type of stem cell	Cell types	Example	Differentiation potency
Totipotent	ESCs	Zygote and the first few cells from divisions after fertilization	High
Pluripotent	ESCs and iPSCs	ESCs and iPSCs	High
Multipotent	ASCs	Mesenchymal stem cells and hematopoietic stem cells	Low
Unipotent	ASCs	Epidermal stem cells and muscle stem cells	Very low

2.2 Pluripotent Stem Cells (PSCs)

PSCs proliferate indefinitely and differentiate into cells of all three germ layers (ectoderm, endoderm, and mesoderm). These two properties of PSCs make them an attractive source for cell therapies of various diseases in human. Historically, PSCs were first produced from the ICM of mouse blastocysts in 1981 (Evans and Kaufman, 1981; Martin, 1981). These cells, named mouse embryonic stem cells (mESCs), have the ability to form teratomas when injected into immunodeficiency mice. Actually, PSCs include ESCs derived from the ICM of blastocyst stage embryos, and iPSCs, which are generated from adult somatic cells that have been genetically reprogrammed using transcription factor combinations that share similar properties with ESCs. Since their initial derivation of human ESCs (hESCs) in 1998 (Thomson et al., 1998). hESCs have been primary candidates for cell-based therapies that aim to restore lost cell and tissue function (Geron et al., 2009). Although, rodents have traditionally served as the primary model organisms in mammalian biology. Mice in particular, offer numerous distinct advantages that render them exceptional candidates for animal modeling. These advantages encompass facile breeding, abbreviated generation intervals, and a notable abundance of offspring. Moreover, mice have long been entrenched as a platform for genome engineering and PSCs technologies, pivotal tools for unraveling intricate molecular mechanisms (Buehr et al., 2008; Lee et al., 2017). Consequently, a multitude of noteworthy discoveries has been documented, some of which have significantly enriched our comprehension of human biology and have catalyzed pharmaceutical advancements. However, recent investigations in both rodent and human contexts have unveiled an augmented disparity between these two species than formerly recognized. Consequently, a prevailing need emerges for an animal model more closely aligned with human biology.

2.3 Naïve and primed states of pluripotency

In rodents, PSCs have the ability to transition between two distinct states: the naïve pluripotent state exemplified by ESCs, and the primed pluripotent state exemplified by epiblast stem cell lines (EpiSCs) (Nichols et al., 2009). Naïve and primed states exhibit dramatic differences, including transcription profile, epigenetic profile, cell cycle regulation, and energy production, which subsequently influence their characteristics and function (Weinberger et al., 2016). A primary criterion for discerning between the naïve and primed states of pluripotency lies in the status of X chromosome activity: female PSCs in the naïve state display two active X

chromosomes, while female PSCs in the primed state have experienced random inactivation of one or both X chromosomes, a pattern consistent with the cell's developmental maturity. In addition, differences in cell morphology between primed and naïve states were also observed. Typically, cells in the naïve state exhibit a dome shape as shown in Figure 2.1, especially when grown on feeder cells, whereas some naïve cells in feeder-free conditions do not show this characteristic (Romayor et al., 2022). Until recently, human PSCs predominantly existed in a primed-like state, dependent on bFGF and activin A for inhibition of differentiation. However, a few rare naïve ESCs have been derived from human blastocysts. (Guo et al., 2016). Achieving this involves using cocktails of growth factors and small molecules (chemical inhibitors of PKC, MEK, and GSK3), which often yield cell lines reputed to be unstable. A more reliable approach entails treating ESCs and iPSCs, which are in the primed state, with resetting factors to restore naïve-type characteristics. The different features between naïve and primed states pluripotency are shown in Table 2.3.

Numerous reports indicated that naïve PSCs have the capability to be cultured as single cells for extended periods. They exhibit a higher rate of cell division and a shorter doubling time compared to primed PSCs, making them more suitable for clonal expansion (Tesar et al., 2007). This characteristic makes naïve pluripotent stem cells well-suited for experimental and medical research purposes, such as testing for biological markers for disease treatment or assessing drug efficacy. However, it has been observed that currently, naïve PSCs are only found in the mESCs and not in the ESCs of other mammals, such as humans and primates, or in iPSCs. These naïve PSCs are of significant interest as they hold potential for research modeling and medical applications.

Table 2.3 Characteristic features of naïve and primed states PSCs.

Property	Naïve state	Primed state
Origin	ICM of early blastocyst	Post-implantation epiblast
Representative examples	mESCs, miPSCs	mEpiSCs, hESCs, hiPSCs
Colony morphology (Cultured on feeder cells)	Compact dome-shaped	Flattened
Expressed genes	High expression of *KLF2, KLF4, KLF5, ZPF42, ESRRB, DPPA3, TFCP2L1, FGF4, TBX3, CDH1*	*DNMT3B, FGF5, POU3F1, MEIS1, OTX2, SOX11, GDF3*
Growth factor dependence	LIF	ACTIVIN, bFGF
Teratomas	Yes	Yes
Chimeric contribution	Yes	No
Single-cell mortality	Low	High
XCI status in female cells	XaXa	XaXi
H3K27Me3 over developmental regulators	Low	High
Global DNA methylation	Hypomethylated	Hypermethylated
Response to LIF/STAT3	Self-renewal	None
Response to FGF/ERK	Differentiation	Self-renewal

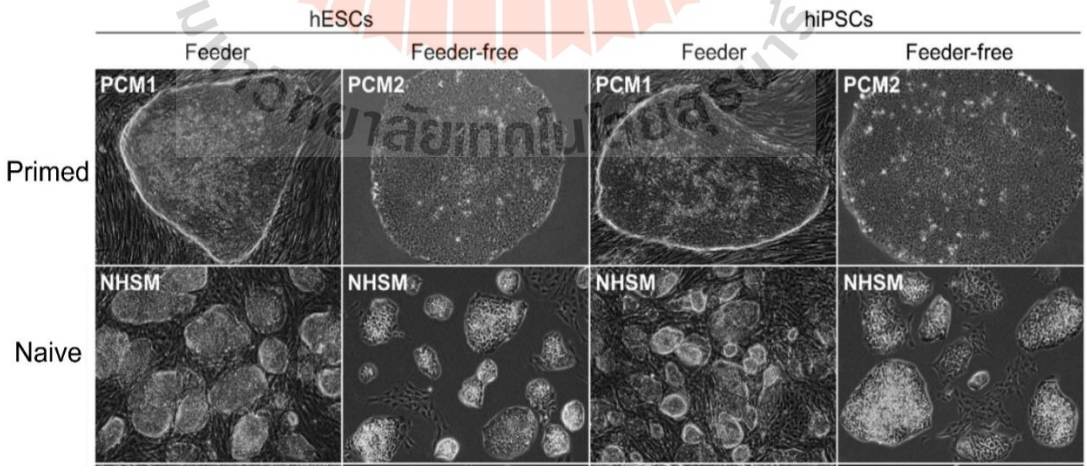

Figure 2.1 The differences in cell morphology between primed and naïve pluripotency of hPSCs cultured in feeder and feeder-free conditions with different converting media (Romayor et al., 2022).

2.4 Rhesus monkey pluripotent stem cells (rhESCs)

RhESCs were first derived from ICM cells of early blastocysts in 1995 (Thomson et al., 1995) These cells can grow on MEFs called “feeder cells” as undifferentiated states form flatter colonies (Thomson & Marshall, 1997). Normally, rhESCs exhibit characteristics of primed pluripotency depending on the activin A/TGFβ/SMAD2-3 and bFGF pathway for self-renewal (Singh et al., 2012; Tesar et al., 2007). bFGF appeared by inducing the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) and MAPK pathways (Hsieh et al., 2011; Lo et al., 2015), whereas the WNT/β-catenin pathway also is indirectly activated by bFGF (Wang et al., 2008). Using any growth factor in the medium is not necessary if they are cultured on feeder cells and LIF is not necessary for the maintenance of rhESCs (Osteil et al., 2016).

For rhESCs, they are positive for alkaline phosphatase activity, expressed the pluripotency includes OCT4 and NANOG transcription factors, SSEA-4, and TRA1-60 surface markers, and also displayed a normal karyotype (42XX or 42XY) (Fang et al., 2014). These cells performed to produce embryoid bodies *in vitro*, differentiated into 3 germ layers, and form teratomas *in vivo* including ectodermal, mesodermal, and endodermal by injection into immunodeficient mice (Xiang et al., 2019). Moreover, rhESCs can culture without feeder cells on a synthetic matrix, with either medium conditioned on mitomycin-treated MEFs or the addition of activin A (Ono et al., 2014).

2.5 Converting primed to naïve state of pluripotency

In particular, PSCs have been cultured either on MEFs feeder cells or in matrix-coated dishes with feeder-CM, occasionally supplemented with bFGF and KOSR. These conventional conditions are known to support the self-renewal of human PSCs in the primed state of pluripotency (Mazid et al., 2022). However, naïve pluripotent state is only found in mESCs derived from ICM of embryo at blastocyst stage. Specifically, the naïve pluripotent state represents a high potential for differentiation into any cell type and holds significant potential for medical research. Several reports describe protocols to generate the naïve state by manipulating Yes-associated protein (YAP), reprogramming with the expression of multiple genes through genome-integrating retroviral and lentiviral expression systems, and resetting transcription factors in the culture medium. (Qin et al., 2016; Takashima et al., 2014; Valamehr et al., 2014). This in turn raised the question of how cells could transition between naïve and primed states, with particular interest in the reverse transition from primed to naïve state. Differences between naïve and primed states pluripotency have been extensively studied, from culture conditions. During growth, the self-renewal of naïve

PSCs depends on LIF/signal transducer and activator of transcription (STAT3) signaling in contrast to primed PSCs, which depends on bFGF and activin A, but not LIF/STAT3 (Nichols and Smith, 2009).

