EPIGENETIC MODIFIERS ENHANCE THE REPROGRAMMING OF RABBIT SOMATIC CELLS AND RABBIT INDUCED PLURIPOTENT STEM CELLS

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ตัวดัดแปลงที่อยู่เหนือพันธุกรรมที่ช่วยส่งเสริมการรีโปรแกรมของเซลล์ร่างกายกระต่ายและเซลล์ต้นกำเนิดไอพีเอสกระต่าย

นางสาววรวลัญช์ สำรวล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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วรวลัญช์ สำรวล : ตัวดัดแปลงที่อยู่เหนือพันธุกรรมที่ช่วยส่งเสริมการรีโปรแกรมของเซลล์ร่างกายกระต่ายและเซลล์ต้นกำเนิดไอพีเอสกระต่าย (EPIGENETIC MODIFIERS ENHANCE THE REPROGRAMMING OF RABBIT SOMATIC CELLS AND RABBIT INDUCED PLURIPOTENT STEM CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. รังสรรค์ พาลพ่าย, 114 หน้า.

เซลล์ต้นกำเนิดไอพีเอสกระต่าย/การรีโปรแกรม/การดัดแปลงโปรตีนฮิสโทน/อินแอคติเวชันโครโมโซม X/สถานะพลูริโพเทนต์

เซลล์ต้นกำเนิดไอพีเอสกระต่าย (rbiPSC) มีลักษณะเฉพาะของไพรม์ (prime) พลูริโพเทนต์ตามที่กำหนดไว้สำหรับสัตว์ฟันแทะและสัตว์เลี้ยงลูกด้วยนม เราทดสอบสมมติฐานที่ว่าการดัดแปลงที่อยู่เหนือพันธุกรรมโดยการยับยั้งการทำงานของ histone deacetylase (HDACi) และ histone 3 lysine 9 methyltransferase (H3K9mei) จะปรับปรุงการรีโปรแกรมในเซลล์ต้นกำเนิดไอพีเอสกระต่าย ให้มีสถานะความเป็นนาอีฟ (naïve) สูงขึ้น ในการศึกษานี้ ได้ทำการรีโปรแกรมเซลล์ต้นกำเนิดไอพีเอส กระต่าย (B19-rbiPSCs) โดยใช้ตัวดัดแปลงที่อยู่เหนือพันธุกรรมและเพาะเลี้ยงเซลล์นี้ในระบบที่ปราศจากเซลล์พี่เลี้ยง วิธีการเพาะเลี้ยงแบบใหม่นี้ส่งผลให้เกิดการกำหนดค่าการแสดงออกของยีนและอีพีเจเนติกใหม่ ซึ่งพิสูจน์ได้จากการให้ผลบวกต่อมาร์คเกอร์ที่จำเพาะต่อความเป็นนาอีฟ ได้แก่ *Oct4, Sox2, Klf4, Stat3, Esrrb, Dppa5, Kdm4a* และเกิดกระบวนการปรับเปลี่ยนฮิสโตนที่เกี่ยวข้องกับสถานะความเป็นนาอีฟ นอกจากนี้การยับยั้งปฏิกิริยาการเติมหมู่เมทิลที่ ฮีสโตน 3 ไลซีน 9 ของ H3K9 methyltransferase และการยับยั้งปฏิกิริยาการดึงหมู่เอทิลออกจากฮีสโตนของ histone deacetylase ยังส่งเสริมการรีโปรแกรมโดยลดระดับของ H3K9 trimethylation (H3K9me3) และเพิ่มระดับของ H3K14 acetylation (H3K14ac) โดยเซลล์ที่ถูกรีโปรแกรมมีเปอร์เซ็นต์ของเซลล์ที่แสดง X inactivation ลดลง ซึ่งสารยับยั้งเหล่านี้สามารถกระตุ้น reactivation ของ X โครโมโซมให้กลับมาทำงานได้

นอกจากนี้ได้ทำการรีโปรแกรมเซลล์ไฟโบรบลาสต์กระต่ายและเซลล์ต้นกำเนิดมีเซ็นไคม์กระต่ายโดยใช้เซนไดไวรัส หรือการถ่าย mRNA ร่วมกับตัวดัดแปลงที่อยู่เหนือพันธุกรรม เซลล์ไฟโบรบลาสต์กระต่ายแสดงการทรานส์ดักชันและประสิทธิภาพการทรานส์เฟคที่สูงกว่าเซลล์ต้นกำเนิดมีเซ็นไคม์กระต่าย กระบวนการรีโปรแกรมเซลล์ไฟโบรบลาสต์กระต่ายด้วยเซนไดไวรัส ที่ประกอบด้วยทรานสคริปชั่นแฟกเตอร์ hKlf4, hOct3/4, hSox2 และ c-Myc ในอาหารเลี้ยงเซลล์ที่เสริมด้วยสารยับยั้งการทำงานของ H3K9 methyltransferase A366 และ HDAC UF010 ได้สร้างโคลนที่เหมือน iPSCs ซึ่งมีการแสดงออกของยีนพลูริโพเทนต์กระต่าย ได้แก่ *Oct4* และ *Nanog* ส่วนการแสดงออกของ *Sox2* นั้นไม่แตกต่างจากการแสดงออกของเซลล์ไอพีเอสกระต่าย นอกจากนี้การ

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WORAWALAN SAMRUAN : EPIGENETIC MODIFIERS ENHANCE THE REPROGRAMMING OF RABBIT SOMATIC CELLS AND RABBIT INDUCED PLURIPOTENT STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 114 PP.

RABBIT INDUCED PLURIPOTENT STEM CELLS/REPROGRAMMING/HISTONE MODIFICATION/X INACTIVATION/PLURIPOTENCY STATUS

Rabbit induced pluripotent stem cells (rbiPSCs) exhibit the characteristic features of primed pluripotency as defined in rodents and primates. The hypothesis that epigenetic modifications by inhibition of histone deacetylase (HDACi) and inhibition of histone 3 lysine 9 methyltransferase (H3K9mei) would enhance reprogramming rbiPSCs toward the naïve state was tested. In the present study, the B19-rbiPSCs was reprogrammed using epigenetic modifiers and cultured them in a resetting medium on feedder-free system. This new culturing protocol resulted in transcriptional and epigenetic reconfiguration, as substantiated by the expression of naïve markers including *Oct4, Sox2, Klf4, Stat3, Esrrb, Dppa5, Kdm4a* and the presence of histone modifications associated with naïve pluripotency. Furthermore, H3K9 methyltransferase inhibition and HDAC inhibition promote reprogramming by downreguration of H3K9 trimethylation (H3K9me3) and upreguration of H3K14 acetylation (H3K14ac). The reprogrammed cells decreased the percentage of cells showing X inactivation, these inhibitors can reactivate the silent X chromosome.

The rabbit fibroblasts (rbFs) and rabbit mesenchymal stem cells (rbMSCs) were also reprogrammed using Sendai virus or mRNA transfection and epigenetic modifiers. The rbFs showed higher transduction and transfection efficiency than rbMSCs. The rbFs reprogramming process with Sendai virus carrying hKlf4, hOct3/4, hSox2 and c-Myc using medium supplemented with H3K9 methyltransferase inhibitor A366 and HDAC inhibitor UF010 generated iPSCs-like clones that expressed rabbit pluripotent transcription factors including *Oct4* and *Nanog*. The expression of *Sox2* was not different to that of B19-rbiPSCs.

Moreover, feeder-free culture medium simplifies the selection and the amplification of reprogrammed clones.

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Student's ignature Worawalan Samrajan
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LIST OF ABBREVIATIONS

DMEM	=	Dulbecco's modified Eagle medium
DMEM/F12	=	Dulbecco's modified Eagle medium/Nutrient mixture F-12
EKB	=	E3, K3, B18 immune evasion factors
FBS	=	Fetal bovine serum
FGF	=	Fibroblast growth factors
GFP	=	Green fluorescent protein
IL6	=	Interleukin-6
IL6R	=	Interleukin-6 receptor
KOSR	=	knockout serum replacement
LIF	=	Leukemia inhibitory factor
MEF	=	Mouse embryonic fibroblasts
MMC	=	Mitomycin C
MOI	=	Multiplicity of infection
NEAA	=	Non essential amino acids
OSKMNL	=	Oct4, Sox2, Klf4, cMyc, Nanog, Lin28 reprogramming factors
PSG	=	Penicillin streptomycin glutamine
qPCR	=	Quantitative polymerase chain reaction
R6	=	IL6 + IL6R

CHAPTER I

INTRODUCTION

1.1 Background

Pluripotent stem cells (PSCs) can exist in two distinct states, designated as the naïve and primed states (Nichols and Smith, 2009). Induced pluripotent stem cells (iPSCs) derived from humans and non-human primates (NHPs) also exhibit primed pluripotency-like characteristics (Nichols and Smith, 2009; Vallier et al., 2005; Wianny et al., 2008). Several studies have reported on the resetting of conventional human embryonic stem cells (hESCs) and iPSCs to naïve-like pluripotency using different combinations of transcription factors (NANOG, KLF2 and STAT3), chemical inhibitors of various kinases (ROCK, GSK3, MEK1/2, SRC, $p38^{MAPK}$, BRAF and JNK) and growth factors (FGF2, LIF and Activin A). The resetted cells, known variously as NHSM (Gafni et al., 2013), 3iL (Chan et al., 2013), 6i/L/A (Theunissen et al., 2014), Reset (Takashima et al., 2014), and TL2i (Chen et al., 2015). These reprogrammed cells display some characteristic features of naïve pluripotent rodent stem cells, including reconfigured transcriptome and epigenome, alterations in mitochondrial respiration (Takashima et al., 2014), loss of FGF2 and ERK dependency (Takashima et al., 2014; Chen et al., 2015), and gain of leukemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (STAT3) dependency (Chan et al., 2013; Takashima et al., 2014; Chen et al., 2015).

In 2006, Takahashi and Yamanaka demonstrated that fibroblasts could be reprogrammed into PSCs by overexpressing four transcription factors, namely Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka.,2006). The resulting “induced pluripotent stem cells” or iPSCs, displayed the cardinal features of their embryonic counterpart, the embryonic stem (ESCs). This major discovery was made in mice, and was soon applied to human with a similar outcome (Takahashi et al., 2007; Yu et al., 2007). Since these pioneering studies, the iPSCs technology was implemented in several other species, including rhesus macaque (Liu et al., 2008), pig (Ezashi et al.,

2009), and rabbits (Honda et al., 2010; Tancos et al., 2012; Osteil et al., 2013). The first rabbit PSCs were produced from New Zealand White rabbit blastocysts in the form of embryonic stem cells (rbESCs) (Fang et al., 2006; Wang et al., 2007). In their undifferentiated state, rbESCs required both fibroblast growth factor 2 (FGF2) and transforming growth factor β (TGF-β) family (activin, nodal) for self-renewal (Wang et al., 2008). These results were confirmed in another study, which showed that activin/nodal signaling through Smad2/3 activation was necessary for maintaining the pluripotent status of rbESCs (Honda et al., 2009).

RbiPSCs exhibit the characteristic features of primed pluripotency. They are dependent on FGF2 signaling and TGF-β (activin, nodal) for self-renewal which showed that activin/nodal signaling through Smad2/3 activations was necessary for maintaining the pluripotent status of rbESCs (Honda et al., 2009; Osteil et al., 2013; Wang et al., 2008; Wang et al., 2007). In the present study, we reprogrammed of primed rbiPSCs toward the naïve state in a process called resetting. Chemical resetting of primed to a naïve pluripotent state is one such method and has come to the forefront as a simple, efficient, and transgene-free method to induce naïve pluripotency. The process involves the transient application of a histone deacetylase inhibitors and histone 3 lysine 9 methyltransferase inhibitor to initiate resetting, followed by the emergence of nascent naïve pluripotent stem cells in supportive conditions, and finally the stabilization and expansion of naïve pluripotent stem cell cultures. Many researches have been performed to increase the efficiency of the resetting process, to reveal the underlying mechanistic events, and allowed the generation of patient and disease-specific rabbit iPSCs, which have the potential to develop into many different or specialized cell types for replacement therapies and disease modeling. This study provides the requisite technical protocols and resources to facilitate routine generation and study of candidate rabbit naïve iPSCs.

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CHAPTER II

LITERATURE REVIEW

2.1 Stem cells

Stem cells have unparalleled characteristics. They are not specialized cells and show the ability of self-replication and differentiation according to the suitable signal (Windley et al., 2005). Stem cells can reproduce itself over and over again through asymmetric cell division, they can produce a newly produced offspring cell preserving the characteristics of the mother cell and another offspring cell that has a different potency and lineage potential, such as a committed progenitor that transiently amplifies to make several offspring (Ilancheran et al., 2009). Stem cells can be classified according to their origin and also, they can be classified according to their potency.

2.1.1 Classification of stem cells on the basis of their origin

2.1.1.1 Embryonic stem cells: ESCs are pluripotent, self-renewing cells that can be derived from both mouse or human blastocysts, they are taken from the very early stages of embryo development after 4-5 days after fertilization (Ying et al., 2003; Lerou, 2011). They can be stored in culture as undifferentiated cell lines because they are capable of self-renewal by symmetric division, and can be stimulated to differentiate into any cell line (Klimanskaya et al., 2006). They can differentiate into endoderm, mesoderm, and ectoderm embryonic germ layers, and also any type of somatic and germline cells. They, therefore, hold a great capacity in tissue regeneration therapy (Zhang and Huang, 2010).

2.1.1.2 Adult stem cells: the second type of stem cells are any stem cells taken from mature tissue; they are found in the tissues of a fully developed child (whole embryo) or adult and can only produce a limited number of cell types. They have limited potential as compared to the stem cells that derived from embryos and fetuses because of the stage of development of these cells (Robinson, 2001). They play a vital role in tissue repair and regeneration. They are referred to

their tissue origin (Gimble et al., 2007). Bone marrow is an abundant source of adult stem cells (Gao et al., 2006).

2.1.1.3 Induced pluripotent stem cells: The third type of stem cell, with properties similar to embryonic stem cells, has emerged. Scientists have engineered these iPSCs by manipulating the expression of certain genes - reprogramming somatic cells back to a pluripotent state (Ulloa-Montoya et al., 2005).

2.1.2 Classification of stem cells on the basis of potency

Stem cells can be classified by the extent to which they can differentiate into different cell types. These four main classifications are totipotent, pluripotent, multipotent, or unipotent.

2.1.2.1 Totipotent: The ability to differentiate into all possible cell types. Only, the zygote formed at egg fertilization and the first few cells that result from the division of the zygote are tolipotent (Cauffman et al., 2009).

2.1.2.2 Pluripotent: The ability to differentiate into almost all cell types. Examples include ESCs and iPSCs that are able to differentiate into derivatives of the three germ layers that are formed in the beginning stages of gastrulation (Ralston and Rossant, 2010).

2.1.2.3 Multipotent: The ability to differentiate into a closely related family of cells. Examples include tissue adult stem cells like hematopoietic stem cells that can become red and white blood cells or platelets (Pretson et al., 2003).

2.1.2.4 Unipotent: The ability to only produce cells of their own type, but have the property of self-renewal required to be labeled a stem cell. Examples include (adult) muscle stem cells (Sage et al., 2008).