In NHPs, PSCs in the primed state have been treated with resetting factors to instate naïve-type characteristics. Fang et al. (2014), pioneered the conversion of primed to naivety in the rhesus monkey (Macaca mulatta) using 4i/L/b medium, supplemented with growth factors (LIF and bFGF), as well as inhibitors PD0325901 (MEK), CHIR99021 (GSK3β), SB203580 (p38MAPK), and SP600125 (JNK). This resulted in the gradual transformation of the cell population into dome-shaped colonies, a characteristic of naïve cells (Fang et al. (2014). Subsequent studies focused on converting primed to the naïve state in cynomolgus monkey (Macaca fascicularis) using various culture media supplemented with different inhibitors, as detailed in Table 2.4.

Table 2.4 Converting of primed NHP PSCs to naïve state pluripotency.

Cell types	Culture medium	Conversion time (Days)	Naïve marker analysis	References
RhiPSCs	4i/L/b	7-10	*TBX3, FOXA2, NR5A2, PRDM14, LIFR, KLF5, REX1*	Fang et al., 2014
*CyESCs	NHSMV	3-4	*LIFR, STAT3, KLF4, GBX2*	Chen et al., 2015
CyESCs	NHSMV/2iLD/ K3cLD/ K5cLD	24	*GDF3, DNMT3L, DPPA5, TBX3, TFCP2L1, CDH1, KLF5*	Honda et al., 2017
**CjESCs	NBK5/2iLD	7-9	*ESRRB, DPPA3, KLF5, LEFTY1, LEFTY2*	Shiozawa et al., 2020
CjESCs	PLAXA	9	*KLF4, KLF17, TFAP2A*	Bergmann et al., 2022
CyESCs	4CL	12	*KLF17*	Li et al., 2023

* Cynomolgus monkey ESCs; CyESCs, **Common marmoset (Callithrix jacchus) ESCs; CjESCs

They have been reported to generate human and NHPs naïve PSCs by improving cocktail culture media with cytokine and growth factors such as LIF, activin A, Gö6983, bFGF, XAV939, and rho kinase inhibitor (ROCKi; Y-27632). (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Yang et al., 2016). Usually, the maintenance a ground state of naïve and regulated the transcription factors through important signal pathways as shown in Figure 2.2 including LIF, bFGF, TGF-beta/BMP, and WNT signaling. activin A has a role play for the maintenance of pluripotency by activating TGF-beta signaling via SMAD2/3 (Guzman et al., 2004). LIF is a small molecule that activates Janus kinase (JAK)/STAT3 signal through LIF signaling pathway. These are replacements for the MEFs to maintain the transcription factors of pluripotency (Niwa et al., 1988). Gö6983 is a small molecule for promoting the stability of pluripotency and stemness. These are involved with protein kinase C inhibitor (PKCi) which also directly affects to inhibits FGF signaling via the Raf/MEK/ERK pathway (Weinberge et al., 2016). XAV939 is a small molecule involved with tankyrase inhibitor (TNKi). They promote self-renewal and prevent their differentiation via WNT/ β -catenin signaling inhibitors (Guo et al., 2017). In addition, Y-27632 is a small molecule that is used to increase the survival of cells after single-cell dissociation in culture (Gauthaman et al., 2010).

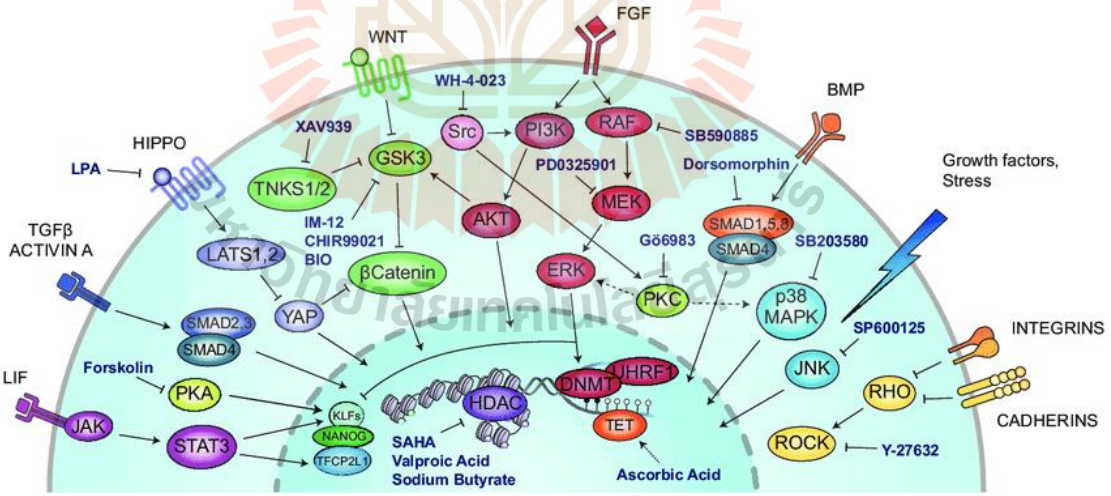

Figure 2.2 Signaling pathways in naïve and primed human pluripotent states. The different cocktails of activates/inhibitors and growth factors used to induce naïve pluripotency (Collier and Rugg-Gunn et al., 2018).

2.6 Epigenetic modification with small molecules for converting primed to naïve state of pluripotency

Epigenetic mechanisms are known to define cell-type identity and function, as well as induce changes in gene expression without altering the DNA sequence. These mechanisms include DNA methylation, histone modifications, and noncoding RNAs such as micro-RNAs and long noncoding RNAs (Basu & Tiwari, 2021). Converting primed to naïve state depending on the molecular mechanism and signaling pathway such as LIF/gp130/STAT3, FGF2/MEK/ERK and WNT/ β -catenin pathway (Zimmerlin et al., 2017). A small molecule is a tool that affects the activation or inactivation of signaling pathways. There are several reports using small molecules such as CHIR99021 and PD0325901 with LIF (2i/LIF) (Lynch et al., 2020) and PD0325901, XAV939, CHIR99021 with LIF (3i/LIF) and also PD0325901, CHIR99021, Gö6983 with LIF (2iLGö) (Guo et al., 2017) and promoted naïve-like ground state via WNT inhibition signaling (Zimmerlin et al., 2016). Moreover, epigenetic manipulation is an efficient alternative method for converting primed to the naïve state. Notably, histone deacetylation inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) were considered a tool of reset primed EpiSCs into naïve ESCs by reconfiguration of the epigenome (Takashima et al., 2014). Some reports using valproic acid (VPA) or sodium butyrate that are small molecules react as HDACi associated which can resetting primed to naïve and displaying naïve marker gene expression and other reports displayed small molecules DNMTi associated such as 5-aza-cytidine, 5-aza-2′-deoxycytidine (5-aza-dC), zebularine, and ascorbic acid (Nakamura et al., 2015).

2.7 CMs production for converting PSCs

From therapeutic application, a cell-free approach based on CM offers several benefits over stem cell-based treatments. Generally, PSCs grow stably on feeder MEFs, which secrete extracellular matrix (ECM) components and growth factors necessary for the growth of undifferentiated cells. However, this system is too difficult to apply in medical applications because it contains other cell types and various molecules released from the feeder MEFs. Xu and colleagues pioneered the growth of PSCs on a feeder-free system using a complex mixture of matrixes such as matrigel, vitronectin, and laminin (Xu et al., 2001). The development of feeder-free systems by producing CM is substantially useful for reducing contamination from mice. Currently, several studies report using feeder-free systems for research and applications in regenerative medicine and drug discovery. (Kanatsu-Shinohara et al., 2011; Nakagawa

et al., 2014; Szczerbinska et al., 2019). Recently, CM can be considered a promising pharmaceutical product in regenerative medicine (Kuo et al., 2021).

2.7.1 CMs derived from MEFs

Conventionally, hESCs were co-cultured with mitotically inactivated MEFs feeder cells, which secretion of essential growth factors, cytokines and ECM components necessary for the growth and maintenance of undifferentiated cells (Villa-Diaz, Ross, Lahann, & Krebsbach, 2013). Due to, the use of MEFs in cell culture may expose the cells to animal pathogens, which is not suitable for therapeutic applications involving human stem cells. Several reports describe different media used to produce MEFs-CM for expand hPSCs. The media are variously termed term, for example, culture medium consist with 15% serum replacement and a combination of growth factors with β1 (TGFβ1), leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), (Amit, Shariki, Margulets, & Itskovitz-Eldor, 2004), fibroblast media as consist DMEM supplemented with 10% FBS and bFGF (Tomishima, 2014), and MEF medium as consist of DMEM supplemented with 10% FBS, 1%NEAA, and 1 mM L-glutamine (Li et al., 2017). In addition, several studies have reported using MEFs-CM in protocols for converting primed PSCs to a naïve state in humans (Isono et al., 2023), mice (Kime et al., 2016), and non-human primates (Chen et al., 2015; Aksoy et al., 2021). These demonstrate that PSCs cultured under feeder-free conditions can be effectively maintained, thereby supporting their use in regenerative medicine.

2.7.2 CMs derived from WJ-MSCs

Over the past three decades, MSCs have become the most frequently used stem cell population in the field of cellular therapy and tissue engineering. Cell-free therapy based on CM derived from MSCs (MSCs-CM) has gained attention in the field of protective and regenerative medicine. In fact, MSCs are multipotent stem cells that can be isolated from different sources, including bone marrow, adipose tissue, dental pulp, umbilical cord, placenta, and amnion. In order to apply human PSC, for regenerative medicine, it should be concern about pathogen contamination before therapeutic application. Several reported successfully produced of CMs from MSCs derived from placenta (Ji et al., 2021), adipose-tissue (Park, 2021), human umbilical cord (Sriramulu et al., 2018), bone marrow (Saleem et al., 2021) and Wharton's jelly (Fong et al., 2014). These can produce various secreted factors like cytokines, growth factors and chemokines, which supporting self-renewal of PSCs.