2.2 Pluripotent stem cells

PSCs were first derived from the inner cell mass (ICM) of mouse embryos in 1981 (Evans and Kaufman, 1981; Martin, 1981). These cells, named ESCs, were shown to be able to colonize the epiblast of host blastocysts and, consequently, to produce chimeric mice in 1984 (Bradley et al., 1984). This ability of mouse ESCs (mESCs) has made it possible to develop transgenesis techniques in this species, facilitating substantial progress in functional genetics. Later on, PSCs were derived from the late epiblast of mouse post-implantation embryos (Brons et al., 2007; Tesar

et al., 2007). These cells, named epiblast stem cells (EpiSCs), display several different features from mESCs, especially with respect to their transcriptome and epigenome, and are notably not able to colonize host blastocysts. At the same time, mouse PSCs were obtained by reprogramming differentiated somatic cells by overexpression of four pluripotency factors, namely Oct4, Sox2, Klf4, and cMyc (Takahashi and Yamanaka, 2006). These cells, named iPSCs, can exhibit features of mESCs or EpiSCs depending on the medium used during their reprogramming (Bar-Nur et al., 2014; Buganim et al., 2014). Therefore, mESCs and EpiSCs epitomize two states of pluripotency existing *in vivo* during early embryonic development-the naive state, corresponding to early epiblast cells from preimplantation embryos, and the primed state, corresponding to late epiblast cells from post-implantation embryos, respectively (Nichols and Smith, 2009). *In vitro* naive PSCs are sustained by the LIF/gp130/STAT3 and bone morphogenetic protein 4 (BMP4)/ALK/SMAD1-5-8 signaling pathways, whereas primed PSCs are supported by the FGF2 and activin A/TGF-ß/SMAD2-3 signaling pathways (Hassani et al., 2019). To date, all PSC lines, ESCs and iPSCs, obtained in non-rodent mammalian species display features of primed pluripotency (Soto and Ross, 2016; Su et al., 2020). Therefore, it seems that non-rodent PSCs are only able to stabilize in culture in the primed state of pluripotency (Savatier et al., 2017). For example, primate PSCs present a transcriptome very different from that of human early blastocyst epiblast cells (Yan et al., 2013), but is rather closer to that of post-implantation late blastocyst epiblast cells according to an analysis in cynomolgus monkeys (Nakamura et al., 2017). The actual research challenge in the PSC field is to reprogram primed cells toward the naive state of pluripotency, in order to obtain and culture more genetically stable cells, which are easier to handle by single-cell dissociation (Lund et al., 2012; Peterson and Loring, 2014) and are more useful for cellular therapies or the production of disease models (Wu and Hochedlinger, 2011; Zeltner and Studer, 2015). The possibility to convert primed PSCs into naive PSCs was first demonstrated in mice by overexpressing pluripotency genes such as *Klf4, Nanog, Stat3, Tfcp2l1,* or *Prmd14*, either alone or in combination (Guo et al., 2009; Silva et al., 2009; Yang et al., 2010; Qiu et al., 2015; Okashita et al., 2016), and by strengthening the signaling pathways sustaining the naive state using inhibitors of mitogen-activated extracellular regulated kinase (MEK)

and glycogen synthase kinase 3 beta (GSK3β) in a medium called 2iLIF (Tsukiyama and Ohinata, 2014; Illich et al., 2016). Such strategies for reprogramming primed PSCs have been extensively studied for human PSCs and have produced naive-like cells with a heterogenous reconfiguration of their transcriptome and epigenome (Hanna et al., 2010; Theunissen and Jaenisch, 2014; Chen et al., 2015; Guo et al., 2017) as well as a variable capacity to produce interspecies chimeras after microinjection into mouse blastocysts (Wu et al., 2015; Masaki et al., 2016). These variations in the molecular and functional characteristics of primate PSCs show that embryonic cells could be stabilized *in vitro* at different stages along a continuum of pluripotency, the ends of which are epitomized by the naive and primed states, respectively (Afanassieff et al;, 2018). Lagomorphs and primates share many similarities in their embryonic development (Madeja et al., 2019), in particular in the timing of the embryonic genome activation at the 8/16-cell stages (Leandri et al., 2009), the timing of the waves of DNA demethylation and methylation (Reis Silva et al., 2011; Reis Silva et al 2012), and the timing of the random inactivation of the second X chromosome (Okamoto et al., 2011). Like their human counterparts, rabbit embryos develop as a flat disc on the surface of the conceptus (Idkowiak et al., 2004), and present the advantage of implanting very late (at E6.75) due to a mechanism similar to that of human embryos (Nicholls et al., 2011). Above all, the gastrulation of rabbit embryos begins before implantation (at E6.0) so that the epiblast remains more easily accessible for experimentation than in rodents (Duranthon et al., 2004). These similarities and particularities make rabbits an interesting model not only for the study of the biology of PSCs, but also to be used to create transgenic animal models of human development and diseases and to improve interspecies chimerism tests.

2.3 Rabbit pluripotent stem cells

2.3.1 Rabbit embryonic stem cells (rbESCs)

Rabbit embryonic cell cultures were described by American teams in 1993 (Giles et al., 1993; Graves and Moreadith, 1993), but the first lines of rbESCs were not published until 15 years later by two teams from China and Japan (Wang et al., 2007b; Honda et al., 2008). RbESCs are derived from ICM cells of early blastocysts

(E3.5–E4.0). They are cultured on feeder cells and form flat colonies (Figure 2.1). The self-renewal of rbESCs depends on the activin A/TGF-ß /SMAD2-3 and FGF2 pathways (Honda et al., 2009; Osteil et al., 2013). FGF2 appears indispensable by inducing the PI3K/AKT and MAPK pathways (Hsieh et al., 2011; Lo et al., 2015), while the WNT/ß -catenin pathway may also be indirectly activated by FGF2 (Wang et al., 2008). It is possible to derive cells without any growth factor in the medium if they are cultured on feeder cells, showing that LIF is not necessary for the maintenance of rbESCs (Osteil et al., 2016). However, the addition of LIF to the culture medium of rbESCs has often been used (Itsieh et al., 2011; Lo et al., 2015; Intawicha et al., 2009), and several studies have described the effect of LIF on the derivation of rbESCs and the induction of LIF-receptor expression (Intawicha et al., 2009; Catunda et al., 2008). Unlike the derivation of rodent ESCs stabilized in the naive state (Ying et al., 2008), MEK and GSK3ß inhibitors do not enhance epiblast cell differentiation *in vivo* (Bontovics et al., 2020) or rbESC derivation *in vitro* (Osteil et al., 2016). In addition, the maintenance of dome-shaped naive-like rbESC colonies in the presence of these two inhibitors requires both FGF2 and feeder cells (Liu et al., 2019).

RbESCs are unstable and their enzymatic single-cell dissociation induces higher rates of cell proliferation than clump passages (Osteil et al., 2016), but generates more chromosomal abnormalities as described for human ESCs (Bai et al., 2015). The inhibitor of the rho kinase (ROCKi), which acts by blocking the apoptotic response induced by cell dissociation (Ohgushi and Sasai, 2011), does not increase the clonogenicity of rbESCs, unlike human ESCs (Watanabe et al., 2007), but leads to their arrest of proliferation and differentiation (our own unpublished data). This difference can be explained by the high expression levels of *ROCK1* and *ROCK2* genes in rbESCs (Schmaltz-Panneau et al., 2014), which may be essential to maintain cell pluripotency by eliminating cells engaged into differentiation.

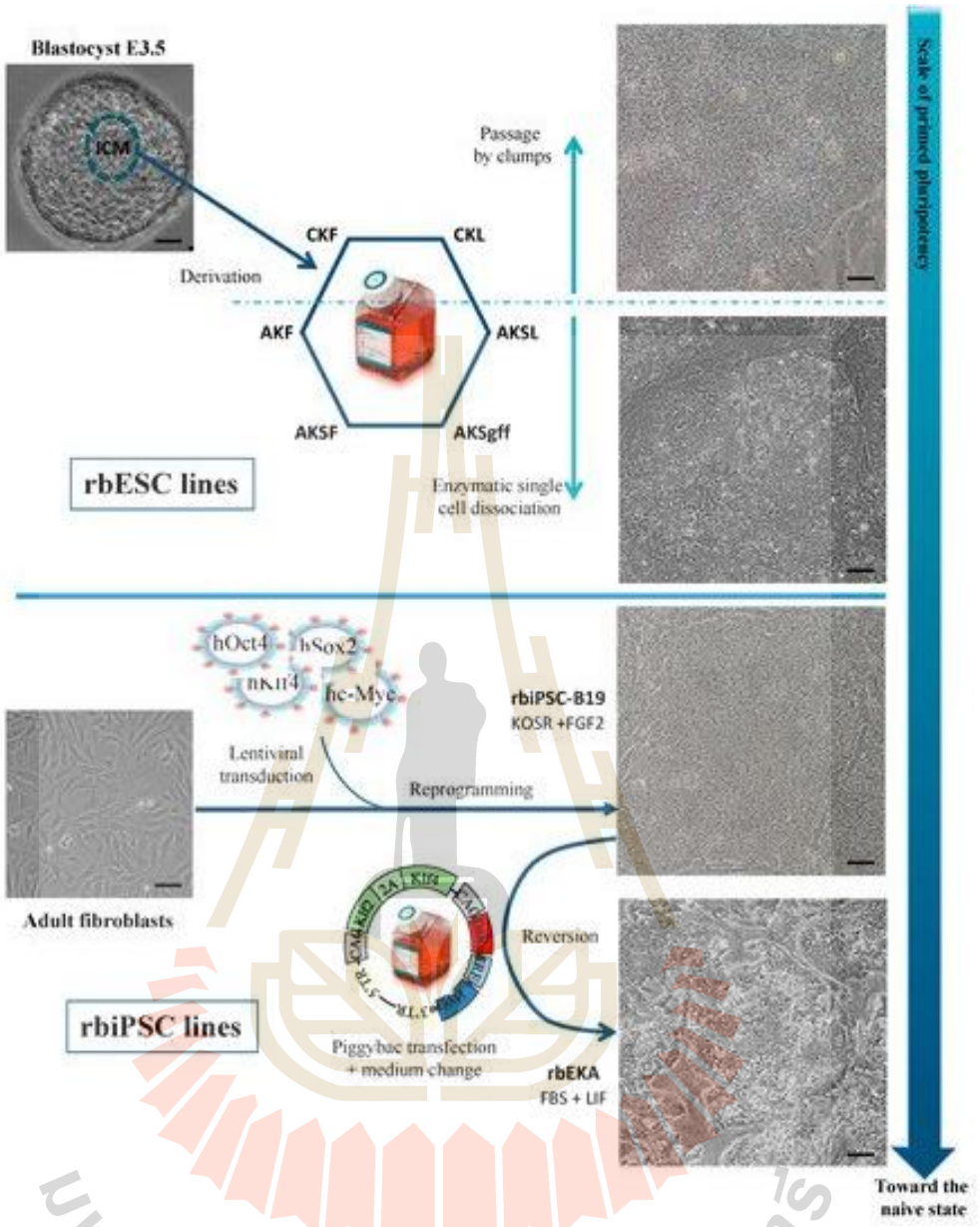

Figure 2.1 Schematic representation of the rabbit pluripotent stem cell lines produced by the rabbit research team of the SBRI and placed on a scale of primed pluripotency. Methods and media are indicated. CKF: clumps/KOSR/FGF2; CKL: clumps/KOSR/LIF; AKF: accutase/KOSR/LIF; AKSL: accutase/KOSR+FBS/LIF; AKSF: accutase/KOSR+FBS/LIF; AKSgff: accutase/KOSR+FBS/growth factor free; rbEKA: rabbit enhanced KLF activity. Scale bar=30 um. (Samruan et al., 2020).

2.3.2 Rabbit induced pluripotent stem cells (rbiPSCs)

RbiPSCs were produced by reprogramming rabbit somatic cells (adult liver and stomach cells or adult and embryonic fibroblasts) using the classical combination of the four human genes (*hOCT4, hSOX2, hKLF4, hc-MYC*) overexpressed by murine leukemia virus (MuLV)-based retroviral vectors (Osteil et al., 2013; Honda et al., 2010; Tancos et al., 2017; Phakdeedindan et al., 2019). RbiPSC lines show reactivation of endogenous genes of the pluripotency core, *Oct4, Sox2,* and *Nanog,* as well as silencing of all four transgenes; they are therefore fully reprogrammed (Osteil et al., 2013). Like rbESC lines, they form flat colonies (Figure 2.1) and self-renew in the presence of FGF2, knockout serum replacement (KOSR), and mitomycin-treated mouse embryonic fibroblasts (MEFs) producing activin A, as feeder cells. The use of serum and LIF-based medium did not allow the reprogramming of adult ear fibroblasts (Osteil et al., 2013). Attempts to directly reprogram rabbit fibroblasts into the naive state used, in addition to the four usual human transgenes, LIF, KOSR, MEK, and GSK3β inhibitors as well as forskolin, a protein kinase C (PKC) agonist shown to reinforce *Klf2* and *Klf4* expression in human PSCs (Hanna et al., 2010). The resulting female rbiPSC lines displayed reactivation of the second X chromosome, formed dome-shaped colonies, exhibited improved growth and clonogenicity rate, and required both LIF/STAT3 and PI3K/AKT pathways to self-renew (Jiang et al., 2014). However, these lines were not fully reprogrammed since their naive-like state depended on the expression of the four transgenes only maintained in presence of doxycycline. This obstacle, posed by the inability of iPSCs to silence exogenous reprogramming factors and to depend on them for self-renewal, is often observed in domesticated animals (Su et al., 2020).

2.3.3 Pluripotent state of rbiPSC

Both rbESC and rbiPSC lines express the cardinal markers of PSCs (Osteil et al., 2013).They are positive for alkaline phosphatase activity (Wang et al., 2007b). They express the pluripotency-associated *Oct4* and *Nanog* transcription factors, as well as the SSEA-1, SSEA-4, TRA1-60, and E-cadherin cell surface markers (Intawicha et al., 2009; Honda et al., 2010). They also display a normal karyotype (42XX or 42XY) (Wang et al., 2007b; Osteil et al., 2013), produce embryoid bodies *in vitro* (Honda et al., 2008; Phakdeedindan et al., 2019), and form teratoma containing

tissues of ectodermal, mesodermal and endodermal origin upon injection under the kidney capsule in severe combined immunodeficient (SCID) mice (Osteil et al., 2013; Honda et al., 2010). However, rbPSCs can only be stabilized in the primed state of pluripotency. The suppression of FGF2 and/or culturing without feeder cells lead to their differentiation. Thus, these cells can be cultured only on a synthetic matrix, with either medium conditioned on mitomycin-treated MEF or addition of activin A (Osteil et al., 2016; Tapponnier et al., 2017). In the same way, rbPSCs differentiate in the presence of inhibitors of SMAD2-3, TGF-ß -receptor, or FGF-receptor (Honda, Hirose, and Ogura, 2009; Wang et al., 2008). Transcriptomic comparisons of rbPSCs with rabbit epiblast cells from E4- and E6-stage embryos show overexpression of 17 genes involved in the WNT/ß-catenin signaling pathway, including TCF4, LEF1, and WNT5A (Schmaltz-Panneau et al., 2014). This result is consistent with the decrease in the rate of rbESC multiplication after inhibition of the WNT pathway by a Frizzled-1 antagonist or an anti-Wnt3a antibody (Wang et al., 2008). This effect is associated with an increase in phosphorylation of ß-catenin and SMAD1-5-8, as well as a reduction in expression of SSEA-4 pluripotency marker, indicating cell commitment to differentiation. The WNT pathway is involved in maintaining the balance between self-renewal and differentiation of both mouse and human ESCs (Sokol, 2011; Munoz-Descalzo, Hadjantonakis, and Arias, 2015; Zhao and Jin, 2017). It is therefore also important in sustaining the pluripotency of rbPSCs, although the 2iLIF medium allowing the self-renewal of rodent PSCs and containing the GSK3ß inhibitor CHIR99021 did not allow the stabilization of rbESCs. RbPSCs form flat colonies characteristic of their primed pluripotent state (Figure 2.1). They express 173 genes involved in cytoskeletal organization and function compared to rabbit epiblast cells at the E4 and E6 stages (Schmaltz-Panneau et al., 2014). Among them are gene-encoding filamins (FLNA and FNLC) and vinculin (VCL), which function to stabilize the actin cytoskeleton and its anchoring to the plasma membrane. These molecules promote cell-to-matrix interactions rather than cell-to-cell adhesions, and therefore may interfere with colony morphology. These proteins are also more expressed in EpiSCs than in mESCs (Osman et al., 2010; Frohlich et al., 2013). Interestingly, a comparison of rbESC and rbiPSC lines produced from the same breed of New Zealand white rabbit showed different features that bring rbiPSC lines closer to the

naive state of pluripotency than rbESC lines (Osteil et al., 2013). These characteristics mainly concern their proliferation rate, their resistance to unicellular dissociation, their global transcriptome, and their expression of markers specific to the primed and naive pluripotency states (Afanassieff et al., 2020). The variation of these characteristics allows the classification of rbPSC lines on a graduated scale of primed pluripotency with rbESCs at one end (most primed state) and rbiPSCs at the other end (closer to the naive state) (Figure 2.1).