CHAPTER III
MATERIALS AND METHODS

3.1 Ethics statement and reagents

This study used hWJ-MSCs of which was approved by the Ethics Committee for Research Involving Human Subjects, Suranaree University of Technology (EC-66-73). Chemicals used in this research were purchased from Sigma-Aldrich Corporation. The cells culture medium was purchased from Gibco and plastic cell culture devices were procured from SPL life sciences (Gyeonggi-do, Republic of Korea).

3.2 Primed rhESCs culture and passage

Primed rhESCs were obtained from Yerkes National Primate Research Center, Atlanta, GA30329, United States. The cells were cultured with the standard method as previously reported (Putkhao et al., 2013). Briefly, the cells were grown on mitomycin-C treated OF1-MEFs at the density of $2x10^5$ cells in ESC medium which consists of KnockoutTM DMEM (KO-DMEM; Gibco, 10820-010) supplemented with 20% Knockout serum replacement (KOSR; Invitrogen, 10820-010), 1x MEM non-essential amino acids (NEAA, Invitrogen, 21114050), 4 ng/mL bFGF (Sigma, SRP3043), 1% Glutamax (Invitrogen, 35050-061) and 0.1% of 10,000 units/mL penicillin and 10,000 g/mL streptomycin (P/S; Lonza Bioscience, 17-602E). The cells were incubated at 37°C under humidified atmosphere of 5% CO_2 in air. The cell culture medium was changed after 3 days of culture, and then it was changed every day thereafter. When the cells had grown, colonies with good morphology (flat colonies, clear-edged colonies, and homogenous cells) were passaged and expanded by being mechanically cut into small cell clumps using capillary needle. The cell clumps were then plated on the top of feeder layers (OF1-MEFs). The medium was changed every day and the passage was made every 5 - 6 days. Then, the pluripotency and primed characteristic were analyzed by ICC and RT-qPCR using specific pluripotency markers (OCT4, NANOG and SOX2) and primed markers (TBXT and OTX2) respectively.

3.3 Feeder cell preparation

OF1-MEFs used in this study were obtained from INSERM, Stem Cell and Brain Research Institute U1208, F-69500 Bron, France. Passage 4 of OF1-MEFs were thawed and cultured on 100 mm culture dish in standard culture medium (OF1 medium) as previously described by Aksoy et al., 2021. The OF1 medium consisting of 1x DMEM supplemented with 10% FBS, 1% penicillin-streptomycin-glutamine (PSG; Invitrogen, 10378-016) and 1% NEAA. Next, the cells were cultured for 3-4 days, depending on the confluent of cells. When the cells reach 80-85% confluent, the cells were treated with 5 mL of MMC medium (OF1 medium supplemented with 5 μg/mL mitomycin-C; MMC) and then incubated at 37°C under humidified atmosphere of 5% CO_2 in air for 3 h. After that, the medium was removed and washed three times with 10 mL of phosphate buffer saline (PBS(-)). Then 1 mL of 1x trypsin was added to a dish and incubated for 5 min at 37°C. After incubation, the complete medium (α-MEM, 10% FBS, 2mM L-glutamine and 0.1% P/S was added into culture dish to stop the enzymatic reaction and pipette cells suspension into 15 mL conical tube containing 5 mL of complete medium. After that, the cells were centrifuged at 1,200 rpm for 5 min, then removed supernatant and resuspended the cell pellet in 1 mL of OF1 medium. Ten μL of cells suspension were counted using a hematocytometer under an inverted microscope and then the cells were plated onto gelatin-coated dishes which was previously prepared by added 1 mL of 0.1% gelatin coated on a 35 mm culture dish before use for 15 min. The gelatin was removed and then transferred 1 mL of cells suspension onto 35 mm culture dish at a density of $2x10^5$ cells and cultured in OF1 medium. The cells were incubated overnight at 37°C under humidified atmosphere of 5% CO_2 in air before used for 24 h.

3.4 Preparation of CMs

3.4.1 CM produced from OF1-MEFs

OF1-MEFs were thawed and cultured on 100 mm culture dish with OF1 medium. When cells have grown 85% confluence, cells were treated with 5 mL of MMC and then incubated at 37°C under humidified atmosphere of 5% CO_2 in air for 3 h. The OF1-MEFs were then passaged, counted, and seeded at a concentration of approximately $4x10^6$ cells on a 100 mm culture dish, allowed to reach 70% to 80% confluence. The medium was then changed to 25 mL N2B27 basal medium, included 50% Neurobasal medium (Invitrogen, 21103-049), 50% DMEM-F12 (Invitrogen, 21331-020), 1% PSG, 1% B27 supplement (Thermo Fisher Scientific, 17504-044), 0.5% of N2 (homemade) and 0.01% of β-mercaptoethanol, supplemented with 20 ng/mL bFGF,

and the cells were cultured for 72 h. Finally, CM was collected and filtered with 0.22 μm, and stored at -20°C. These CM which produced from OF1-MEFs regimen are hereafter referred to as OF1-MEFs-CM.

3.4.2 CM produced from hWJ-MSCs

3.4.2.1 hWJ-MSCs isolation and expansion

The human umbilical cords were obtained from Maharat Nakhon Ratchasima Hospital (Nakhon Ratchasima, Thailand) with mother's informed consent. The cords, which were approximately 7 - 10 cm in length, were washed with PBS (-) then hWJ-MSCs were isolated from the umbilical cord and cultured as previously described by Tanthaisong et al. (2017). Briefly The gelatinous Wharton's Jelly tissues were collected and sliced into small pieces. These pieces were placed in 100 mm culture dishes and grown in alpha modification of Eagle's medium (α-MEM), enriched with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS, will hereafter be referred to as complete medium. The MSCs were expanded until passage 3, cryopreserved with 10% dimethyl sulfoxide (DMSO) in culture media, and then stored in liquid nitrogen.

3.4.2.2 hWJ-MSCs culture for CM preparation

hWJ-MSCs were thawed and cultured in standard culture medium as previously described by Somredngan et al., 2023. Briefly, passage 4 of hWJ-MSCs were cultured on 100 mm culture dish in complete medium. When cells have grown 85% confluence, cells were treated in complete medium supplemented with 5 mL complete medium supplemented with MMC and then incubated at 37°C under humidified atmosphere of 5% CO_2 in air for 3 h. The hWJ-MSCs were then passaged, counted, and seeded at a concentration of approximately $3x10^6$ cells on a 100 mm culture dish, allowed to reach 70% to 80% confluence. The medium was then changed to 25 mL N2B27 basal medium the same as OF1-MEFs-CM production, and the cells were cultured for 72 h. Lastly, CM was collected and filtered with 0.22 μm, and stored at -20°C. These CMs which produced from hWJ-MSCs regimen are hereafter referred to as hWJ-MSCs-CM.

3.5 Converting of primed rhESC to naïve state

The primed rhESCs were converted on feeder-free condition using laminin521 coated dished. Firstly, the cells were selected a good colony and cut into a small clumps cell using mechanical passage as described in section 3.2. After that clump of cells was put into 15 mL conical tube and washed the cells once with PBS (-) and then cells were centrifuged at 1,000 rpm for 5 min. Subsequently, PBS (-) was

removed and cells were dissociated into single cells using 1 mL of 1× of TrypLE™ (Invitrogen, A12177-01), incubated at 37°C, 5% CO_2 for 5 min. The dissociated cells were then seeded onto a dish coated with 5 g/mL of laminin521 in the conversion medium cocktail VALGöX, which consisting of either hWJ-MEFs-CM or OF1-MEFs-CM supplemented with ascorbic acid, activin A, LIF, Gö6983 and XAV939. Then the cells were cultured at 37°C, 5% O_2 under humidified atmosphere of 5% CO_2 in air. The medium was changed daily, and cells were passaged every 2-3 days. On Day 9, the medium was switched to ALGöX (removed ascorbic acid), and the cells were cultured until Day 21. Lastly, protein expression was investigated at day 9 and day 21 using ICC with pluripotent markers, naïve markers, and epigenetic markers. Additionally, gene expression was examined using RT-qPCR. The schematic diagram of converting primed to the naïve state of rhESCs is shown in Figure 3.1

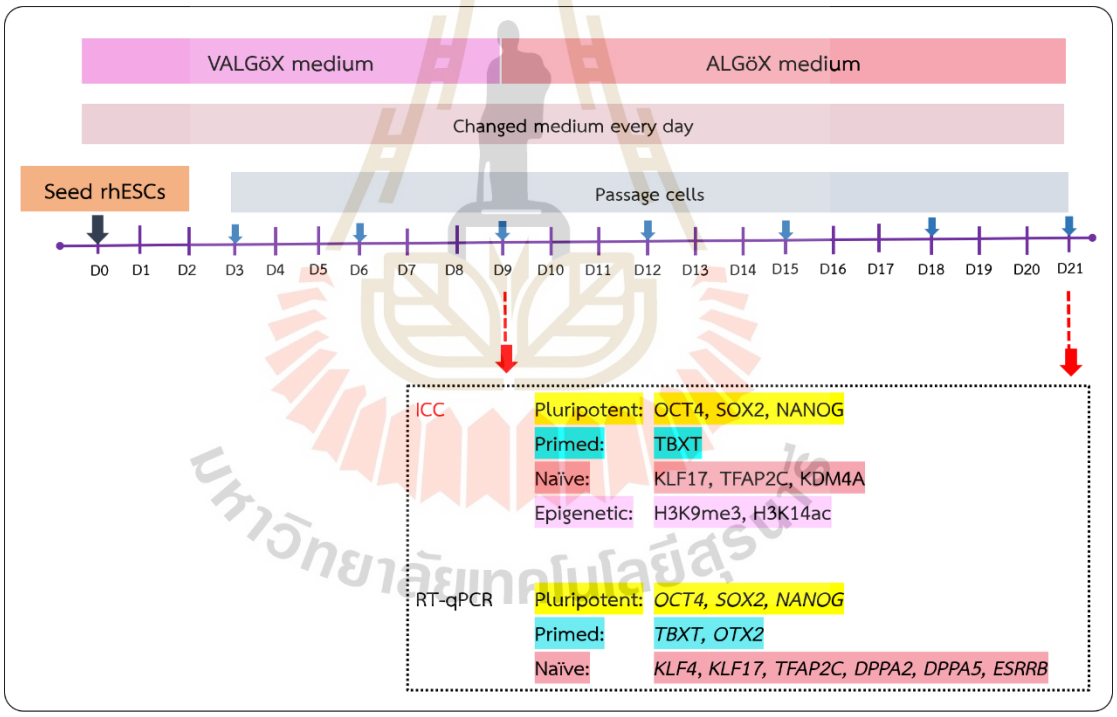

Figure 3.1 Schematic diagram of converting primed to the naïve state of rhESCs.

3.6 Analysis of protein expression using Immunocytochemistry (ICC)

Primed colonies and naïve-like cells were seeded on glass cover slips with diameter 15 mm (Thermo Fisher Scientific) on OF1-MEFs feeder cells at density $5x10^4$ cells on gelatin-coated 4 wells plate before staining. Cell colonies were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). Then the cells were

washed three times for 5 min in PBS (-) at RT and permeabilized in 0.5% Triton X-100 for 30 min and then washed with PBS (-) 3 times for 10 min of each. After washing cells in PBS (-), cells were kept in blocking solution 2% bovine serum albumin (BSA) for 1 h at RT and incubated overnight at 4°C with primary antibodies (Table 3.1). For the preparation of primary antibodies, it was added in 2% BSA with 1/200 dilution. After incubation overnight, primary antibody was removed and washed with PBS (-) 3 times and hold in PBS (-) for 10 min. Then the cells were incubated with secondary antibodies (anti-Rabbit Alexa Fluor 488, anti-Goat Alexa Fluor 594 and anti- Sheep Alexa Fluor 488) in 2% BSA with 1/500 dilution at room temperature for 1 h in dark condition. After that secondary antibody was removed and washed with PBS (-) 3 times and hold in PBS (-) for 10 min. The cells were stained with DAPI in PBS (-) with 1/5000 dilution and also mounted with mounting medium prolong antifade (Invitrogen, P10144). Then the cells were observed under fluorescence inverted microscope (Eclipse TE 300, Nikon Imaging Japan Inc.) with NIS-Elements D program (Nikon Imaging Japan Inc., Tokyo, Japan). Fluorescent intensities were quantified using Basic Intensity Quantification with FIJI software.

3.7 Analysis of gene expression using RT-qPCR

Total RNA was isolated using FavorPrep TM Tissue Total RNA Mini Kit (Favorgen Biotech Corp., Taiwan). RT-qPCR analysis was performed with KAPA SYBR ® FAST qPCR Master Mix (Kapabio systems, USA). The gene expression analysis was performed using QuantStudio™ 5 Real-Time PCR System in the following conditions: a hot start at 95°C for 3 min followed by 40 cycles (denaturation at 95°C for 15 sec, annealing for 30 sec and elongation at 72°C for 30 sec and finalized by elongation at 72°C for 5 min. Melting curve analysis was also performed to the specificity of the specific primers (Table 3.2). Data were analyzed using Microsoft excel according to $2^{-\Delta\Delta Ct}$ method and values were normalized to the housekeeping gene (*GAPDH*).

3.8 Statistical Analysis

GraphPad version 5 (GraphPad Software, San Diego, CA, USA), was used for statistical analysis and the data performed as the mean ± S.D. Data were analyzed using one-way ANOVA followed by Tukey's test for multiple comparisons. Statistical significance was considered at $p<0.05$. All experiments were analyzed at least in triplicate.

Table 3.1 List of primary antibody and dilution used for ICC analysis.

Type of markers	Primary antibody	Dilution	Company Cat. No
Pluripotency markers	Rabbit anti-OCT4	1:200	Invitrogen U.S.A. PA116943
	Rabbit anti-NANOG	1:200	Cell signaling 4903S
	Goat anti-SOX2	1:200	R&D systems AF2018
Primed specific markers	Rabbit anti-TBXT	1:200	Cell signaling 81694S
Naïve specific markers	Goat anti-TFAP2C	1:50	R&D system AF5059
	Rabbit anti-KLF17	1:200	Sigma-Aldrich HPA024629
	Sheep anti-KDM4A	1:50	R&D system AF6434
Epigenetic markers	Rabbit anti-H3K9me3	1:200	Abcam ab8898
	Rabbit anti-H3K14ac	1:200	Abcam ab52946

Table 3.2 Primer sequences and annealing temperatures used for RT-qPCR analysis.

Gene type	Gene	Primer Sequence (5'-3')	Annealing temperature
Housekeeping gene	*GAPDH*	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	54
Pluripotency genes	*OCT4*	F: ATGTGGTCCGAGTGTGGTTC R: AGCGGCCTCAAAATCCTCTC	57
	NANOG	F: AGTCCTGCTTGCAGTTCCAG R: TCAGGTTGCATGTTCGTGGA	55
	SOX2	F: TCTTGGTTCCATGGGTTCGG R: CTGGAGTGGGAGGAAGAGGT	57
Primed specific genes	*TBXT*	F: CCTTCAGCAAAGTCAAGCTCACC R: TGAACTGGGTCTCAGGGAAGCA	56
	OTX2	F: CAAAGTGAGACCTGCCAAAAAGA R: TGGACAAGGGATCTGACAGTG R: TGAGCGTCACAAACTCCTCC	56

Table 3.2 Primer sequences and annealing temperatures used for RT-qPCR analysis. (Continued)

Gene type	Gene	Primer Sequence (5'-3')	Annealing temperature
Naïve specific genes	*TFAP2C*	F: GTTCTCAGAAGAGCCAAGTCG R: TCGGCTTCACAGACATAGGC	56
	KLF17	F: CCTTACCGCTGCAACTACGA R: ATAGGGCCTCTCACCTGTGT	57
	KLF4	F: CCCTACCTCGGAGAGAGACC R: GGATGGGTCAGCGAATTGGA	58
	DPPA2	F: GTACGCCTGCAGTTTCATGC R: TCTATGCCTGGGGATGGGAA	55
	DPPA5	F: GGTCGTGGTTTACGGTTCCT R: AGTTTGAGCATCCCTCGCTC	56
	ESRRB	F: TGCCCTATGACGACAAGCTG R: TGAGCGTCACAAACTCCTCC	58

CHAPTER IV
RESULTS

4.1 Primed rhESCs culture

RhESCs were cultured and expanded on OF1-MEFs feeder cells using ESC medium. The primed rhESC colonies had well-defined boundaries, displayed a flat shape with clear edges, and exhibited compactness similar to the morphology of rhESCs in previously reported (Mitalipov et al., 2006). The healthy colonies were isolated and passaged onto fresh feeders manually for an additional 5-10 passages to generate sufficient cell numbers for cryopreservation and characterization (Figure 4.1A). RhESCs expressed key pluripotent markers, including OCT4, NANOG, SOX2 and TBXT as detected by ICC. Note that only colonies of rhESCs tested positive for pluripotency markers, whereas feeder cells tested negative (Figure 4.1B).

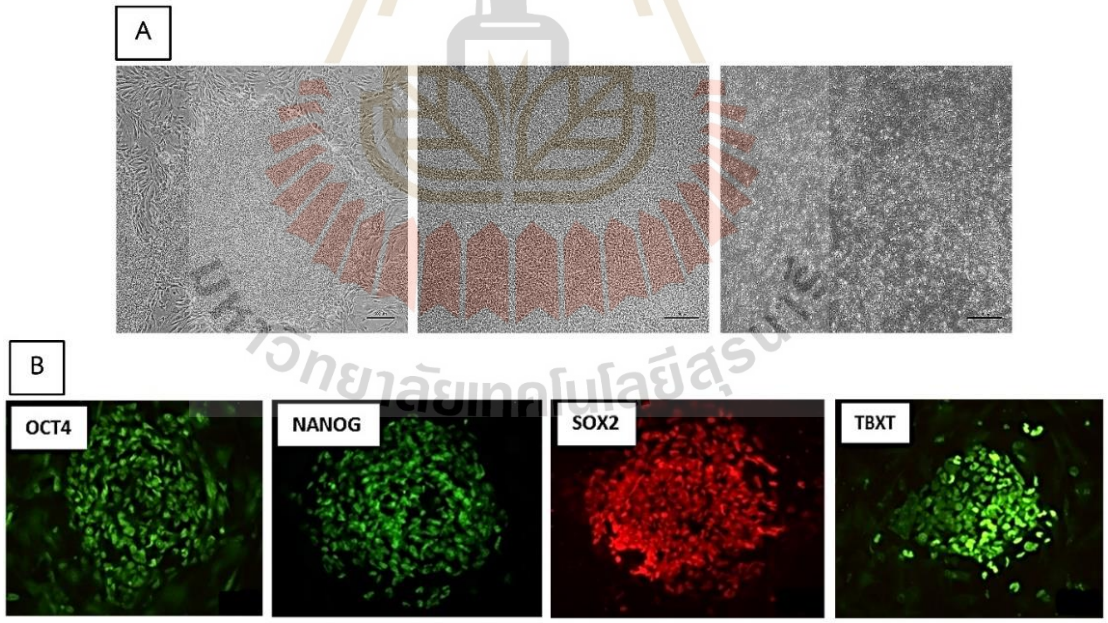

Figure 4.1 The morphology of primed rhESCs cultured on OF1-MEFs feeder cells is shown at magnifications of 4X (left), 10X (middle), and 20X (right) in (A). Pluripotency markers detected by ICC are shown at 20X magnification in (B).