2.3.4 Reprogramming of rbPSC toward the Naïve state of pluripotency

As in humans, the reprogramming of primed rbPSCs toward the naive state was addressed by mimicking the protocols used in rodents and was based on the overexpression of pluripotency genes associated with the use of media sustaining the naive state. To date, two studies have been published in this area. The first used overexpression of the *hOCT4* gene and the 2iLIF medium supplemented by KOSR, forskolin, and kenpaullone, a potent inhibitor of several cell-cycle complexes, such as CDK/cyclin (Honda et al., 2013). In the second, The rabbit team of SBRI made use of the *hKLF2–hKLF4* gene duo with a serum/LIF-based medium to produce the cells named rbEKA, for rabbit enhanced KLF activity (Tapponnier et al., 2017) (Figure 2.1). Both studies showed an improvement in some properties of the reprogrammed rbPSCs, such as increased differentiation capacity (Honsho et al., 2015) or reprogramming of the transcriptome, epigenome, and miRNome (Tapponnier et al., 2017). As previously seen with the methods used to directly produce naive-like rbESCs (Liu et al., 2019) and rbiPSCs (Jiang et al., 2014), the techniques tested improve the pluripotency of primed rabbit cells, but without reaching a stable naive state, nor the capacity to produce germline-competent chimeras. In addition, all of these rbPSC lines present highly variable properties due to the heterogeneity of the rabbit strains used, the variability in the production techniques and media used, and the characterization criteria tested. Therefore, how rbPSCs can be stabilized in the naive state of pluripotency remains a question open to new research. However, the molecular pathways responsible for pluripotency in this species, although closer to those of primates than rodents, present some idiosyncrasies that have yet to be adequately characterized (Frankenberg, 2015).

2.4 Signaling pathways regulating pluripotency

Self-renewal and pluripotency of stem cells are two key defining stem cell properties by extrinsic signals mediated by an endogenous pluripotency gene regulatory network consisting of core transcription factors (TFs), such as *Oct4, Sox2,* and *Nanog* (Rizzino, 2009; Young, 2011; Theunissen and Jaenisch, 2014), epigenetic modifiers and regulating kinase signaling pathways. Maintaining stemness of mouse and human PSCs relies on distinct extrinsic signaling pathways including LIF/STAT3, FGF/extracellular signal-regulated kinase (ERK) pathway, phosphoinositide 3-kinase (PI3K)/AKT, Wnt/GSK3, and TGF-β signaling. Pluripotency maintenance in ESCs and iPSCs are provided by inhibiting the signaling pathways governing the differentiation potential of the stem cells (Akberdin et al., 2018). Hierarchy of pluripotency gene regulatory network (PGRN) displayed in Fig 2.2.

2.4.1 LIF signaling

LIF is a signaling molecule plays an important role in the maintenance of mouse ESCs (Ying et al., 2008). LIF is expressed in the trophectoderm from mouse pre-implantation embryo, while transcripts of LIF receptors Lifr and Gp130 are found in ICM, suggesting that LIF signaling may contribute to the pluripotency of the ICM cells (Nichols et al., 1996). LIF stimulated activation of the Janus kinase (JAK)/STAT3 signaling pathway has been described as a replacement to the MEFs (Williams et al., 1988; Smith et al., 1988). However, LIF can also engage a number of other intracellular signaling pathways, including PI3K, ERK and even YES-YAP (Hirai et al., 2011; Niwa et al., 2009; Tamm et al., 2011). LIF-mediated signals downstream of *Stat3* are transmitted via transcription factors such as *Tfcp2l1*; constitutive *Tfcp2l1* expression can be effectively substituted for LIF or *Stat3*. *Tfcp2l1* directly binds to promoters of *Oct4, Sox2, Nanog* and plays a role as a transcriptional activator of the core pluripotency network (CPN) (Do et al., 2013; Martello et al., 2013; Ye et al., 2013). However, *Stat3* is acting upstream of the *Oct4*, may be a direct transcriptional target of *Oct4, Sox2 and Nanog* (Du et al., 2009; Yin et al., 2015).

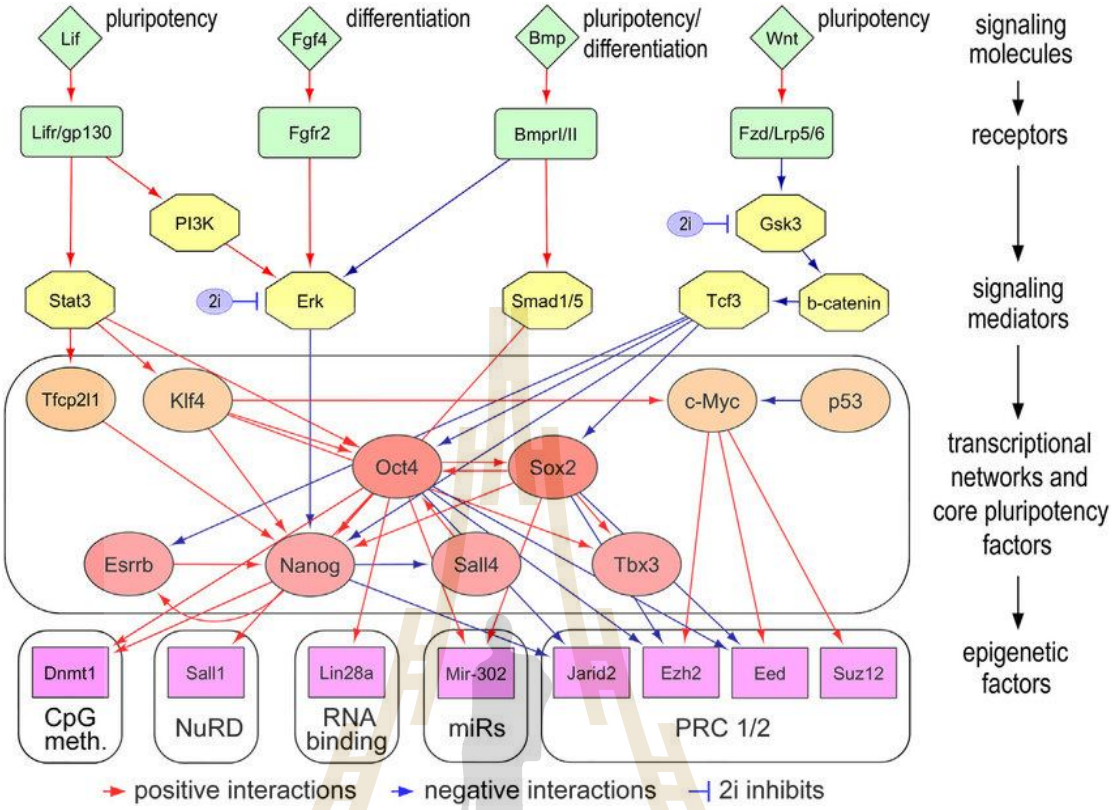

Figure 2.2 Hierarchical organization of pluripotency gene regulatory network. (Papatsenko et al., 2018).

2.4.2 WNT signaling

WNT signaling is known to promote self-renewal in ESCs by preventing their differentiation to more mature epiSCs (Morgani et al., 2017). WNT regulates transcription of the core pluripotency factors by inhibition of GSK3, via interaction of ß-catenin with transcriptional repressor *Tcf3* (Sokol, 2011). Using this or related mechanism ß-catenin also up-regulates *Stat3*, which otherwise is a major effector of LIF and activator of *Oct4* in ESCs (Hao et al., 2006). Beside transcriptional interactions, ß-catenin can also interact with adhesion molecules, such as E-cadherins. In their turn, E-cadherins are suggested to be evolutionary conserved targets of core pluripotency factors, such as *Oct4* (Wagner and Zwaka, 2013); reprogramming studie in mouse revealed that E-cadherin can partially replace *Oct4* (Redmer et al., 2011). These findings point to a possible functional redundancy or a feedback control between the core factors and the adhesion molecules. Presumably, interactions

between E-cadherins and β-catenin can sequester β-catenin, thus reducing signal transduction through WNT pathway. So, loss of cadherin-mediated cell adhesions can promote β-catenin release and emulate elevation of WNT pathway signaling levels. Providing that WNT responses are dose-dependent (Morgani et al., 2017), this may have various effects on pluripotency, depending on cell type (mESCs/ EpiSCs/hESC), and particular levels of accumulated β-catenin (Kurek et al., 2015).

2.4.3 FGF signaling

The inhibiting MAPK/ERK signaling pathway activation downstream of FGF signaling using small molecule inhibitors increased ESCs stability and stemness. FGF4 is also actively produced by ESCs *in vitro*, and it is a direct target of the core pluripotency factors *Oct4, Sox2* and *Nanog* (Nichols et al., 1998). FGF4/ERK signaling pathway activation is crucial for mESCs multi-lineage differentiation; removal of FGF4 blocks ESCs differentiation towards neural and mesodermal lineages (Kunath et al., 2007). However, in the presence of LIF, addition of exogenous FGF4 to mouse ESCs can promote proliferation (Kook et al., 2013). FGF signaling in ESCs may be mediated via ERK, PI3K and Jak-Stat pathways (Lanner and Rossant, 2010). ERK1/2-mediated signaling has a repressive regulatory effect on *Nanog* and a negative impact on pluripotency. While the ERK-mediated FGF signaling is targeting *Nanog* and *CPN*, *Fgf* gene itself appears among the targets of *Nanog* the core pluripotency factors.

2.4.4 TGF-β /BMP signaling

TGF-β/activin/nodal signals via SMAD2/3 are also associated with pluripotency and required for the maintenance of the primed hESCs and mouse epiblast (Guzman-Ayala et al., 2004; James et al., 2005). TGF-β signaling plays an essential role in blastocyst formation in the early mouse embryo (Han et al., 2010). Two major pathways engaged in both human and mouse ESCs include Bmp4 signaling mediated by Smad1/5/8 and Nodal/Activin signaling mediated by Smad 2/3 (Watabe and Miyazono, 2009). In mouse ESCs low levels of Bmp4 in the presence of LIF signaling promote pluripotency states (Galvin-Burgess et al., 2013; Ying et al., 2003), while in the absence of LIF, Bmp4 promotes differentiation (Di-Gregorio et al., 2007). In the presence of LIF Bmp4 can relieve ERK-mediated repression of *Oct4* and suppress differentiation (Morikawa et al., 2016).

2.5 Epigenetic modification and gene regulation

2.5.1 Type of epigenetic modification

Epigenetic modifications of gene expression include DNA methylation, chromatin structures and histone modifications. The impacts of epigenetic modification on the gene expression status have been explained in the following sections (Table 2.1) (Godini et al., 2018).

2.5.1.1 Histone modifications

Histones are involved in DNA packaging via direct protein-DNA interactions. Chromatin structure changes as a result of dynamic processes involving post-translational modifcations (PTMs) at the histone N-terminal tails. Various PTMs including histone acetylation, methylation and phosphorylation as well as less known ubiquitylation, deamination and sumoylation, which affect chromatin packaging and availability to the gene transcription machinery (Rothbart and Strahl, 2014); (Fig. 2.3).

1) Histone acetylation

Histone acetylation occurs on lysine residue of histone 3 and 4 and to less extent on H2A. Acetylation generally alter the charges of lysine side chains from a positive to a null charge (Bannister et al., 2002). This modification potentially weakens the protein-DNA interactions and open structure of chromatins to an active chromatin conformation (Euchromatin) thereby affect the gene expression (Bell et al., 2016). There are two types of enzymes responsible for acetylation/ deacetylation of histones. Histone acetyl-transferase (HATs) and histone deacetylase (HDACs) considered to be activator and silencers of gene expression (Bannister et al., 2002). Histone acetylation is required for embryonic differentiation, where cell fate after division is determined (Dovey et al., 2010).

2) Histone methylation

Histone methylation mainly occurs on lysine (K) and arginine (R) residues. Similar to histone acetylation, methylation is also a reversible reaction (Bannister et al., 2002). Histones methyl transferases (HMT) regulating the methylation of histone on difference residuces. The removal of methyl group could be achieved through different activities including either conversion of methylated arginine into citrulline (Cuthbert et al., 2004; Wang et al., 2004) or direct

demethylation as observed for H3R2 and H4R3 (Chang et al., 2007). Direct demethylation carries out by different enzymes such as lysine demethylases, for removal of methyl group from H3K4me1 and H3K4me2 (Shi et al., 2004) and JMJD2 complex, for removal of tri-methyl from H3K9 and H3K26 (Whetstine et al., 2006).

Methylation of H3K4 and H3K36 induces open histone structure and active gene expression, H3K36me3 is found in the body of the expressive genes (Ernst et al., 2011). On the other hand, methylation of H3K9 and H3K27 are generally associated with the heterochromatin and repressed genes transcription (Perez-Lluch et al., 2015).

3) Histone phosphorylation

Phosphorylation affect charges of histones and thereby interfere with the protein-DNA interactions. The structure of chromatin would be affected by adding a phosphate group to the hydroxyl group of the side chains and increase in the overall negative charges of the histone tails. Histone phosphorylation occurs on serine, threonine and tyrosine residues at the histones N-terminal ends (Sawicka and Seiser, 2012; Rossetto et al., 2012). The level of phosphorylation is determined by the activity of kinases and phosphatases, phosphorylate the histone formation. In stem cells, histone phosphorylation is involved in chromatin remodelling and repairs the damaged DNA during reprogramming process (Sawicka and Seiser, 2014; Srinageshwar et al., 2016).

2.5.1.2 DNA modifications

Generally, DNA methylation is involved in genomic imprinting (Li et al., 1993; Paulsen and Ferguson-Smith, 2001), silencing of gene expression (Ma et al., 2014; Lin et al., 2014), suppression of retrotransposons (Nagamori et al., 2015), X chromosome inactivation (Sharp et al., 2011; Cotton et al., 2015) and chromatin organization (Lim and Maher, 2010; Mattout et al., 2015; Guo et al., 2016) (Figure 2.3). All regions of DNA have potential to be methylation in the promoter (Calo and Wysocka, 2013) and enhancer regions could significantly impact the gene expression (Heyn et al., 2016). Dysregulation in methylation on the promoters is linked to aberrant gene expression that in turn could causes silencing of tumour suppressor genes and activation of oncogenes. However, this view has been challenged by recent studies (Wagner et al., 2014; Moarii et al., 2015).

Table 2.1 Histone modifications and their possible impacts on the gene expression either activation or repression (Godini et al., 2018).

Histones modifications	**H1**	**H2A**	**H2B**	**H3**	**H4**
Methylation	Lys26 (Rep)	None	Lys5 Lys27	Lys4 (Act) Arg2 Arg8 (Rep) Lys9 (Rep) Lys14 Arg17 (Act) Lys23 Lys27 (Rep) Lys36 (Act) Lys79 (Act)	Arg3 (Act) Lys20 (Act) Lys59 (Rep)
Acetylation	None	Lys5 (Act) Lys9 Lys12 Lys15	Lys5 (Act) Lys12 (Act) Lys15 (Act) Lys20 (Act)	Lys4 Lys9 (Act) Lys14 (Act) Lys18 (Act) Lys23 (Act) Lys27 (Act) Lys56	Lys5 (Act) Lys8 (Act) Lys12 (Act) Lys16 (Act) Lys20
Phosphorylation	Ser27 (Act)	Ser1 (Rep) Ser139	Ser14 Ser32 Ser36	Thr3 (Act) Ser10 (Act) Thr11 Ser28 Tyr41 Thr45	Ser1 (Act) His18
Ubiquitylation	None	Lys119	Lys120	None	None
Biotinylation	None	Lys9 Lys13	None	Lys4 (Act) Lys9 (Act) Lys18 (Act)	Lys12

Methylation of cytosine residues by DNA methyltransferase represses transcription and switches genes off. The addition of acetyl groups to histones by histone acetylase activates transcription and switches gene on. Histone modifications represent chemical modifications of residues on histones structures. Two main chemical groups affecting histones are acetyl and methyl groups which lead to opening and closing histone structures, respectively. DNA modification is another important epigenetic modification that happens by methylation of cytosine base of CpG islands. These modifications have several consequent effects on cellular functions, including gene expression, genomic imprinting, and chromatin organization. The third important epigenetic modification is chromatin remodeling which involved in determining of euchromatin or heterochromatin structures of chromosomes.