4.2 Conversion primed rhESCs to naïve state in VALGöX medium

To study and compare the features of naïve-like cells, we treated primed rhESCs using two different base CMs (OF1-MEFs-CM and hWJ-MSCs-CM) supplemented with small molecules cocktails. The conversion of primed rhESCs was carried out for 21 days on feeder-free condition. To compare the conversion of primed rhESCs to a naïve state between OF1-MEFs-CM and hWJ-MSCs-CM, primed rhESCs colonies were dissociated into small clump cells and cultured for the first 9 days in converting medium included ascorbic acid (Vit C), LIF, activin A, PKC inhibitor (Gö6983), and tankyrase inhibitor (XAV939) which are hereafter referred to as VALGöX medium. After this initial period, the cells were cultured in ALGöX medium. The results showed differences in colony morphology, with spread colonies observed on day 9 and day 21 (Figure 4.2). The size of nucleolus of colonies of the converted cells in OF1-MEFs-CM was smaller than that the converted cells in hWJ-MSCs-CM.

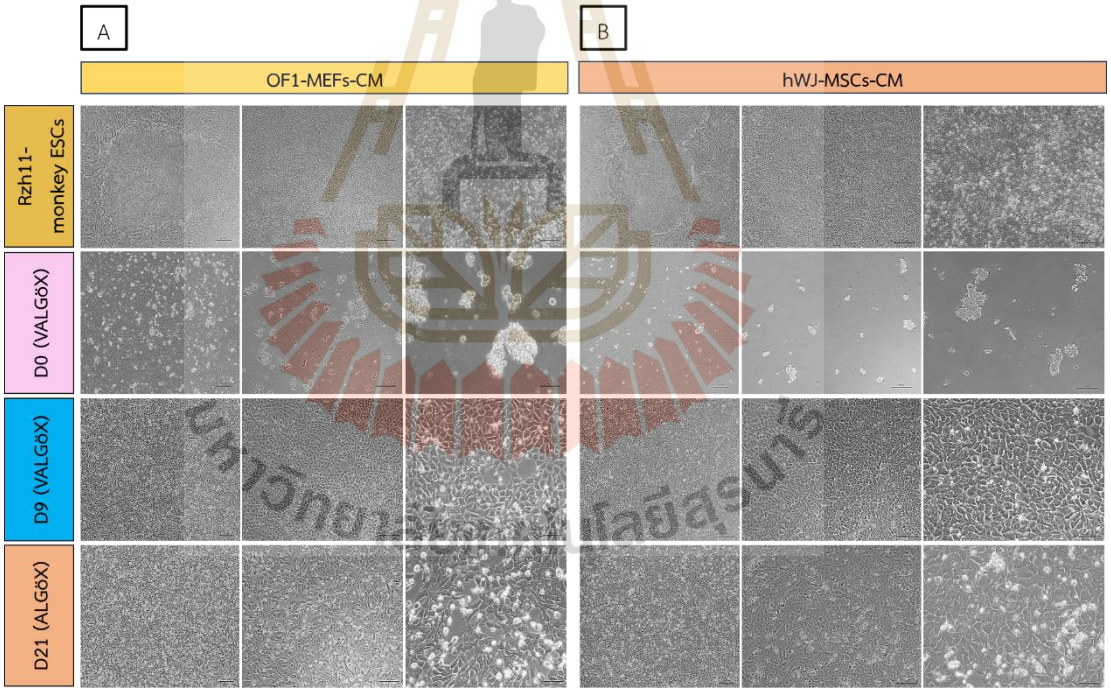

Figure 4.2 The difference of morphology during conversion primed to naïve state of rhESCs in different CMs is shown using (A) OF1-MEFs-CM and (B) hWJ-MSCs-CM at magnification 4X (left), 10X (middle), and 20X (right)

4.3 Primed and naïve-like cells characterization

Subsequently, we assessed the pluripotency status by performing ICC and RT-qPCR to detect several pluripotency markers in both primed and naïve state. The primed rhESCs were used as the control group to compare with naïve-like cells. The results revealed that primed rhESCs lines presented of all positive pluripotency markers. (OCT4, NANOG, and SOX2) and strongly expressed a specific primed marker, TBXT (Figure 4.3).

In terms of the comparison of ICC staining, the conversion of primed rhESCs to naïve-like cells was assessed while culturing them in OF1-MEFs-CM and hWJ-MSCs-CM in VALGöX medium for the first 9 days, followed by a switch to ALGöX medium. The results showed that the core pluripotency markers, OCT4 and NANOG, were expressed at lower levels in the converted cells of both CMs compared to the control group on both day 9 and day 21. In contrast, SOX2 was expressed at higher levels than the control group in the converted cells of OF1-MEFs-CM on day 9 and hWJ-MSCs-CM on day 21. Remarkably, the naïve-like cells in both CMs showed a significantly lower expression of TBXT compared to the control group (Figure 4.3). Regarding, naïve markers, including TFAP2C and KLF17, were more highly expressed in the hWJ-MSCs-CM condition on day 21 compared to the control group. In contrast, the results showed that KDM4A markers were highly expressed in the converted cells in both CMs on day 9 and day 21 compared to the control group (Figure 4.4). Moreover, we observed that cells viability decreases in both the OF1-MEFs-CM and hWJ-MSCs-CM conditions on day 21, with a more pronounced decrease in the culture medium without ascorbic acid (ALGöX).

For the ICC of epigenetic markers. We then examined two different epigenetic marks, permissive (H3K14 acetylation) and repressive (H3K9 trimethylation), in primed rhESCs converted in VALGöX medium by using OF1-MEFs-CM and hWJ-MSCs-CM to evaluate their epigenetic reconfiguration upon conversion process. All two epigenetic markers could be detected with the expected nuclear distribution (Figure 4.5A and B). We observed a decrease in the protein level of the H3K9 trimethylation in all cells that converted with VALGöX medium compared with control group, which was confirmed by imaging and quantification (Figure 4.5C). This result indicated that a reduction in methylation on histone proteins, consistent with the characteristics of a naïve state. H3K14 acetylation was strongly expressed in all cells that converted with VALGöX medium compared with control group, suggesting an increased in acetylation on histone protein, consistent with the characteristic of naïve state. Our result suggest

that the cells converted with VALGöX medium have improved the epigenetic reprograming, making them suitable for transitioning from primed to naïve state.

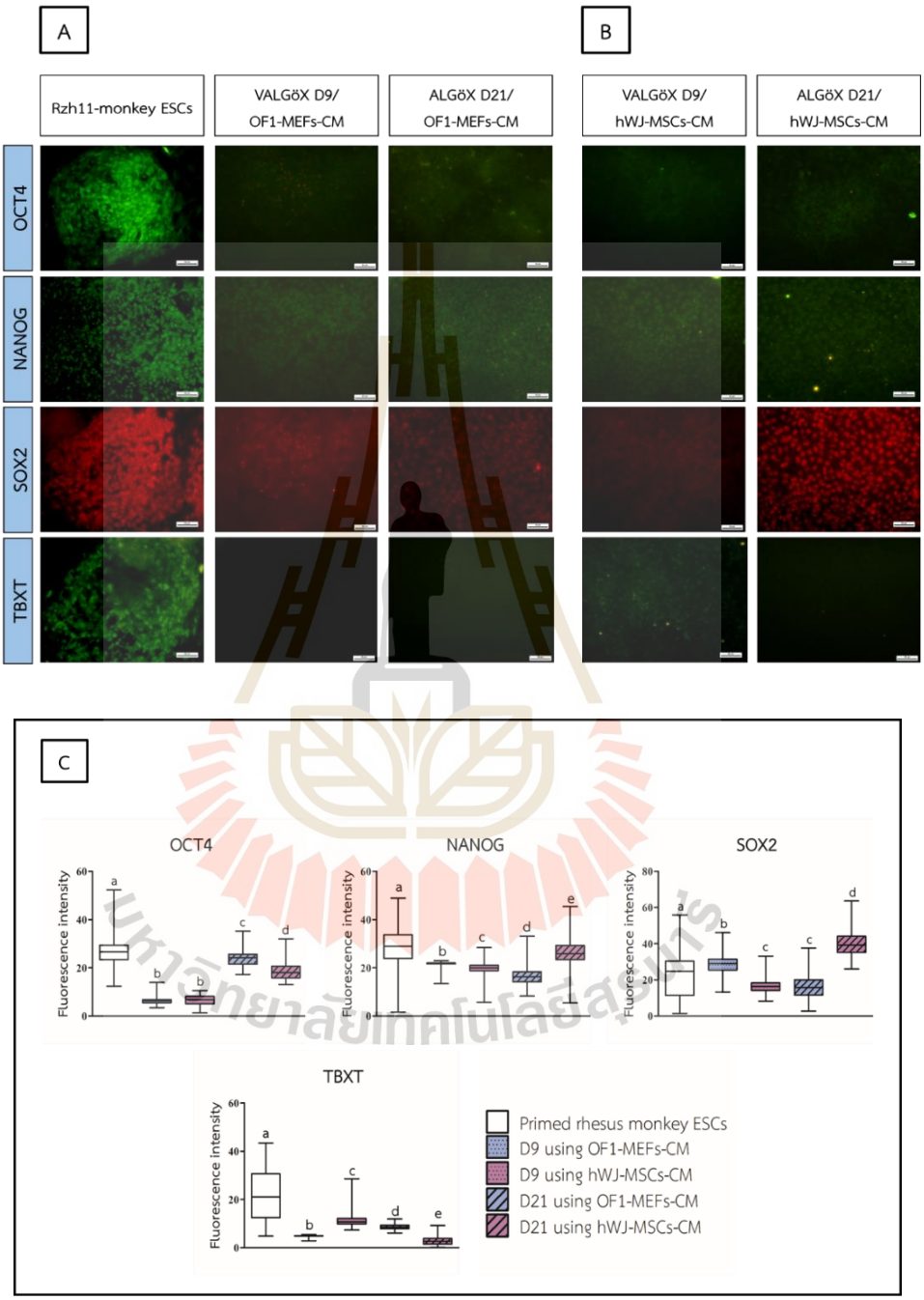

Figure 4.3 ICC imaging of pluripotency markers and primed marker during conversion from primed to naïve state of rhESCs between (A) OF1-MEFs-CM and (B) hWJ-MSCs-CM. (C) Intensity analysis. Data were shown as mean ± S.D. with different lower-case letters, and are significantly different at $p < 0.05$.

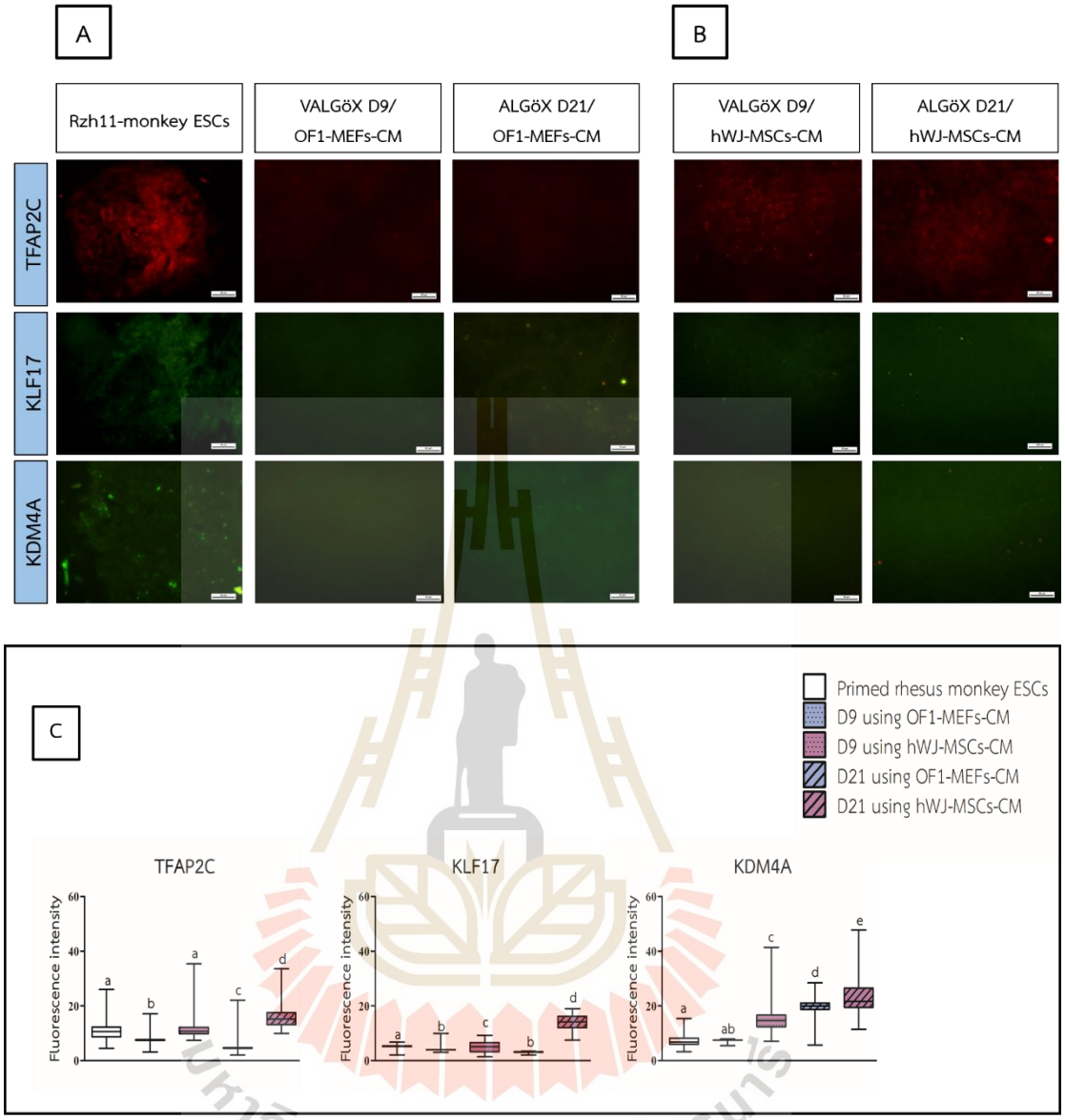

Figure 4.4 ICC imaging of naïve markers during conversion from primed to naïve state of rhESCs between (A) OF1-MEFs CM and (B) hWJ-MSCs CM. (C) Intensity analysis. Data were shown as mean ± S.D. with different lower-case letters, and are significantly different at $p < 0.05$.

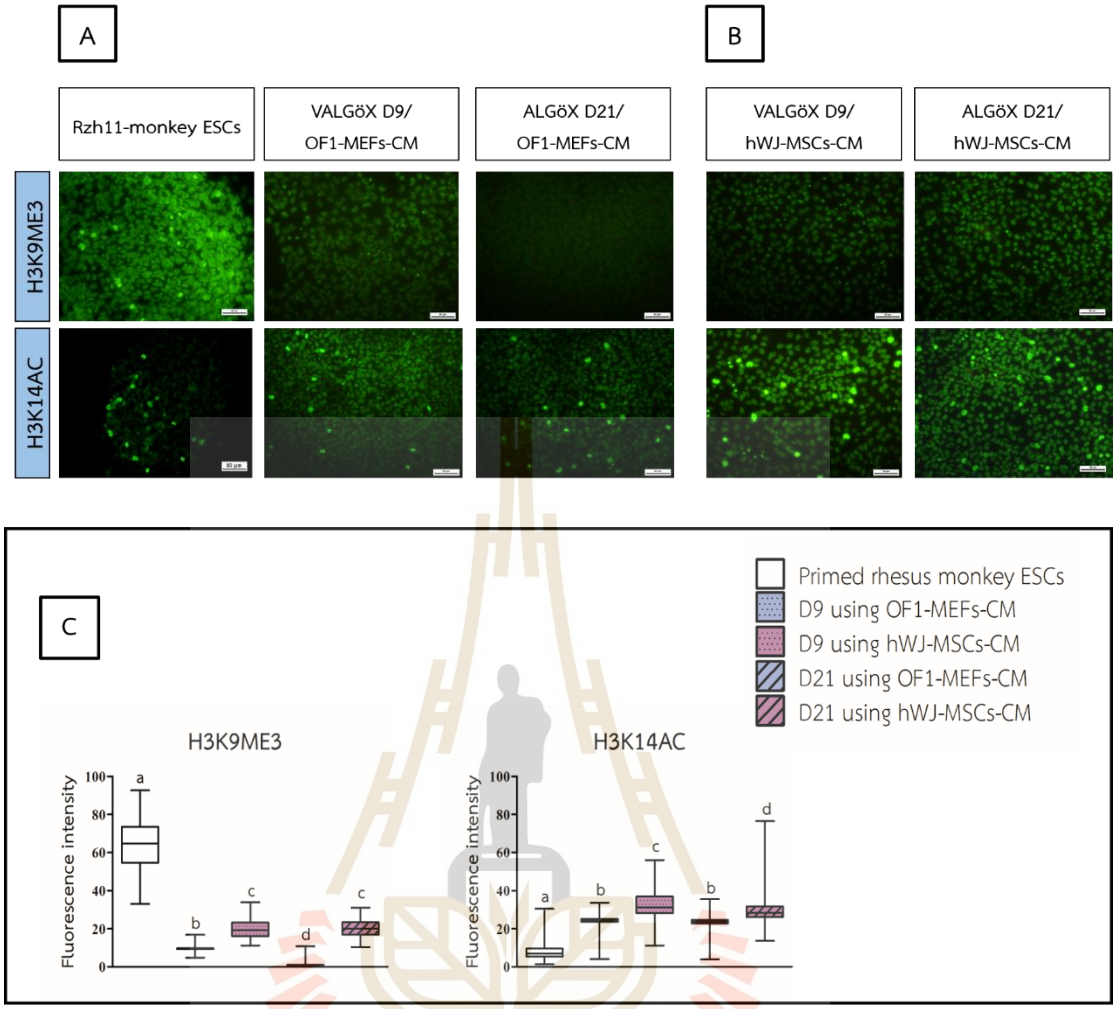

Figure 4.5 ICC imaging of epigenetic markers during conversion from primed to naïve state of rhESCs between (A) OF1-MEFs CM and (B) hWJ-MSCs CM. (C) Intensity analysis. Data were shown as mean ± S.D. with different lower-case letters, and are significantly different at $p < 0.05$.

Considering the RT-qPCR data, pluripotent markers were expressed, indicating the continued pluripotency of the stem cells, as well as the expression of primed markers (Figure 4.6A). Regarding the results of pluripotent markers, we found that *SOX2* was expressed at higher levels in the converted cells compared to the control at both day 9 and day 21 in both CMs, whereas *OCT4* and *NANOG* were decreased. However, primed markers, including *TBXT* and *OTX2*, exhibited lower expression compared to the control group (Figure 4.6A).