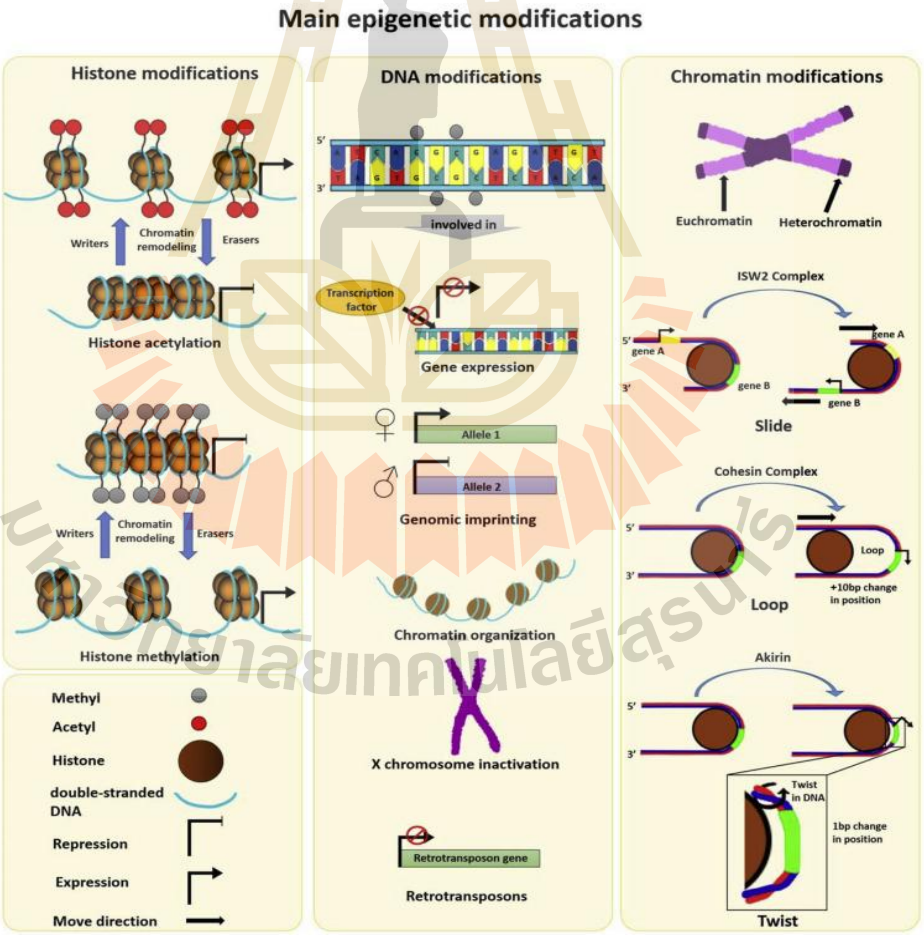

Figure 2.3 A schematic illustration of main epigenetic modifications in cells. (Godini et al., 2018).

2.5.1.3 Chromatin remodelling

DNA is packed in chromatin structures and also provides gene regulation by controlling the accessibility of transcription to the genetic code. There are two types of chromatins include heterochromatin and euchromatin. Euchromatin associated to more relaxed and transcriptionally active, while heterochromatin associated to condensed and repress the gene transcription (Narlikar et al., 2013). Interactions between enzymes involved in chromatin remodelling and histone modifications regulate the chromatin structure (Luo and Dean, 1999). Chromatin remodelling complexes which either moving, ejecting or restructuring nucleosomes. These complexes regulate gene expression via reposition (slide, twist or loop) nucleosomes along the DNA. These actions remove or replaces histone molecules, producing nucleosome-free regions on DNA for gene activation (Wang et al., 2007a). Chromatin remodelling is involved in many key cellular processes, including transcriptional regulation, DNA repair, apoptosis and replication. The main enzymes involved in remodelling the chromatin include cohesion complex, ISW2 complex and Akirin (Hota and Bruneau, 2016; Nowak et al., 2012; Deindl et al., 2013).

2.5.2 Epigenetic modifications and gene regulation

Gene expression depends on the interaction between multiple proteins and transcription factors. Therefore, any components interfering with these interactions could affect gene expression. For example, scanning promoter sequences by many transcription factors (TFs) would be essential to start transcription, while DNA methylation would restrict accessibility to the promoters and thereby down-regulates the gene expression. It is well documented that methyl-CpG binding proteins are involved in transcription repression (Bird and Wolffe, 1999). Contradictory to these observations, methylation has also been observed in the promoter region of active genes (Suzuki and Bird, 2008). This would challenge the old view of direct role of methylation in gene repression. Hence, it appears that epigenetic blockage is an approach to permanently silence a gene or whole chromosome (X-inactivation) in the cell (Li et al., 1993).

In addition to transcriptional events, the exit from or entry into pluripotency involve changes in the epigenetic landscape, including chromatin remodeling and DNA methylation (Kobayashi and Kikyo, 2015). Many components responsible for

altering the epigenetic landscape are under the control of the transcriptional networks, either directly or indirectly (Tsai et al., 2012; Ura et al., 2008; Wu et al., 2014). In this view, the epigenetic regulation occupies lower position in the PGRN network, at least with respect to CPN transcription factors (Figure 2.2). During differentiation, a transition from open to close chromatin configuration may be achieved via the action of Polycomb group repressive complexes (PRC); PRC1 and PRC2 can deposit H3K27me3 repressive mark on histone H3 (Leeb et al., 2010). Protein complexes from Trithorax group are involved into the reverse transition from closed to open chromatin by depositing H3K4me3 activation mark (Ang et al., 2011; Guenther et al., 2010; Kingston and Tamkun, 2015). The core pluripotency transcription factors can directly or indirectly regulate chromatin modification/ remodeling systems. Thus, *Oct4* and *Sox2* negatively regulate Ezh2, as well as certain other components of PRC2 (Wu et al., 2014), (see Figure 2.2). Control of DNA methylation on gene promoters containing CpG islands represents another layer of epigenetic regulation essential for pluripotency. Components of the methylation system such as DNA methyltransferase Dnmt1 are responsible for maintaining DNA methylation patterns during pluripotency and are directly controlled by *Oct4* and *Nanog* (Tsai et al., 2012). Elements of de novo DNA methylation (Dnmt3b) are integrated into a network, controlled by *Oct4, Sox2* and *Nanog* (Tan et al., 2013). Taken together, epigenetic factors, such as PRC/Trithorax complexes, Nucleosome Remodeling Deacetylase complexes (NuRD) (Kaji et al., 2006; Liang et al., 2008; Rais et al., 2013), and DNA methylation systems are under direct or indirect control of transcriptional networks in general and CPN in particular. Typically, during cell differentiation, irreversible epigenetic changes (commitment) come into the play later than the activation of the corresponding transcriptional programs (priming). This may suggest that the epigenetic levels occupy somewhat lower hierarchical positions and respond to differentiation (input) stimuli after transcriptional networks (Pera and Tam, 2010) (see Figure 2.2).

2.5.3 Epigenetic regulation in ESCs and iPSCs

The pluripotent state of ESCs is enforced by epigenetic factors closely linked to the pluripotency transcription factor network (Young, 2011; Orkin and Hochedlinger, 2011). Resetting the epigenetic state of somatic cells to that of ESCs is

one of the ultimate tasks for the reprogramming factors in iPSC generation. The epigenetic factors involved in maintaining the pluripotency of ESCs must be activated through the reprogramming process. Furthermore, epigenetic modulating strategies must be used to overcome the inherent somatic epigenetic state. Therefore, some epigenetic factors may function specifically to erase somatic epigenetic statuses.

ESCs display distinctive chromatin features related to its unique properties. The chromatin in ESCs is in an open state, with more accessible chromatin domains and less heterochromatin foci. In contrast, highly condensed heterochromatin foci are prevalent in lineage-committed somatic cells (Meshorer et al., 2006; Efroni et al., 2008). Consistent with this, genome-wide distribution of repressive histone modifications is less prevailing in ESCs, compared with differentiated cells (Hawkins et al., 2010; Wen et al., 2009) and active histone modifications are more abundant in ESCs (Efroni et al., 2008; Krejci et al., 2009).

Reprogramming of somatic cells, through ectopic expression of four transcription factors (Oct4, Sox2, Klf4 and c-Myc), would result in generation of iPSCs. It has been shown that reprograming is the result of resetting gene expression patterns as well as altering DNA methylation and histone modifications states in the original somatic cells (Lewitzky and Yamanaka, 2007). During iPSC generation, the somatic cell chromatin needs to be reorganized to an ESC-like state with loosely organized heterochromatin and abundant euchromatin modifications (Fussner et al., 2011; Mattout et al., 2011). It appears that the chromatin reorganization events take place in a coordinated and sequential manner. Rearrangement of the heterochromatin, characterized by the presence of histone H3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein (HP1), precedes the activation of *Nanog*, while enrichment of euchromatin marks occurs concurrently with *Nanog* activation (Mattout et al., 2011). Consistently, heterochromatin is rearranged and becomes dispersed when partially reprogrammed cells are converted to iPSCs by dual inhibition of MEK and GSK3 (Fussner et al., 2011). Thus, chromatin reorganization from the somatic state to an ESC-like one seems to be required for the activation of pluripotency circuitry. However, such a drastic chromatin rearrangement appears to have a substantial latency in the reprogramming process. Detailed characterization of

these changes at the molecular level is difficult due to the low percentage of somatic cells that can be successfully converted to iPSCs.

Histone-modifying enzymes play important roles in regulating ESC identity and the iPSC generation process. Histone modifications are thought to function by either directly affecting higher-order chromatin configurations or mediating chromatin-related processes through recruiting specific binding proteins (Kouzarides, 2007). Histone acetylation can potentially open up chromatin by neutralizing the positive charge of histone lysine residues. Consistent with this function, histone acetylation is highly enriched in ESCs compared with differentiated cells (Efroni et al., 2008; Krejci et al., 2009), indicating that it contributes to the open chromatin state in ESCs. Consistently, treatments of HDAC inhibitors have been shown to enhance nuclear dynamics, reduce differentiation propensity (Melcer et al., 2012) and support the self-renewal program in ESCs (Ware et al., 2009).

Histone methylation is closely linked to transcription. Methylations on different residues and sometimes to different degrees (i.e. tri-, di- or mono-) represent differential transcriptional statuses. H3K9 methylation is associated with transcription silencing and heterochromatin formation. Genome-wide localization studies have shown that the genomic domains marked with H3K9me3 are substantially expanded in differentiated cells compared with ESCs (Hawkins et al., 2010), and long-range silenced genomic regions marked by H3K9me2 are also increased upon differentiation (Wen et al., 2009). It has been shown that, upon exiting the pluripotent state, H3K9-specific HMT Kmt1c (also called G9a) contributes to the silencing of the Oct4 locus by forming heterochromatin structure and recruiting the *de novo* DNA methylation machinery (Epsztejn-Litman et al., 2008). While Kmt1c plays a role in differentiation-induced silencing, H3K9me3/2-specific histone demethylases (HDMs), Kdm3a and Kdm4c (also called Jhdm2a/Jmjd1a and Jhdm3c/Jmjd2c, respectively) are essential for maintaining the ESC identity. Knocking down either Kdm3a or Kdm4c in ESCs blocks ESC self-renewal and leads to differentiation. In ESCs, Kdm3a regulates a distinct set of pluripotency genes, including *Tcl1, Tcfcp2l1* and *Zfp57*, while Kdm4c contributes to the activation of Nanog (Loh et al., 2007). H3K27me3 is a repressive modification placed by PRC2. Much attention has been drawn to its role in ESCs for its involvement in the

“bivalent” domain, which is coined by the coexistence of the repressive mark H3K27me3 (Bernstein et al., 2006). Genes that harbor the bivalent domain are transcriptionally silenced in ESCs, suggesting a potentially dominant role of H3K27me3. In ESCs, genes with bivalent domain include a substantial number of differentiation-related genes targeted by the core pluripotency factors (Azuara et al., 2006; Bernstein et al., 2006). Consistently, bivalent differentiation-related genes are bound by PRC2 components in ESCs (Bracken et al., 2006; Boyer et al., 2006). Recruitment of PRC2 to these targets are directed by the PRC2-associated protein Jarid2 or Mtf2 (Landeira et al., 2010; Li et al., 2010; Walker et al., 2011). In addition, part of the targets repressed by PRC2 are further occupied by PRC1, which establishes another repressive mark, histone H2AK119 ubiquitylation (H2AK119ub) (Bracken et al., 2006; Landeira et al., 2010; Ku et al., 2008). The recruitment of PRC1 to these loci is thought to fortify the gene repression during differentiation (Ku et al., 2008).

While iPS cells have been shown to be similar to ES cells, several articles have suggested that iPS cells differ from ES cells in their gene expression profiles (Chin et al., 2009), persistence of donor-cell gene expression (Ghosh et al., 2010; Marchetto et al., 2009), and differentiation abilities (Feng et al., 2010; Hu et al., 2010). It has been reported that, following the reprogramming of iPSCs, epigenetic memory is inherited from the parental cells (Kim et al., 2010; Polo et al., 2010; Lister et al., 2011; Doi et al., 2009; Ohi et al., 2011). Despite the fact that iPSCs exhibit many features of the ESCs, an epigenetic memory of the original somatic cells, might still remain in their genomes (Papp and Plath, 2011). Surprisingly, they harbour altered DNA methylation pattern and histone modifications when compared to ESCs. This indicates an incomplete erasure of epigenetic memory in iPSCs and such somatic memory may reverse the whole processes of induction as indicated by reversal of the whole reprogramming process by removing these factors (Prilutsky et al., 2014).

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CHAPTER III

EPIGENETIC REPROGRAMMING OF RABBIT INDUCED PLURIPOTENT STEM CELLS TOWARD THE NAÏVE STATE

3.1 Abstract

Rabbit induced pluripotent stem cells (rbiPSCs) possess the characteristic features of primed pluripotency as defined in rodents and primates. We tested the hypothesis that epigenetic modification by inhibition of histone deacetylase (HDACi) and inhibition of histone 3 lysine 9 methyltransferase (H3K9mei) would enhance reprogramming rbiPSCs toward the naïve state. In the present study, we reprogrammed B19-rbiPSCs using epigenetic modifiers and cultured them in a resetting medium on feedder-free system. This new culturing protocol resulted in transcriptional and epigenetic reconfiguration, as substantiated by the expression of naïve markers including *Oct4, Sox2, Klf4, Stat3, Esrrb, Dppa5, Kdm4a* and the presence of histone modifications associated with naïve. Furthermore, H3K9 methyltransferase inhibition and HDAC inhibition promote reprogramming by downreguration of H3K9 trimethylation (H3K9me3) and upreguration of H3K14 acetylation (H3K14ac). The reprogrammed cells showed decreased percentage of cells showing X inactivation, these inhibitors can reactivate the silent X chromosome.

3.2 Introduction

PSCs can exist in two distinct states, designated as the naïve and primed states (Nichols and Smith, 2009). Induced pluripotent stem cells (iPSCs) derived from humans and NHPs also exhibit primed pluripotency-like characteristics (Nichols and Smith, 2009; Vallier et al., 2005; Wianny et al., 2008). Several studies have reported on the reversion of conventional hESCs and iPSCs to naïve-like pluripotency using different combinations of transcription factors (NANOG, KLF2 and STAT3), chemical inhibitors of various kinases (ROCK, GSK3, MEK1/2, SRC, $p38^{MAPK}$, BRAF and JNK) and growth factors (FGF2, LIF and Activin A). The reprogrammed cells, known variously as

NHSM (Gafni et al., 2013), 3iL (Chan et al., 2013), 6i/L/A (Theunissen et al., 2014), Reset (Takashima et al., 2014), and TL2i (Chen et al., 2015). These reprogrammed cells display some characteristic features of naïve pluripotent rodent stem cells, including reconfigured transcriptome and epigenome, alterations in mitochondrial respiration (Takashima et al., 2014), loss of FGF2 and ERK dependency (Takashima et al., 2014; Chen et al., 2015), and gain of leukemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (STAT3) dependency (Chan et al., 2013; Takashima et al., 2014; Chen et al., 2015).

RbiPSCs exhibit the characteristic features of primed pluripotency. They are dependent on FGF2 signaling and growth factors of the TGF-β (activin, nodal) for self-renewal which showed that activin/nodal signaling through Smad2/3 activations was necessary for maintaining the pluripotent status of rbESCs (Honda et al., 2009; Osteil et al., 2013; Wang et al., 2008; Wang et al., 2007). In the present study, we reprogrammed of primed rbiPSCs toward the naïve state in a process called resetting. Chemical resetting of primed to a naïve pluripotent state is one such method and has come to the forefront as a simple, efficient, and transgene-free method to induce naïve pluripotency. The process involves the transient application of a histone deacetylase inhibitors and histone 3 lysine 9 methyltransferase inhibitor to initiate resetting, followed by the emergence of nascent naïve pluripotent stem cells in supportive conditions, and finally the stabilization and expansion of naïve pluripotent stem cell cultures.