With the comparison of naïve markers expression between the conversion in OF1-MEFs-CM and hWJ-MSCs-CM. Our results found that hWJ-MSCs-CM conversion at day 9 and day 21 exhibited higher levels of all naïve markers including *KLF4*, *KLF17*, *ESRRB*, *TFAP2C*,

DPPA2, and *DPPA5* compared to control group. However, some markers, including *KLF4*, *KLF17*, *TFAP2C*, and *DPPA2*, showed decreased expression on day 21. In contrast, the conversion using OF1-MEFs-CM exhibited significantly higher levels of *DPPA5* compared to hWJ-MSCs-CM on both day 9 and day 21 (Figure 4.6B). These results suggest that the efficiency of each conditioned medium varies in terms of the expression of naïve markers at different stages.

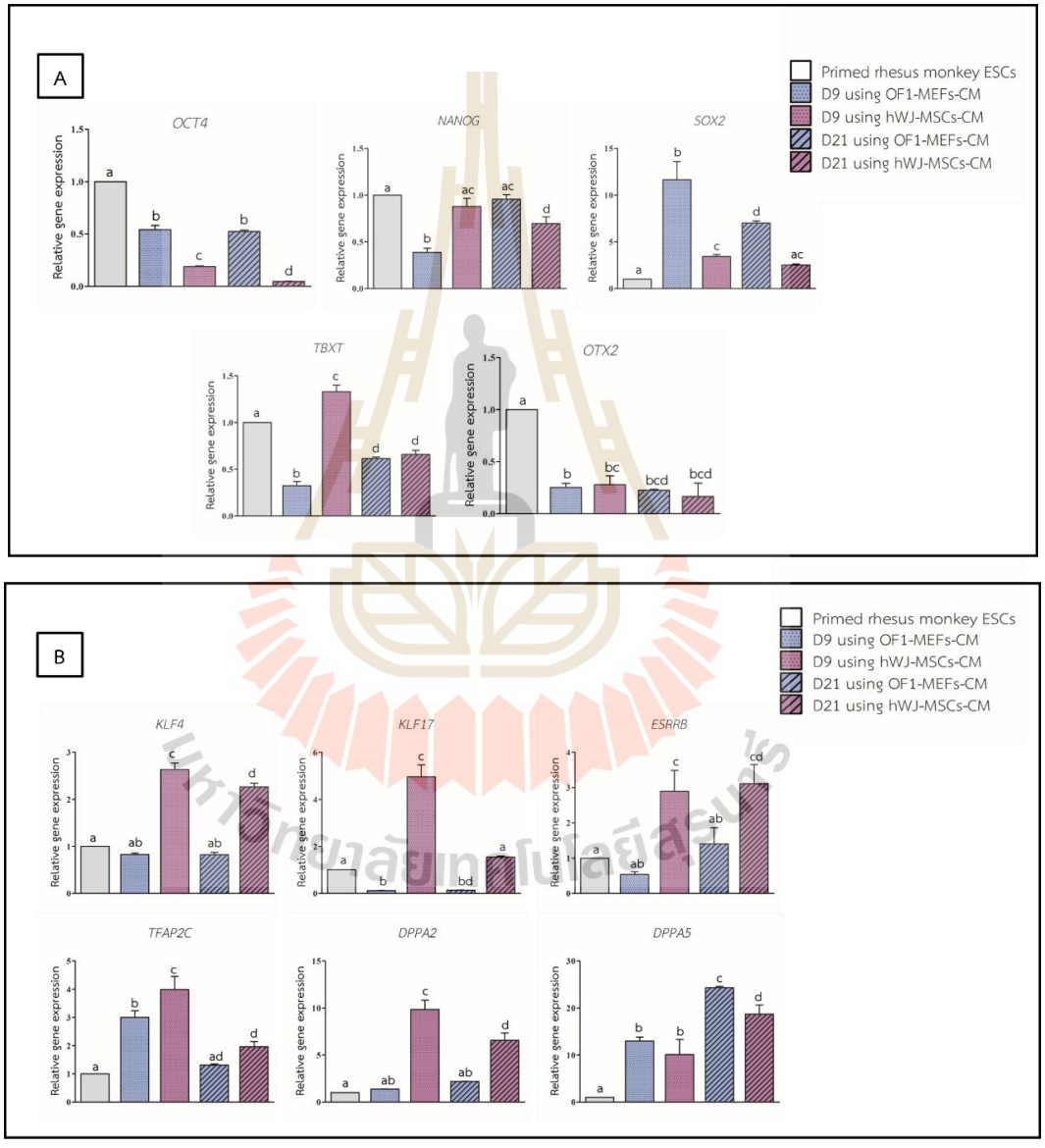

Figure 4.6 Gene expression analysis during conversion from a primed to a naïve state of rhESCs between (A) OF1-MEFs CM and (B) hWJ-MSCs CM. The target gene was normalized to *GAPDH* as a reference gene and calculated the relative expression compared with each group. Data were shown as mean ± S.D. with different lower-case letters, and are significantly different at $p < 0.05$.

CHAPTER V
DISSCUSSION

In this study, we produced and compared the effectiveness of CMs from OF1-MEFs, known as a gold standard, and hWJ-MSCs for converting rhESCs from primed to naïve state. Here, we attempted to manufacture hWJ-MSCs-CM preparation, which is aimed for useful in medical applications in the future. In establishing robust standards for the production of particularly culture media, this study used hWJ-MSCs which were characterized by standard method in our laboratory before their utilization in CM production (Somredngan et al., 2023). The CMs were prepared using N2B27 medium, which supports cell proliferation and self-renewal in ESC cultivation (Ying et al., 2008) and has been used several times as a base medium for naïve PSCs culture (Guo et al., 2016; Qin et al., 2016; Rostovskaya et al., 2019). However, previous reports have identified different culture media for preserving strong pluripotency markers and achieving stable ground-state pluripotency. (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Yang et al., 2016). Here, we converted primed rhESCs to naïve state carried out using N2B27 based CM supplemented with cocktail of small molecules and growth factors including ascorbic acid (Vit C), LIF, activin A, PKC inhibitor (Gö6983), and tankyrase inhibitor (XAV939) under feeder-free cells condition. In the conversion process, primed rhESCs were firstly cultured in ALGöX with ascorbic acid (VALGöX) for 9 days, then switched to ALGöX for 12 days. When comparing the morphology of converted cells under feeder-free conditions, colonies of converted cells at day 9 and day 21 tend to spread out more and form fewer compact colonies compared to the more defined and compact morphology see in feeder-dependent cultures. This difference is likely due to the lack of feeder cells that provide extracellular matrix components and growth factors essential for maintaining the tight, colony-like structure (Moody, 2013; Stover & Schwartz, 2011). Moreover, we observed that the size of nuclei of converted cells in hWJ-MSCs-CM was bigger than that in OF1-MEFs-CM. This evidence might suggest that hWJ-MSCs-CM is a rich source of chemokines and trophic factors that can support self-renewal and cell proliferation (Liang et al., 2021).

Subsequently, the cells were evaluated the characteristics of naïve-like cells using ICC and RT-qPCR. Here, we reported that able to generate CM from human

tissue and to investigate the efficiency of this culture medium that could be used to reprogram primed cells into naïve-like cells of rhESCs on feeder-free conditions. In terms of ICC analysis, we observed that converted cells during the second period on day 21 in hWJ-MSC-CM indicating a desirable transition to the naïve state during conversion, with had high expression of naïve markers including TFAP2C, KLF17, and KDM4A (Table 5.1). Regarding, naïve gene expression, it confirms that cells have been correctly converted from primed to naïve state, as the expression of *KLF4, KLF17, ESRRB, TFAP2C, DPPA2,* and *DPPA5* in naïve conditions using hWJ-MSCs-CM at day 9 (Table 5.2). In contrast, the detection of primed markers *TBXT* and *OTX2* showed a loss of gene expression in converted cells in both CMs. This high expression in the initial period can indicate an active state of the cells transitioning from a primed to a more differentiated state before potentially losing this expression as they move towards a naïve state of pluripotency later on indicating a transition to a naïve state. However, *TBXT* exhibited significantly higher gene expression compared to the control during the initial period on day 9 of converted cells in hWJ-MSC-CM, which later decreased. In term of pluripotency, converted cells in hWJ-MSC-CM during the initial period on day 9 showed a rapid reduction in the expression of OCT4, NANOG, and *SOX2*. This is consistent with OCT4's role in primarily activating pluripotency and self-renewal genes while repressing differentiation promoting genes in coordination with other pluripotency factors and coregulators (Shi & Jin, 2010). Despite experiencing a loss of pluripotency during the initial period on day 9, it is interesting to observe an increase in the expression of pluripotency markers in the converted cells in hWJ-MSC-CM during the second period on day 21. Moreover, result from RT-qPCR showed the high gene expression of *SOX2* which, suggesting that these cells have retained some aspects of pluripotency.