3.3 Materials and methods

3.3.1 Reagents

All chemical compounds and cell culture reagents were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA), Thermo Fisher Scientific (Waltham, MA, USA), Tocris Bioscience (Avonmouth, Bristrol, UK), Bio-techne, R&D system and Novus Biologicals (Minneapolis, Minnesota, USA), Abcam (Cambridge, UK), Cell signaling (Danvers, Massachusettes, USA), Molecular Probes (Eugene, Oregon, USA) and MedChem Express (Bar Hill, Cambridge, United Kingdom), respectively. The cell culture ware was obtained from Corning Life Sciences (Tewksbury, Massachusetts, USA), unless stated otherwise.

3.3.2 B19-rbiPS cells

B19-EOS cells were produced by somatic cell reprogramming of adult rabbit fibroblasts with the human transcription factors OCT4, SOX2, c-MYC, and KLF4, followed by infection with a lentiviral vector, L-SIN-EOS-C(3)-EiP (EOS) (Hotta et al., 2009), and subsequent selection of EOS-expressing cells using puromycin (Osteil et al., 2013). In the EOS vector, the GFP and puromycin resistance genes are under the transcriptional control of a minimal early transposon promoter and trimer of CR4 enhancer of mouse Oct4. The CR4 enhancer overlaps a distal enhancer whose activity is associated with naïve-like pluripotency (Yeom et al., 1996). RbiPSCs were cultured on mitomycin C treated MEFs in "A" medium composed of DMEM/F12 medium supplemented with 20% knockout serum replacement, 1% non-essential amino acids (NEAA), 1% of a solution of 10,000 U/ml penicillin + 10,000 U/ml streptomycin + 29.2 mg/ml L-glutamine (PSG), 1 mM sodium pyruvate, and 10 mM ß-mercaptoethanol. This medium was freshly supplemented with 10 ng/ml FGF2 before use. rbiPSCs were routinely dissociated into single-cell suspensions after treatment with 0.05% trypsin–EDTA.

3.3.3 Feeder preparation

Mouse embryonic fibroblasts (MEFs) were prepared from -12.5day-old embryos from the OF 1strain (Charles River) as described by Afanassieff et al (2014). Frozen-thawed a vial of OF1 MEFs and plate the cells in three 100-mm culture dishes, each containing 10 mL of fresh fibroblast medium composed of DMEM medium supplemented with 10% fetal bovine serum, 1% NEAA, 1% PSG, and 10 mM ß-mercaptoethanol. The cells were cultured at 37°C under humidified 5% CO_2 in air for 72 h. On day 4, replaced the culture medium over MEFs with 5 mL of 5 ug/mL mitomycin-C. The cells were incubated at 37°C under humidified 5% CO_2 in air for 2–3 h and removed the mitomycin-C, then washed five times with 5 mL of phosphate buffer saline (PBS). Then, to each dish, added 1 mL of 0.05% trypsin–EDTA and incubated for 5 min at 37°C. After incubation, added 1 mL of fibroblast medium to each dish to stop the enzymatic reaction. The cell were dissociated by repeated pipetting. After that the cell suspension was transferred into a 15-mL Falcon tube containing 10 mL of fibroblast medium, and centrifuged for 5 min at 300xg, and resuspended the cell pellet in 10 mL of fresh medium. The cells were counted using

a Countess™ 3 Automated Cell Counter (Thermo Fisher Scientific) and plated onto gelatin-coated dishes at a density of $1.6x10^4$ cells/cm^2. The cells were incubated overnight at 37°C under humidified 5% CO_2 in air before use. Inactivated MEFs (MEF-MMC) must be used within 3 days.

3.3.4 Chemical resetting protocol

3.3.4.1 N2B27 conditioning medium (N2B27CM) preparation (Guo et al., 2017)

MEF-MMC at concentration of $4x10^6$ cells in fibroblast medium were plated on dish (diameter 10 cm) and incubated at 37°C under humidified 5% CO_2 in air at lease 3 h before used. After incubation, cells were washed with PBS before adding N2B27 $_{basal\ medium}$ 25 ml/dish supplemented with 20 ng/ml FGF2 and incubation at 38°C under humidified 5% CO_2 and 5% O_2. After 24 h incubation, N2B27CM was collected before adding new fresh N2B27 $_{basal\ medium}$ 25 ml/dish supplemented with 20 ng/ml FGF2 (repetition 3 times in one original MEF-MMC dish). N2B27CM was kept at 4°C until the third collection of CM, then filtered and stored at -20°C before used. The composition of N2B27 $_{basal\ medium}$(500 ml) is 48.7% of DMEM/F12, 48.7% of Neurobasal, 1% of B27, 0.5% of N2 (home made), 0.02% of ß-mercaptoethanol, and 1% of PSG.

3.3.4.2 "A" conditioning medium (ACM) preparation

Rabbit induced pluripotent stem cells medium or "A"$_{medium}$ 25 ml/dish supplemented with 20 ng/ml FGF2 was added on MEF-MMC ($4x10^6$ cells) and incubated at 38°C under humidified 5% CO_2 and 5% O_2. The same procedure as for the N2B27CM was used to produce, filtered and stored ACM.

3.3.4.3 Resetting medium and supplement (modified by Guo et al., 2017)

1) Chemical resetting medium 1 (CRM1)

ACM supplemented with 10 ng/ml human LIF (hLIF, home made) and 1 mM valproic acid (VPA, Sigma 1069-66-5).

2) ALGÖX resetting medium (ALGÖX)

N2B27CM supplemented with 10 ng/ml Activin A, 10 ng/ml hLIF, 2.5 uM GÖ6983 (GÖ, Bio-techne 2285), and 2.5 uM XAV939 (XAV, Sigma 3004).

3) AR6GÖX resetting medium (AR6GÖX)

N2B27CM supplemented with 10 ng/ml Activin A, 10 ng/ml IL6 + 20 ng/ml IL6-Receptor (R6, home made), 2.5 uM GÖ6983 and 2.5 uM XAV939.

4) VALGÖX resetting medium (VALGÖX)

ALGÖX supplemented with 250 ug/ml L-ascorbic acid (Vitamin C, Sigma A8960).

5) AALGÖX resetting medium (AALGÖX)

ALGÖX supplemented with 2 uM A366 (MedChem Express HY-12583).

6) Histone deacethylase inhibitors: 1 mM VPA (Sigma 1069-66-5), 2 uM BRD4354 (BRD, Tocris 6010), and 5 uM UF010 (UF010, Tocris 5588).

7) Methylation inhibitors and demethylation: 2 uM A366 (H3K9 methyltransferase inhibitor), and 250 ug/ml Vitamin C (DNA demethylation).

8) Signaling inhibitors: 0.25 uM SB590885-BRAFi (MedChem express HY-10966), 10 uM SB203580-P38i (MedChem express HY-10256), and 4 uM SP600125-JNKi (MedChem express HY-12041).

3.3.4.4 Resetting medium and supplement (modified by Guo et al., 2017)

The process can be separated into three steps including initiation of resetting with medium containing HDAC inhibitors, emergence of reprogrammed cells, stabilization of reprogrammed cells.

1) A comparative study between CRM1 and VALGÖX.

For resetting, cells were grown on 6-well culture plate pre-treated with matrigel (Beckton Dickinson, New Jersey, USA). Cells were dissociated into single cells by incubation with 0.05% trypsin-EDTA. Cells were resuspended in CRM1 or VALGÖX supplemented with VPA (HDAC inhibitor) and plated at density $1x10^5$ cells/well of 6 well-plate. Cells were cultured under humidified 5% O_2, 5% CO_2 at 38°C for 2 days. Fresh medium was replaced daily and at day 2 cells in both groups were cultured with VALGÖX for another 4 days. Then cells were passaged (P0) and cultured in VALGÖX and passaged every 3 days (P1 and P2), with medium changed daily. At days 6, 9 and 12, reprogrammed cell pellet were kept for gene expression analysis (Fig. 3.1A).

2) Study the effect of signaling inhibitors.

Same timing and processes, cells were applied to analyze the effects of signaling inhibitors. At first 2 days, cells were cultured in VALGÖX supplemented with VPA. After that medium was replaced with VALGÖX without any supplement or supplemented with single signaling inhibitors (SB590885, SB203580 or SP600125), (Fig. 3.3A).

3) Study the effect of HDAC inhibitors.

Cells were grown and cultured using the same previous protocol. Except that cells were resuspended in VALGÖX supplemented with single HDAC inhibitors (VPA, BRD4354 or UF010) and two days later, medium was replaced by VALGÖX supplemented with signaling inhibitor (SP600125), (Fig. 3.5A).

4) A comparative study between DNA demethylation (Vitamin C) and H3K9 methylation inhibitor (A366) in initiate resetting.

Always using the same protocol, cells were grown in ALGÖX medium supplemented with either Vitamin C or A366. The HDAC inhibitors (VPA, BRD4354, or UF010) were used at the first 2 days and then replaced by the signaling inhibitors (SP600125), (Fig. 3.7A).

5) Study the effect of epigenetic modifiers (A366, BRD4354 or UF010) in ALGÖX or AR6GÖX.

In this last study, the effect of epigenetic modifiers (A366, BRD4354 or UF010) was examined by adding them to ALGÖX or AR6GÖX medium. ALGÖX or AR6GÖX medium supplemented with epigenetic modifiers were used to culture the cells at the first 4 days and then cultured the cells without the epigenetic modifiers. Reprogrammed cells at days 4 and 14 were analyzed by RT-qPCR and Immunofluorescens (Fig. 3.9A).

3.3.5 Analysis of the expression of pluripotency gene by RT-qPCR

Total RNA was extracted from cell pellets using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription and specific target amplification were performed using Applied Biosystems™ High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Real-time PCR (qPCR) was performed using the StepOnePlus real-time PCR system and Fast SBYR® Green Master Mix (Applied Biosystems, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Following 40 amplification cycles,

melt-curve analysis was used to verify that only the desired PCR products had been amplified. PCR efficiency for both target and reference genes was determined from the relative quantitative values for calibrator normalized target gene expression using StepOnePlus Software V2.3 (Applied Biosystems). Expression of the target genes was normalized to that of the *rabbit TATA-box binding protein (Tbp)* gene. All primers used for RT-qPCR are shown in Table 3.1 (Tapponier et al., 2017 and Osteil et al., 2013).

Table 3.1 List of primers used for gene analysis by RT-qPCR.

Gene	Forward primer (5'>3')	Reverse primer (5'>3')
*Tbp**	CTTGGCTCCTGTGCACACCATT	ATCCCAAGCGGTTTGCTGCTGT
Oct4	GCAGCAGATCAGCCACATC	AACAGTCACTGCTTGATCGTTTG
Nanog	CACTGATGCCCGTGGTGCCC	AGCGGAGAGGCGGTGTCTGT
Sox2	GAGAACCCAAGATGCACAAC	CCGTCTCCGACAAAAGTTTCC
Klf4	TCCGGCAGGTGCCCCGAATA	CTCCGCCGCTCTCCAGGTCT
Stat3	CTTCCTGCAAGAGTCCAATGTTC	GATTCGGGCAATCTCCATTGG
Esrrb	CGTGGAGGCCGCCAGAAGTA	TCTGGCTCGGCCACCAAGAG
Kdm4a	GCCGCTAGAAGTTTCAGTGAG	GCGTCCCTTGGACTTCTTATT
Dppa5	GAGGTGCTGCAGGTACAG	GCTCAATGTAAGGGATTCGAGAAC
Ooep	GGTGGCGGACTTGATCTTTG	GTGCGTGGACCGTGATTTC
Cdh3	GCCCCGCCCTATGACTCCCTAT	GAAGCGGCTGCCCCACTCG
Tbxt	AGCAAAGTCAAGCTCACCAATAAG	TCCGGGGCTCATACTTGTG
Otx2	CGCCTTACGCAGTCAATGG	GAGCGCTTCCAGCACATC

* Reference gene

3.3.6 Analysis of pluripotency markers (Tapponier et al., 2017)

For immunofluorescens, the B19-EOS rbiPS cells were grown on cover slips pre-treated with gelatin and coated with feeder cells. For reprogrammed cells were grown on cover slips pre-treated with matrigel. The cells were fixed with %4 PFA in PBS at room temperature for 20 min, permeabilized with %0.5 Triton X-100, and washed 3 times (10 min each) in PBS. Non-specific binding sites were blocked

using PBS supplemented with %2 bovine serum albumin for 1 h. The cells were then incubated overnight at °4C with primary antibodies (rabbit anti-H3K14ac, 1:400 dilution, # 52946Abcam; rabbit anti-H2AK119ub, 1:400 dilution, #8240 Cell signaling; rabbit anti-H3K9me3, 1:400 dilution, #ab8898 Abcam; sheep anti-KDM4A, 1:100 dilution, #AF6434 R&D system; mouse anti-SOX2, 1:400 dilution, #4900 Cell signaling; mouse anti-ESRRB, 1:100 dilution, #PP-H6705-00 Novus Biologicals; goat anti-OTX2, 1:100 dilution, #AF1979 R&D system; rabbit anti-TBXT, 1:1600 dilution, # 81694 Cell signaling). After three times rinsed with PBS and hold with PBS 10 min, the cells were incubated with fluorochrome-conjugated secondary antibodies (anti-rabbit, anti-mouse, anti-goat, or anti-sheep coupled with AlexaFluor 555 or 647; Molecular Probes) at RT for1 h in dark condition. After three times rinsed with PBS and hold with PBS 10 min, DNA was counterstained using DAPI1) :5000 dilution, Molecular Probes). Cover slips were mounted with mounting medium M1289 (sigma), the cells examined with a confocal laser-scanning microscope (DM 6000 CS SP5; Leica), using excitation laser wavelengths -475, -555and -647nm. Acquisitions were performed using an oil immersion objective (40X/0.75 1.25, PL APO HCX and 1.5 x zoom factor; Leica). Tiled scans were automatically acquired using LAS AF software (Leica). Images were acquired with a resolution of 1,024 x 1,024, a slow laser speed (400 Hz) and a frame average of 3 for high quality images. Fluorescent intensities were quantified using Basic Intensity Quantification with FIJI software. At least 3 repeats of each immunofluorescens were performed and more than 100 cells analyzed.

3.3.7 Statistical analysis

Data are represented as arithmetic mean ± SD. P. values only less than 0.05 ($P<0.05$) were considered significant. Error bars indicate standard errors of the means.

3.4 Results

3.4.1 A comparative study between CRM1 and VALGÖX+VPA in initiation of reprogramming

The reprogramming process of B19-rbiPSCs toward the naïve state can be initiated with CRM1 : ACM supplemented with HDAC inhibitors (VPA) and the cytokine leukaemia inhibitory factor (LIF) or VALGÖX : N2B27 conditioning medium

supplement with DNA demethylase (vitamin C), TGF-β/Activin signaling (Activin A), LIF, Protein kinase C inhibitor (GÖ6983), and Tankyrase inhibitor (XAV939) supplemented also with VPA and then the culture could be maintained with VALGÖX without inhibitors (Fig. 3.1A).