During reprogramming of human primed cells, DNA methylation is globally reduced to a level similar to that reported for human ICM (H. Guo et al., 2014). This is considered a crucial process for the elimination of epigenetic memory in the naïve state of pluripotency (Lee et al., 2014). The process of reducing H3K9me3 and inducing H3K14ac is likely to facilitate reprogramming by promoting a more open chromatin configuration, characteristic of naïve cells (Nichols & Smith, 2009). In all experiment groups that were cultured in VALGöX medium, we also observed a strong increase of H3K14 acetylation, while H3K9 trimethylation was weakly expressed in all experiment group that were cultured in VALGöX medium compare to control group. This result indicated that ascorbic acid (DNA demethyltransferase) had induced upregulation of active marks as H3K14 acetylation and downregulated of negative

marks like H3K9 trimethylation, this marks is related to the process of gene silencing (Takashima et al., 2014). Naïve pluripotency is associated with low DNA methylation and an abundance of active histone modification at the promoter regions of developmental genes (Leitch et al., 2013; Leitch et al., 2016; Hayashi et al., 2008). Noticeably, we identified differences in naïve marker expression between OF1-MEFs-CM and hWJ-MSCs-CM. Our results demonstrated that conversion of naïve-like cells from hWJ-MSCs-CM was more efficient than from OF1-MEFs-CM. These results suggest varying CMs efficiencies in terms of naïve marker expression at different stages. Based on this evidence, we disclosed that hWJ-MSCs-CM holds promise for the development of a cell-free product to convert primed state PSCs into a naïve state. However, if we need to improve the pluripotency of converted rhESCs, especially OCT4 and NANOG expression, it might be necessary to increase the conversion period in ALGöX to more than 21 days.

Table 5.1 A summary table of protein expression using ICC based on specific markers of primed and naïve state.

Class of markers	Specific markers	rhESCs	OF1-MEFs-CM		hWJ-MSCs-CM	
			D9	D21	D9	D21
Pluripotent markers	OCT4	+++	+	++	+	++
	NANOG	+++	++	++	++	++
	SOX2	+++	+++	++	++	+++
Primed marker	TBXT	+++	+	++	++	+
Naïve markers	TFAP2C	++	+	+	++	+++
	KLF17	++	+	+	+	+++
	KDM4A	++	++	+++	+++	+++
Epigenetic markers	H3K9ME3	+++	+	+	+	+
	H3K14AC	+	+++	+++	+++	+++

+++ strong protein expression, ++ moderate protein expression, + low protein expression

Table 5.2 A summary table of gene expression using RT-qPCR based on specific genes of primed and naïve state.

Class of markers	Specific markers	rhESCs	OF1-MEFs-CM		hWJ-MSCs-CM	
			D9	D21	D9	D21
Pluripotent markers	*OCT4*	+++	++	++	+	+
	NANOG	+++	+	+++	+++	++
	SOX2	+	+++	+++	++	++
Primed markers	*TBXT*	+++	+	++	+++	++
	OTX2	+++	+	+	+	+
Naïve markers	*KLF4*	+	+	+	+++	+++
	KLF17	++	+	+	+++	++
	TFAP2C	++	+++	+++	++	++
	ESRRB	++	++	++	+++	+++
	DPPA2	+	+	+	+++	++
	DPPA5	+	++	+++	++	+++

+++ strong gene expression, ++ moderate gene expression, + low gene expression

CHAPTER VI
CONCLUSION

The transition of primed to the naïve state of ESCs is crucial when selecting the appropriate cell culture media to enhance the efficiency of reprogramming them into a fully naïve state. In the present study, we evaluated the potential of using rhESCs as an animal model and compared the efficacy of two different culture conditions from OF1-MEFs-CM and hWJ-MSCs-CM, in converting primed rhESCs to a naïve state. Our findings indicate that the conversion of primed rhESCs to a naïve-like state involves significant changes in the expression of pluripotency markers, cell viability, and epigenetic reconfiguration. Different CMs, specifically OF1-MEFs-CM and hWJ-MSCs-CM, demonstrated varying efficiencies in inducing and maintaining the naïve state. Notably, hWJ-MSCs-CM was generally more effective in expressing naïve markers compared to the gold standard OF1-MEFs-CM. Additionally, WJ-MSCs-CM showed better epigenetic reprogramming, characterized by increased H3K14 acetylation and decreased H3K9 trimethylation, which are indicative of a more naïve state. The findings suggest that WJ-MSCs-CM might be superior alternative for converting primed rhESCs to a naïve state, not only maintaining the naïve state and enhancing epigenetic reprogramming but also providing practical and ethical advantages. Furthermore, WJ-MSCs possess immunomodulatory properties and carry a reduced risk of triggering an immune response, which enhances their potential for safe and effective use in future clinical applications. However, in this work, improvements are needed to enhance the stability and maintenance of naïve PSCs. Achieving this advancement could be pivotal for future regenerative medicine applications, providing a more effective and reliable method for maintaining naïve pluripotency.

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APPENDIX

SOLUTION PREPARATION

1. Reagent for cell culture

1.1 ESC medium

Mix the reagent as follow:

- KO-DMEM (Gibco, 10820-010)	80	mL
- 20% KOSR (Invitrogen, 10820-010)	20	mL
- 1x NEAA (Invitrogen, 21114050)	1	mL
- 1x Glutamax (Invitrogen, 5050-061)	1	mL
- 0.1% of P/S (Lonza Bioscience 17-602E)	100	µL

1.2 Complete medium

Mix the reagent as follow:

- α-MEM (Gibco, 11900-016)	89	mL
- FBS (Gibco, 10270-106)	10	mL
- L-glutamine	1	mL
- 0.1% P/S	100	µL

1.3 OF1 medium

Mix the reagent as follow:

- DMEM (Gibco, 11995-065)	88	mL
- 10% FBS (Gibco, 10270-106)	10	mL
- 1% PSG (Invitrogen, 10378-016)	1	mL
- 1x NEAA (Invitrogen, 21114050)	1	mL

2. Reagent for N2B27 basal medium for produce conditioned medium

2.1 100x N2 supplement (homemade)

Mix the reagent as follow:

- DMEM-F12 (Gibco, 11320-074)	7.187	mL
- 2.5 mg/mL Insulin (Sigma, I6634)	1	mL
- 10 mg/ml Human apo-transferrin (Sigma, T1147)	1	mL
- 0.00198 mg/ml Progesterone (Sigma, P8783)	0.033	mL
- 1.6 mg/ml Putrescine dihydrochloride (Sigma, P5780)	0.1	mL
- 0.00052 mg/ml Sodium selenite (Sigma, S5261)	0.01	mL

- 7.5% BSA Fraction V solution (Gibco, 15260-037) 0.67 mL

2.2 N2B27 basal medium

Mix the reagent as follow:

- 50% Neurobasal medium (Invitrogen, 21103-049) 243.7 mL
- 50% DMEM-F12 (Invitrogen, 21331-020) 243.7 mL
- 1% PSG 5 mL
- 1%B27 (Thermo Fisher Scientific, 17504-044) 5 mL
- 0.01% β-mercaptoethanol (Sigma, 11061-68-0) 100 µL
- 0.5x N2 supplement 2.5 mL

3. Reagent for converting primed to naïve state of rhESCs

3.1 250mM L-Ascorbic acid (Sigma, A8960)

Dissolve 0.723 g. of L-Ascorbic acid in 1 mL of MQ water. Then filtrate with 0.22 µm filter and aliquoted to small eppendorf tube and store at -20°C.

3.2 VALGoX medium

Mix the reagent as follow:

- Conditioned medium 1 mL
- 250 µM L-Ascorbic acid 1 µL
- 10 ng/mL Activin A (Peprotech, 120-14E) 0.5 µL
- 1X human LIF 1 µL
- 1.25 µM Go6983 (Bio-techne, 2285) 0.125 µL
- 2.5 µM XAV939 (Sigma, X3004) 0.5 µL

3.3 10mM of Y-27632 dihydrochloride; Rock inhibitor (Abcam ab120129)

Dissolve 10 mg. of Y-27632 dihydrochloride in 3.12 mL of MQ water filtered. Then aliquoted to small eppendorf tube and store at -20°C.

3.4 Laminin521-coated (Invitrogen U.S.A. A29248)

Dilute LN521 stock solution at 5 µg/mL in 1x PBS- (50 µL of LN521 stock solution + 950 µL of 1x PBS (-)), and add 1 mL of LN521 per 35mm dish. Incubate 1 h at 37°C before use.

4. Freezing medium

Mix the reagent as follow:

- α-MEM 6 mL
- 20% FBS (Gibco, 10270-106) 2 mL
- 20% Dimethyl sulfoxide (DMSO) 2 mL
- 0.1% P/S 10 µL

5. Other reagents

5.1 50 µg/mL of MMC stock solution (Sigma, M4287)

Dissolve 2 mg. of MMC powder in 40 mL of α-MEM. Then filtrate with 0.22 µm filter and aliquoted to small eppendorf tube and store at -20°C.

5.2 20 µg/mL of bFGF solution (Sigma, SRP3043)

Dissolve 50 µg. of bFGF in 2.5 mL of 0.1% BSA. Then aliquoted to small eppendorf tube and store at -20°C.

BIOGRAPHY

Ratree Moorawong was born in Nakhon Sawan, Thailand on June 9th, 1998. She finished her high school from Chumsaeng Chanutid School in Nakhon Sawan. In 2021, She graduated from Naresuan University, Phitsanulok, Thailand, with Bachelor's Degree (B.Sc.) of science in Biology. In August 2021, she started studying master degree in Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. She received scholarship from Suranaree University of Technology, One research One graduate (OROG) under supervision of Assoc. Prof. Dr. Rangsun Parnpai. She has publications and proceedings from her work. First, she is a co-author of the review article titled "An Expedition in the Jungle of Pluripotent Stem Cells of Non-Human Primates," published in the journal Stem Cell Reports (Impact factor 5.9). Second, she presented a part of her research titled "A Comparative Study of Conditioned Medium During the Conversion from Primed to Naïve State in Rhesus Monkey Embryonic Stem Cells" at the 35th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB2023). Finally, she presented a poster titled "Culture Media Affect the Conversion Efficiency from a Primed to a Naive State of Rhesus Monkey Embryonic Stem Cells" at the Pluripotent Stem Cells International Conference (PSCC2024).