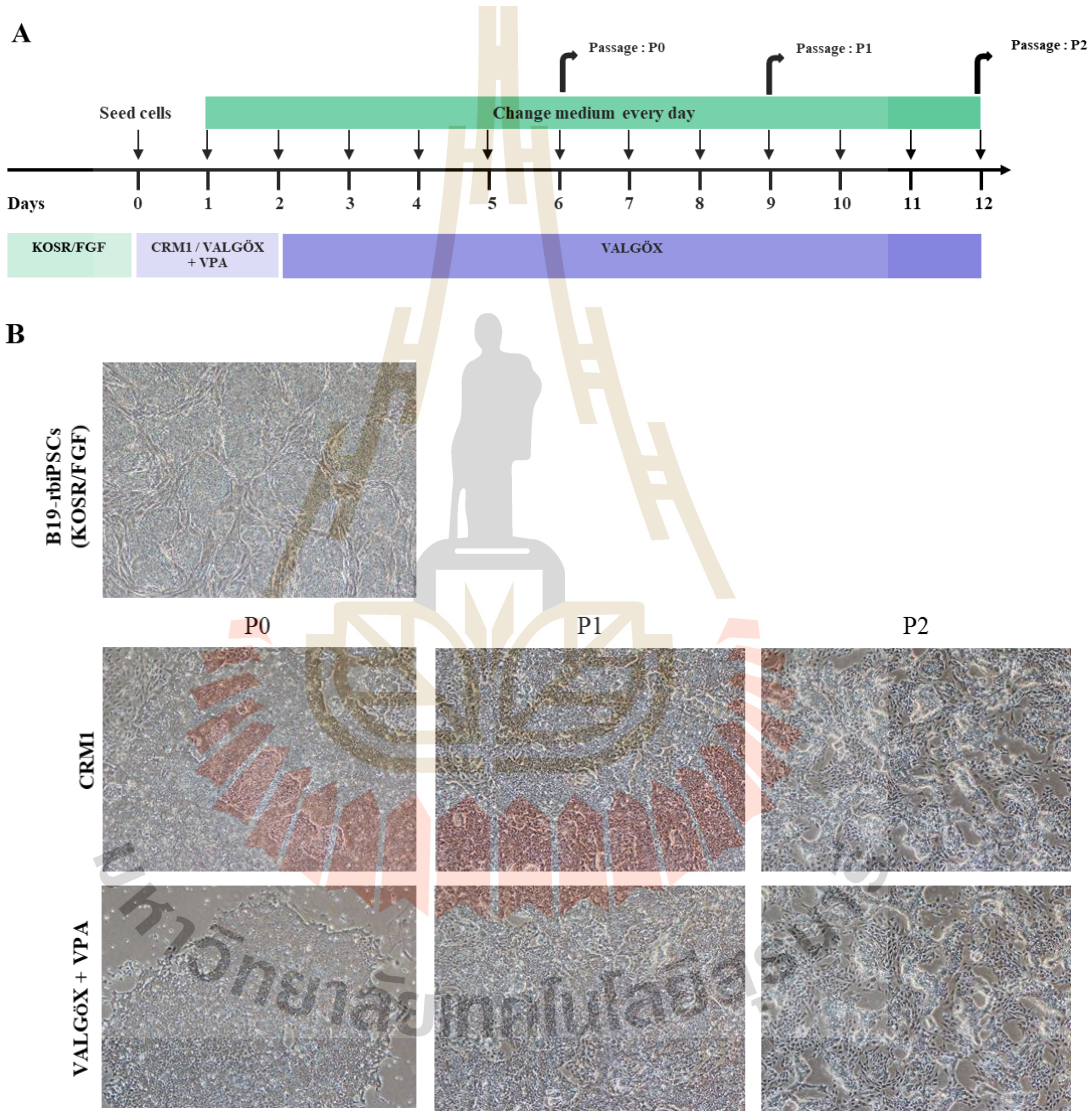

Figure 3.1 Reprogramming B19-rbiPSCs with HDACi (VPA). (A) Schematic of the initiation of reprogramming process with medium containing VPA. (B) Images of B19-rbiPSCs in KOSR/FGF (Control) and first 3 passages of reprogrammed rbiPSCs with VPA. Magnification of 40x.

In the absence of feeders, we found that reprogrammed cells can be expanded in both CRM1 and VALGÖX+VPA media (Fig 3.1B). We subsequently studied the effect of those media containing VPA on the expression of core and naive genes (Fig. 3.2).

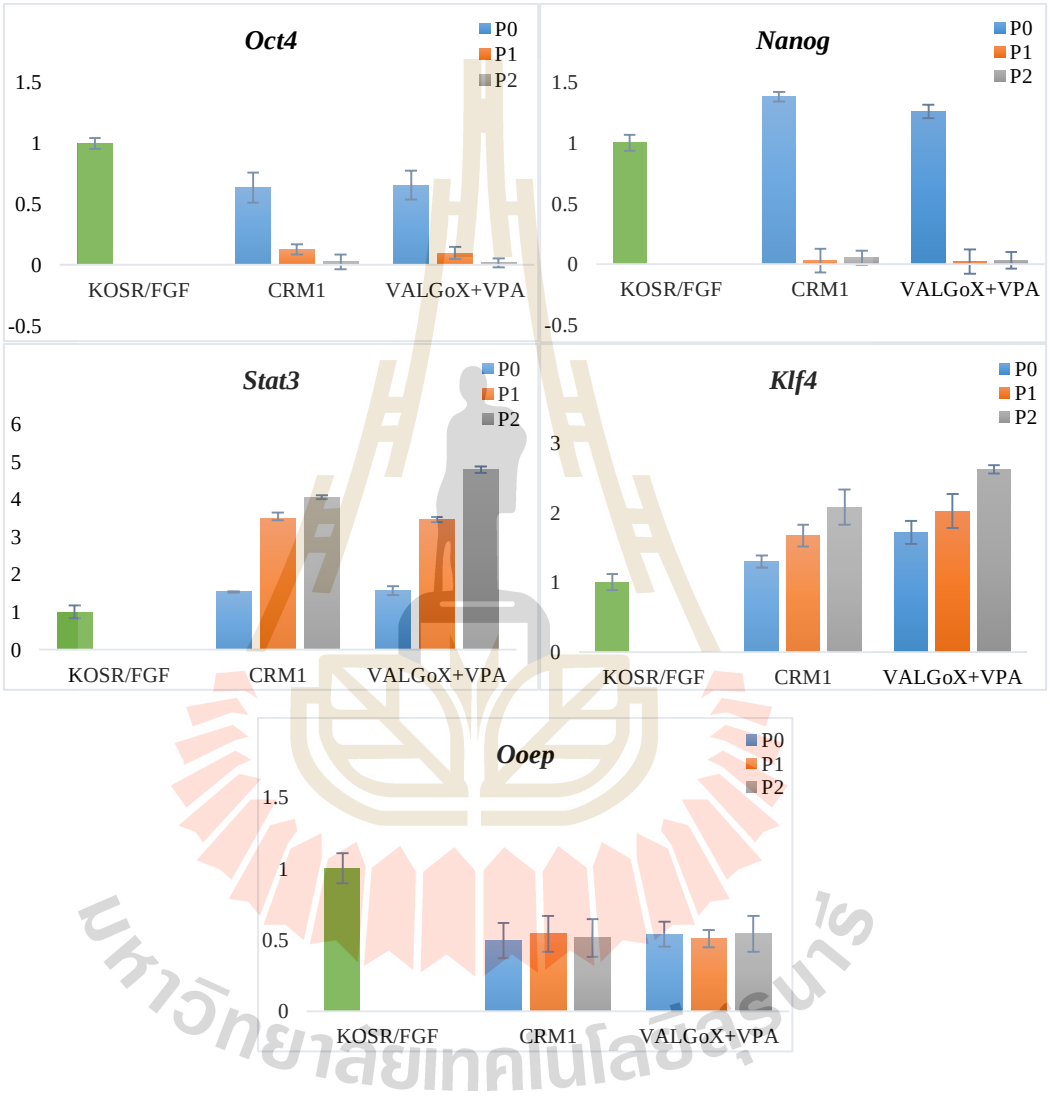

Figure 3.2 RT-qPCR analysis of general (*Oct4* and *Nanog*) and naïve (*Stat3, Klf4* and *Ooep*) pluripotency markers in various reset cell cultures. Error bars indicate SD of three independent experiments.

B19-rbiPSCs reprogrammed in CRM1 and VALGÖX+VPA significantly lower expression of core pluripotent gene (*Oct4*) than control group (B19-rbiPSCs in KOSR/FGF), but no significant difference was found between cells grown in CRM1 or

in VALGÖX+VPA. For the expression of core pluripotent gene (*Nanog*) cells in CRM1 and VALGÖX+VPA at P0 showed significantly higher rate than that control group, but cells in CRM1 and VALGÖX+ VPA no significantly differences. In CRM1 and VALGÖX+VPA cells showed in all 3 passages significantly higher expression of naïve pluripotent genes (*Stat3* and *Klf4*) than control group. While the expression of naïve gene marker (*Ooep*) was significantly lower in CRM1 group comparing with that in control group and was not significantly different with that of VALGÖX+VPA group. We also observed that with using CRM1 for the first 2 days, cells presented more differentiation than using VALGÖX+VPA directly.

3.4.2 The effect of signaling inhibitors

We therefore implemented a revised reprogramming by using initially VALGÖX+VPA then VALGÖX+ signaling inhibitors: BRAF inhibitor (SB590885), p38 inhibitor (SB203580) and Jun N-terminal kinase (SP600125) (Fig. 3.3A).

B19-rbiPSCs reprogrammed in these conditions showed consistent feeder-free expansion with typical naïve-like morphology and gene marker profiles (Fig. 3.3B and 3.4). We found that some reprogrammed cells have improved morphology in the presence of SP600125.

B19-rbiPSCs reprogrammed in all experimental groups had significantly lower expression of *Oct4* than control group. The expression of *Nanog* was significantly higher in VALGÖX+SP600125 group at P0 and P1 comparing with that in control group, while the cells in VALGÖX, VALGÖX+SB590885, and VALGÖX+SB203580 groups presented significantly lower expression of *Nanog* than control group. For the expression of *Stat3*, the cells in all experimental groups showed not significantly differences except for cell in VALGÖX+SB590885, and VALGÖX+SB203580 at P0. The cells in VALGÖX+SP600 at P1 and P2, VALGÖX+SB203580 at P2 showed significantly higher expression of *Klf4* than control group. For the expression of *Ooep*, cells in all groups had significantly lower rates than the control group.

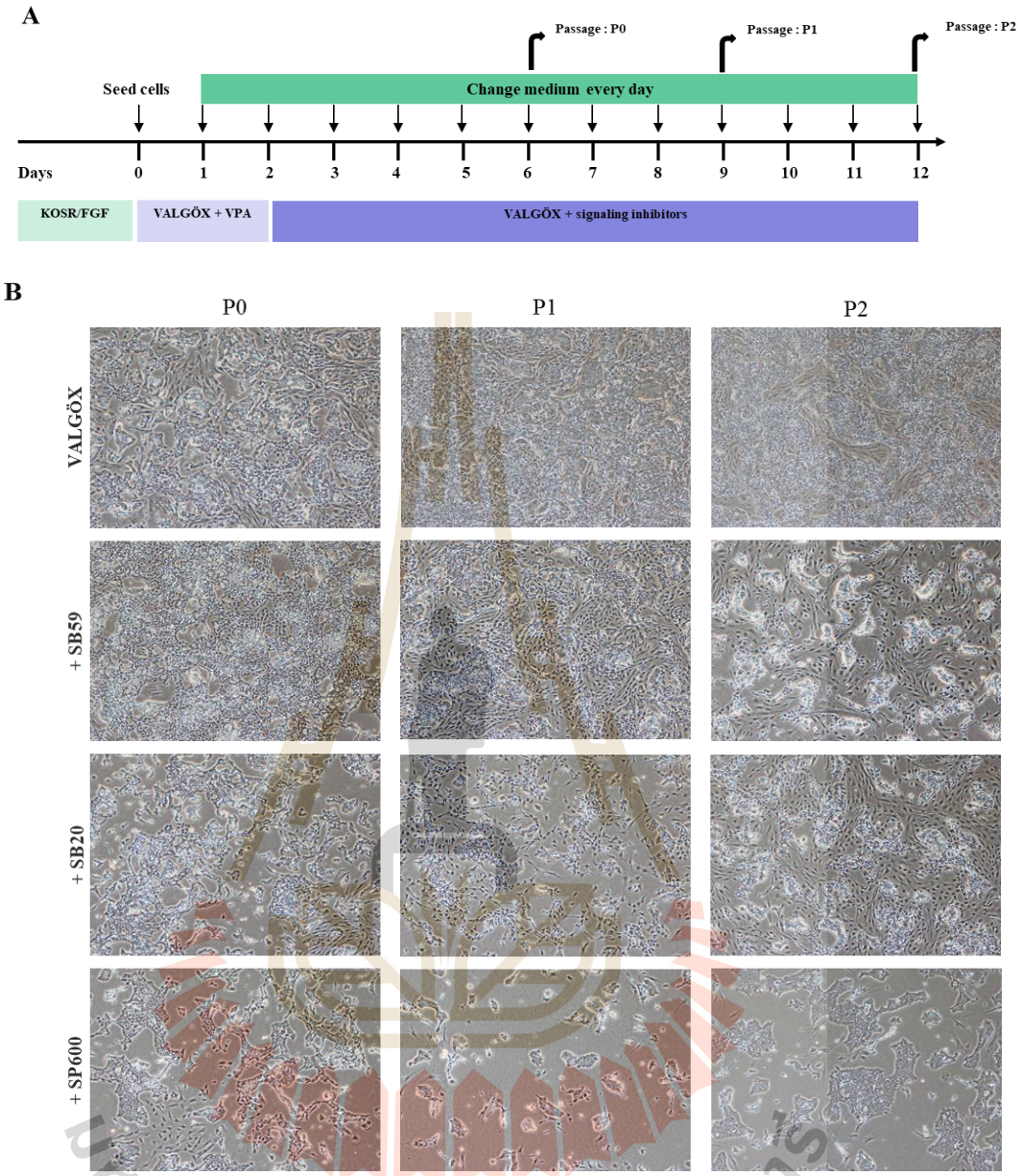

Figure 3.3 Reprogramming B19-rbiPSCs with HDACi (VPA) and signaling inhibitors. (A) Schematic of the emergence and stabilization stages with medium containing signaling inhibitors; BRAFi (SB590885), p38i (SB203580), JNKi (SP600125). (B) Images of first 3 passages of reprogrammed rbiPSCs with VPA and signaling inhibitors. Magnification of 40x.

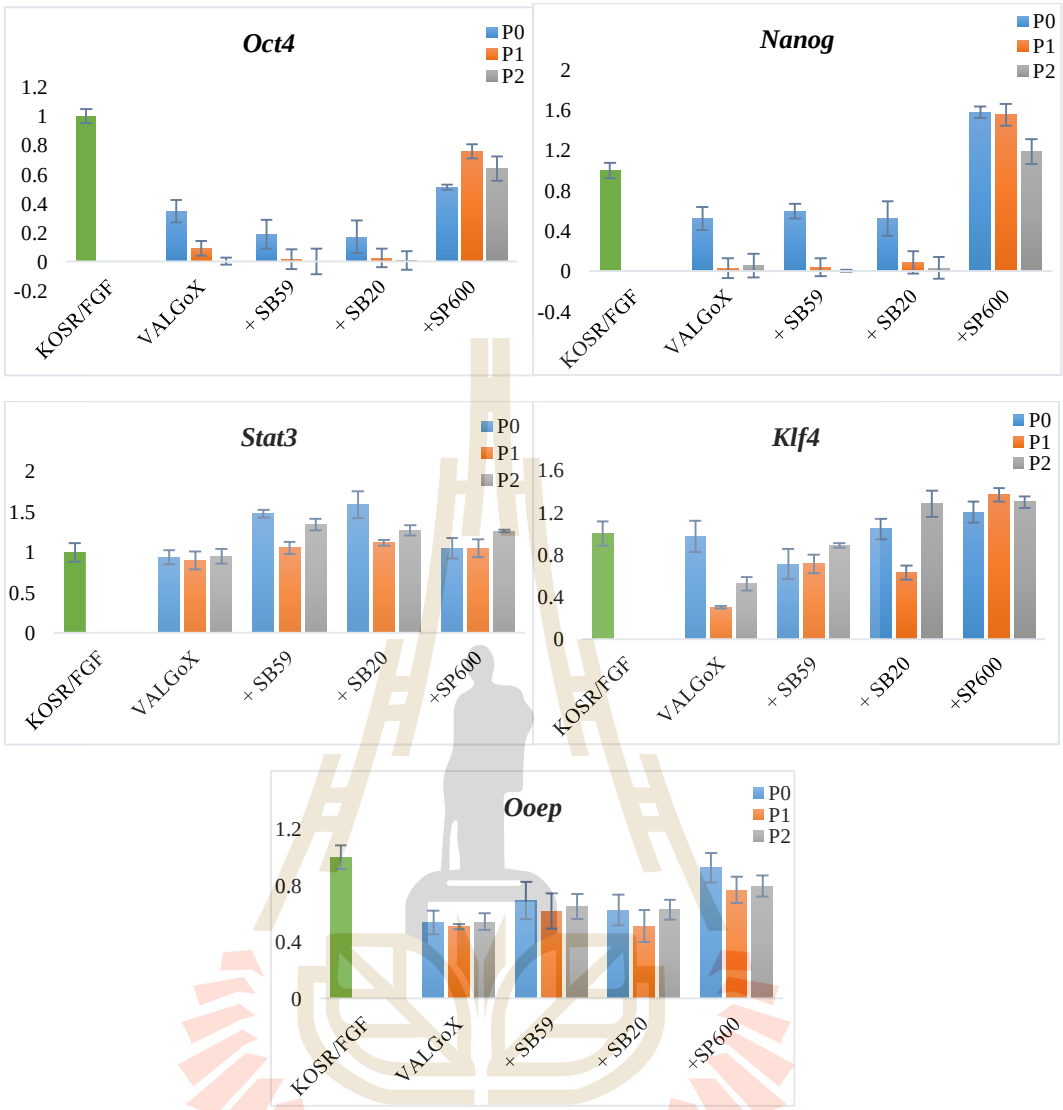

Figure 3.4 RT-qPCR analysis of general and naïve pluripotency markers in various reset cell cultures. Error bars indicate SD of three independent experiments.

3.4.3 The effect of HDAC inhibitors in VALGÖX for initiation of reprogramming

We investigated whether exposure to HDAC inhibitors would promote conversion of rabbit primed cells to the naïve state. We applied valproic acid (VPA), BRD4354 or UF010 during the initiation phase of reprogramming. When cells were treated for 2 days with VALGÖX supplemented with HDACi, then with VALGÖX+SP600125 medium (Fig. 3.5A). We found that reprogrammed cell had

improved morphology in the presence of BRD4354 and UF010 at passage 2 were shown in Fig. 3.5B.

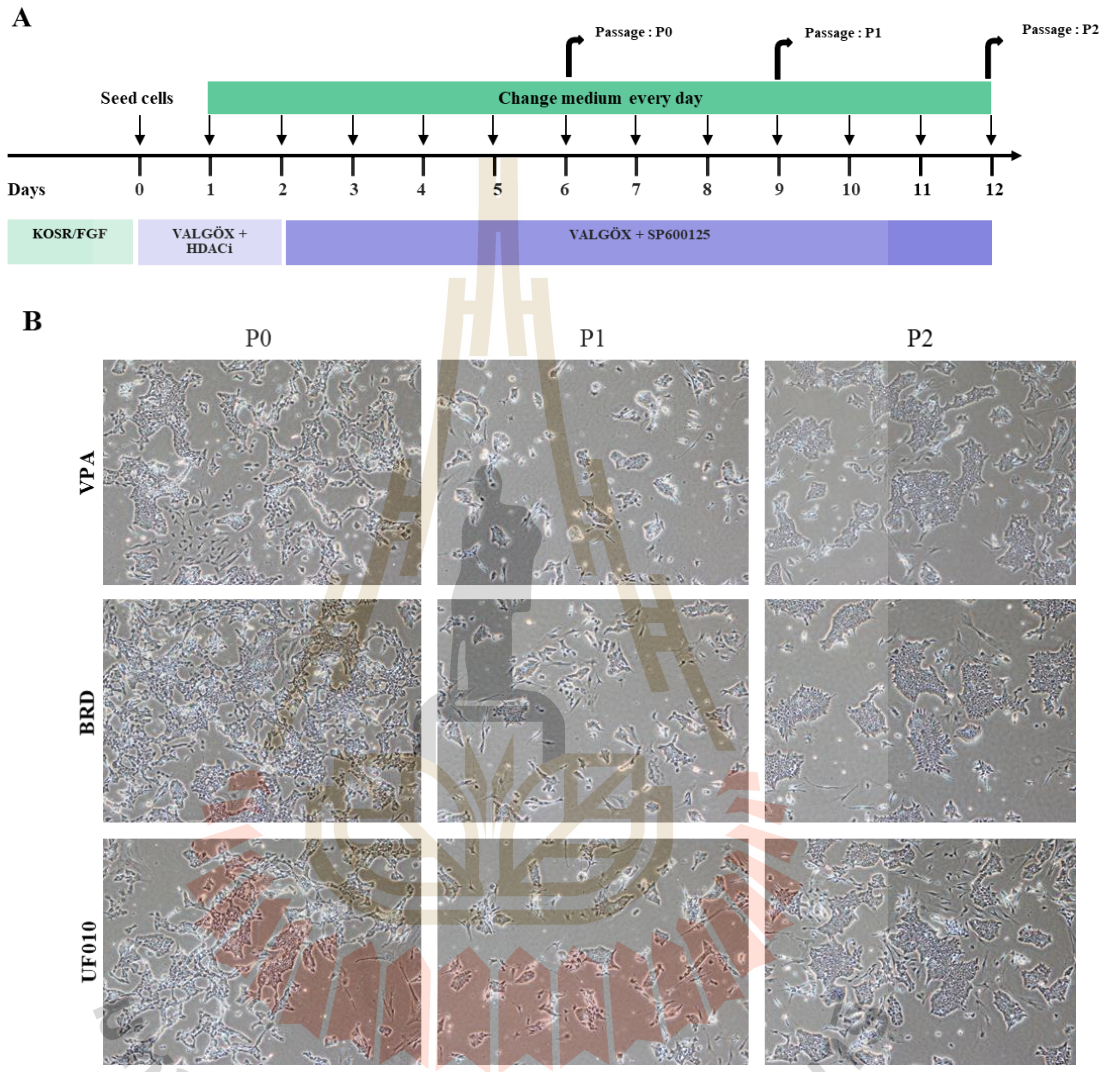

Figure 3.5 Reprogramming B19-rbiPSCs with HDACi. (A) Schematic of the initiation resetting protocol with medium containing HDACi; VPA, BRD4354, UF010 in the initiation phase. (B) Images of first 3 passages of reprogrammed rbiPSCs with VPA, BRD4354, or UF010. Magnification of 40x.

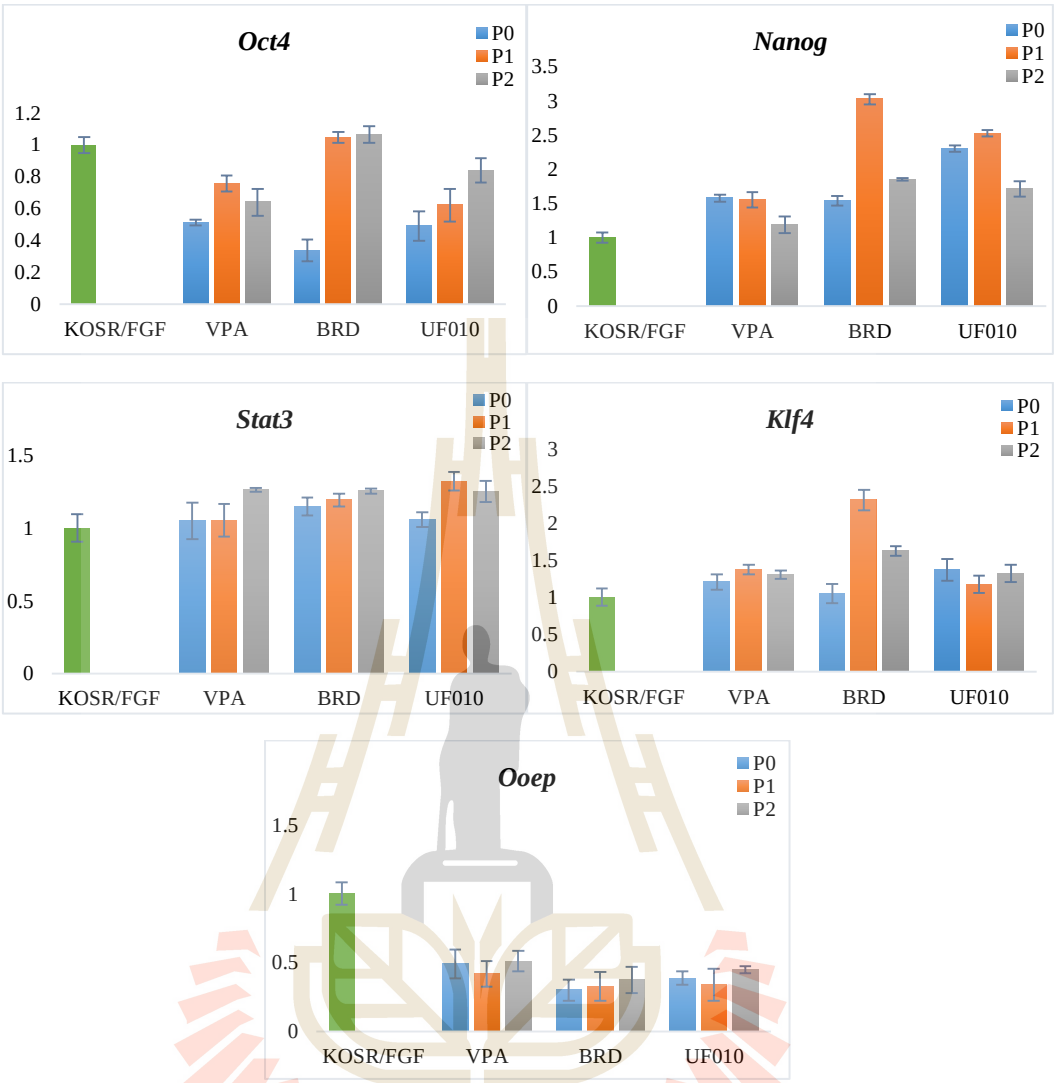

Figure 3.6 RT-qPCR analysis of general and naïve pluripotency markers in various reset cell cultures. Error bars indicate SD of three independent experiments.

We monitored the expression of *Oct4, Nanog,* naïve markers *Stat3, Klf4* and *Ooep* during reprogramming of B19-rbiPSCs with HDAC inhibitors. RT-qPCR analysis (Fig. 3.6) shows that the cells in BRD4354 group at P1 and P2 had significantly higher expression of *Oct4* than control group, but the cells in VPA and UF010 groups presented significantly lower expression of *Oct4* than control group. For the expression of *Nanog*, the cells in VPA, BRD4354 and UF010 groups for all 3 passages had significantly higher expression than control group. The cells in VPA, BRD4354, and

UF010 groups at P2 presented significantly increased expression of *Stat3* when compared with control group. The expression of *Klf4* was significantly higher in BRD4354 group at P1 comparing with that in control group. For the expression of *Ooep*, cells in all groups had significantly lower rates than the control group.

3.4.4 The effect of DNA demethylation (Vitamin C) and H3K9me inhibitors (A366)

We investigated whether DNA demethylase (vitamin C) or H3K9 methyltransferase inhibitor (A366) were required for resetting. Vitamin C or A366 were added in N2B27 conditioning medium with HDAC inhibitors for the first 2 days of reprogramming B19-rbiPSCs (Fig 3.7A and B). We found that some reprogrammed cell showed improved morphology of cells in the presence of A366 with HDAC inhibitors BRD.

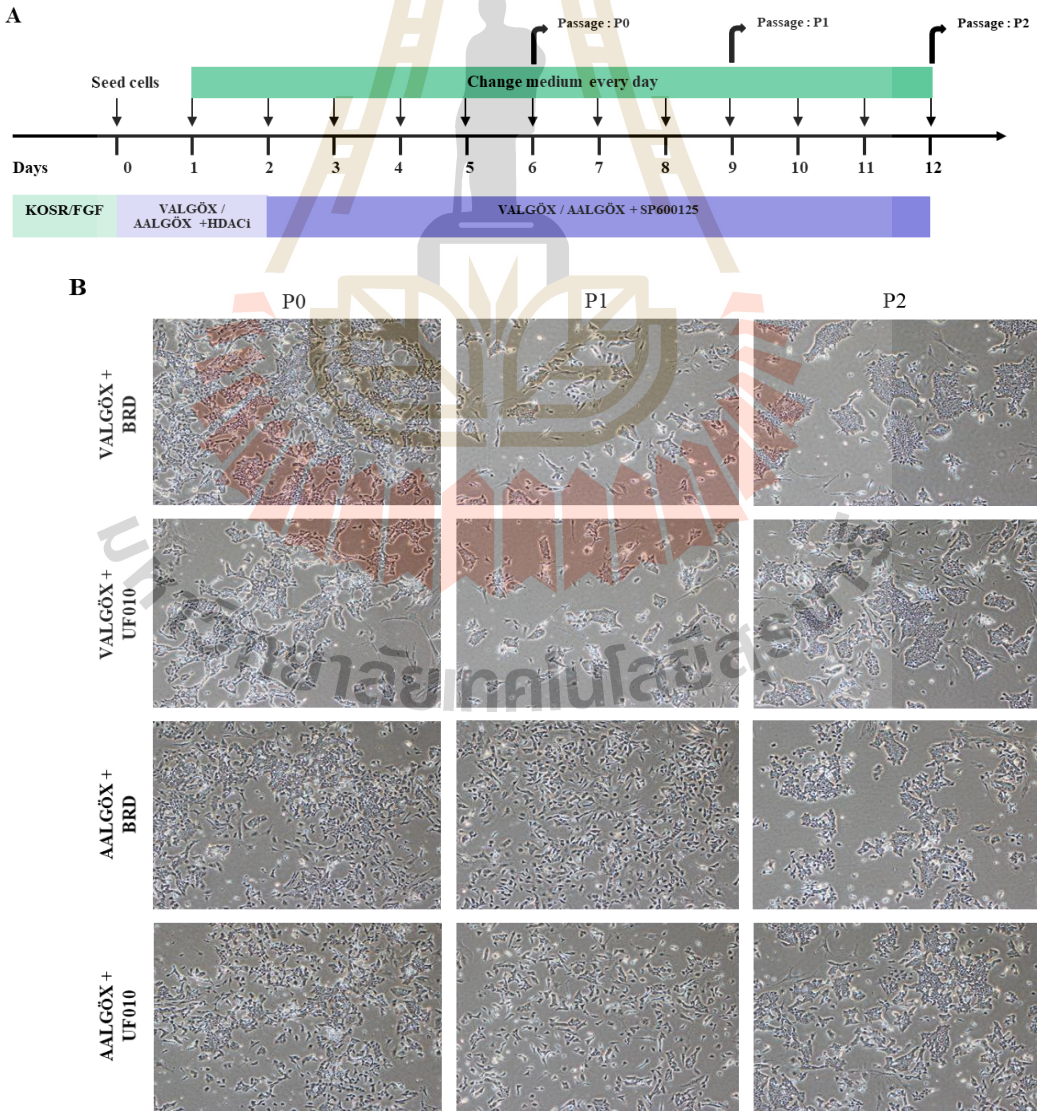

Figure 3.7 Reprogramming B19-rbiPSCs using DNA demethylation (Vitamin C) or H3K9 methylation inhibitor (A366) at the beginning of the process. (A) Schematic of the initiation resetting protocol with medium containing vitamin C or A366. (B) Images of first 3 passages of reprogrammed rbiPSCs with VALGÖX or AALGÖX medium containing HDAC inhibitors (BRD, or UF010). Magnification of 40x.

We examined the mRNA expression of B19-rbiPSCs reprogrammed with DNA methylase (vitamin C) or H3K9 methyltransferase inhibitor (A366) supplemented with HDAC inhibitors (BRD4354, UF010) by RT-qPCR analysis shown in Fig 3.8.

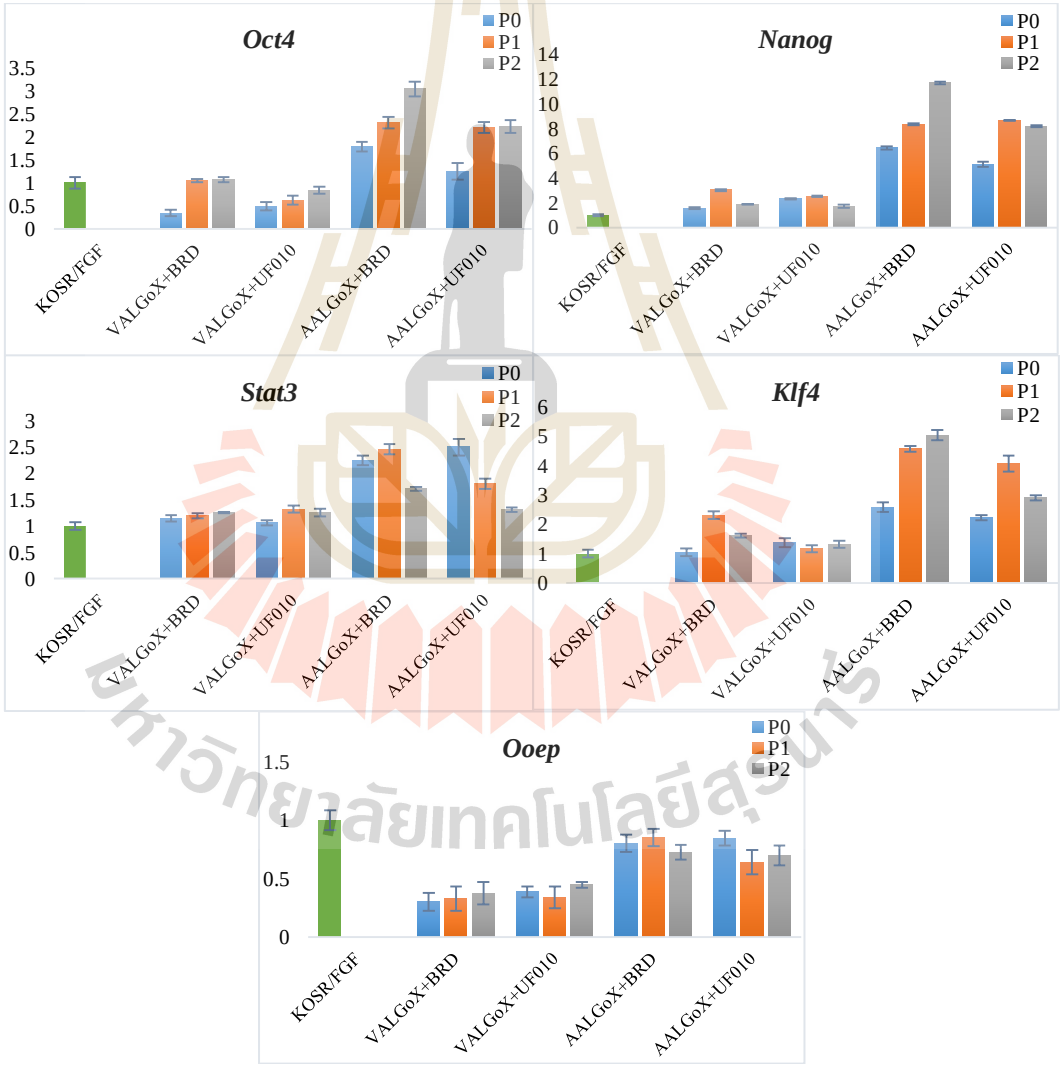

Figure 3.8 RT-qPCR analysis of general and naïve pluripotency markers in various reset cell culture. Error bars indicate SD of three independent experiments.

The cells in ALGÖX supplemented with A366, displayed increased expression of *Oct4, Nanog,* naïve markers *Stat3, Klf4* and *Ooep* than cells cultured in ALGÖX supplemented with vitamin C. While the cells in AALGÖX supplemented with HDAC inhibitors BRD presented significantly higher expression of *Oct4, Nanog,* naïve markers *Stat3* and *Klf4* than cells of control group. In contrast, the cells in all experimental groups cultured in ALGÖX supplemented with vitamin C showed not significantly difference in the expression of *Oct4, Nanog,* naïve markers *Stat3,* and *Klf4* with cells in the control group.

3.4.5 The effect of epigenetic modifiers (A366, BRD4354, UF010) in ALGÖX or AR6GÖX

To gain insights into signaling pathways involved in pluripotency maintenance, B19-rbiPSCs were propagated for 7 passages (14 days) in N2B27 conditioning medium with small molecules that activated STAT3 via gp130 [LIF or R6 (IL6-IL6R)], and also supplemented with inhibitors of H3K9 methyltransferase (A366), TGF-β (Activin A), PKC inhibitors (GÖ6983) and tankyrase inhibitors (XAV939) Fig 3.9A.

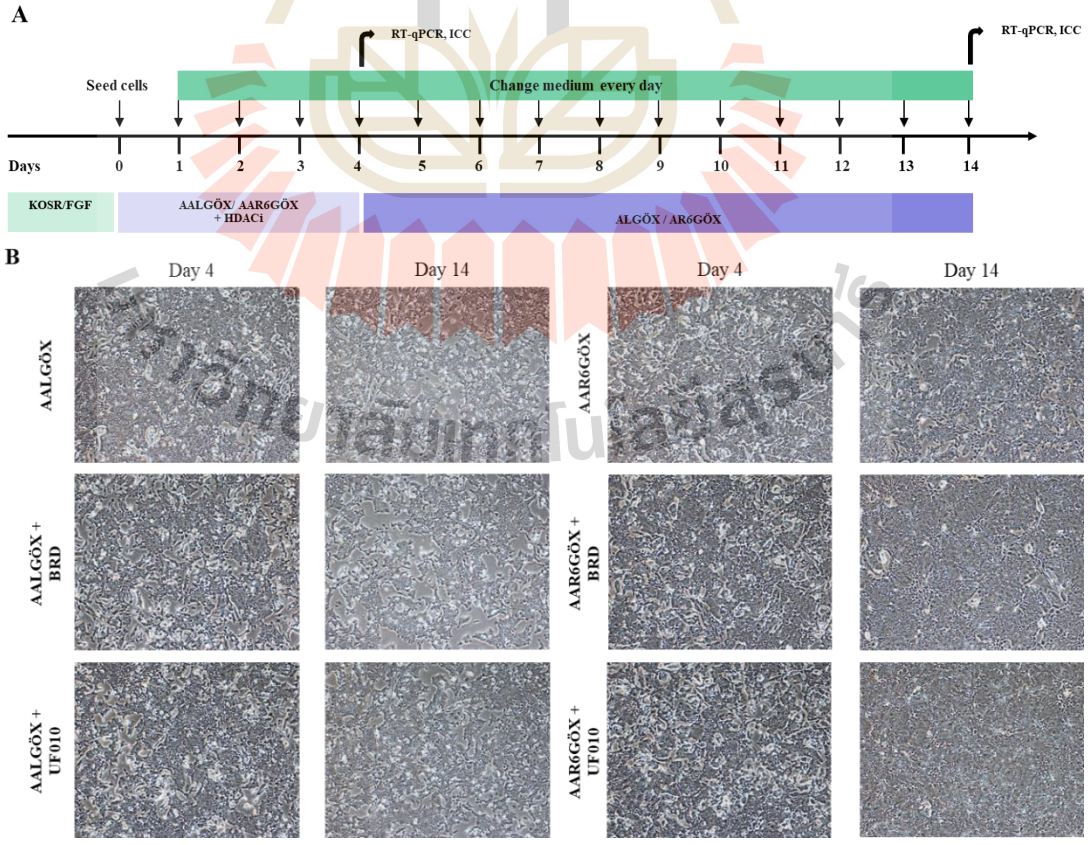

Figure 3.9 Reprogramming B19-rbiPSCs using H3K9 methylation inhibitor and HDACi to initiate the processus in media containing LIF or R6. (A) Schematic of the initiation resetting protocol with medium containing LIF or R6 and supplement with H3K9mei (A366) and HDACi (BRD4354 or UF010). (B) Images of reprogrammed rbiPSCs at day 4 and day 14 in AALGÖX or AAR6GoX with HDACi (BRD4354, or UF010). Magnification of 40x.

We observed that B19-rbiPSCs grown in the AALGÖX medium showed growth rate similar to that of ALGÖX medium (Fig 3.10). Cells grown in the AALGÖX medium supplemented with HDAC inhibitor (BRD4354) showed slightly increased growth rate. In contrast, B19-rbiPSCs propagated in AALGÖX medium containing HDAC inhibitor (UF010) showed dramatically increased growth rate.

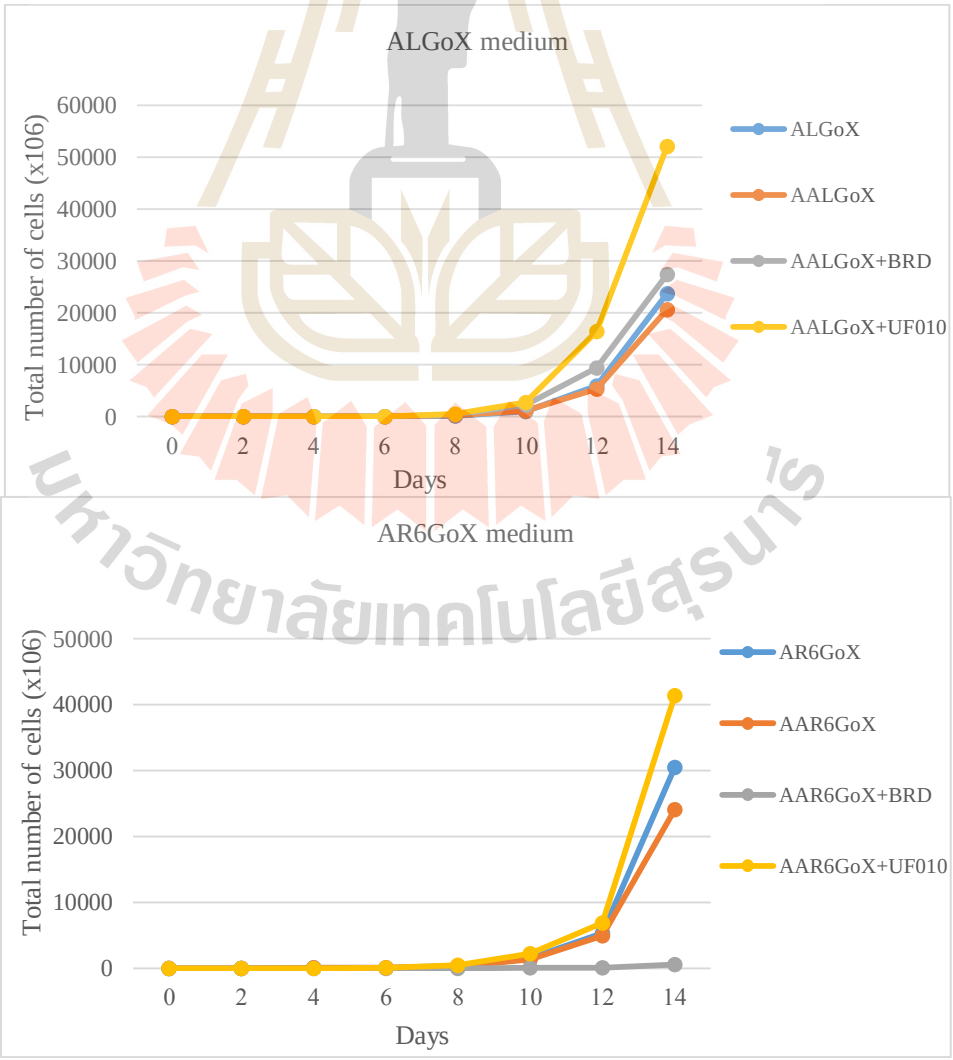

Figure 3.10 Growth rate of B19-rbiPSCs after propagation for 14 day in the ALGÖX or AR6GÖX media with or without H3K9 methylation inhibitor A366 and HDAC inhibitors BRD4354, or UF010.

Propagation of these cells in the AALGÖX medium in the presence of HDAC inhibitors : BRD4354 resulted in increased mRNA expression of *Oct4, Sox2,* naïve markers *Klf4, Esrrb, Kdm4a, Stat3* (Fig. 3.11 and 3.12) and in decreased expression of primed markers *Cdh3, Tbxt, Otx2* (Fig. 3.13).

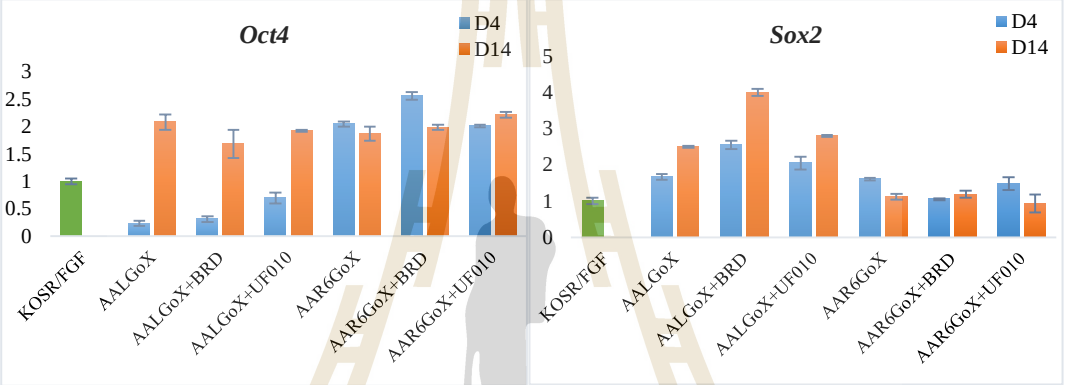

Figure 3.11 RT-qPCR analysis of core pluripotency markers in various cell cultures. Error bars indicate SD of three independent experiments.

At day 4 of reprogramming process, the expression of *Dppa5* was significantly higher in AAR6GÖX with or without HDAC inhibitors comparing with that in AALGÖX with or without HDAC inhibitors and control group. Moreover the cells at day 14 cultured in medium without HDAC inhibitors and H3K9me inhibitor showed significantly decreased *Dppa5* expression when compared with cells at day 4 cultured in medium with HDAC inhibitors and H3K9me inhibitor. The cells in AALGÖX with or without HDAC inhibitors presented significantly higher expression of naïve marker *Stat3* than all experimental groups in AAR6GÖX and control group. These results indicated that LIF were more effective to induced the expression of *Stat3* than R6 (IL6+IL6R).

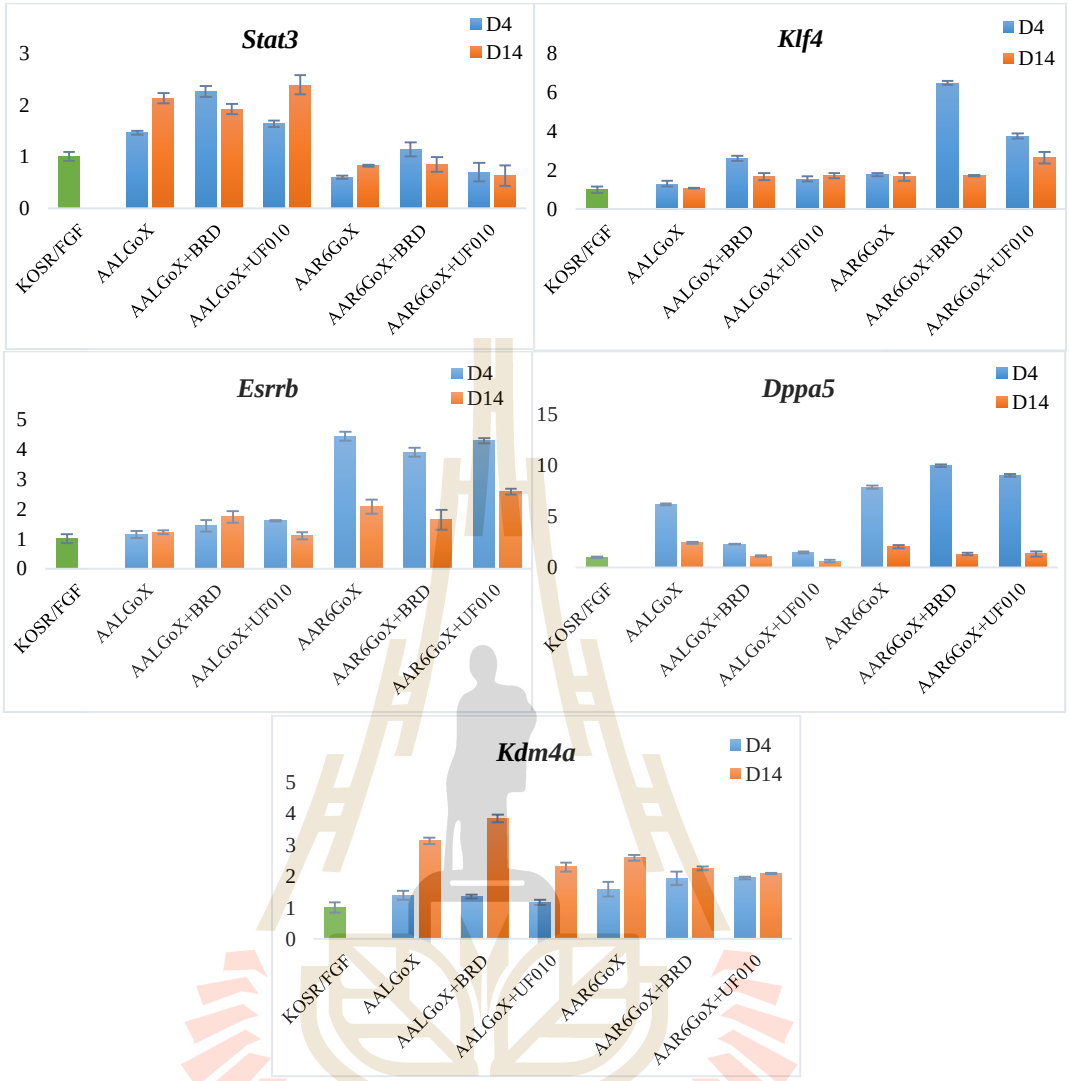

Figure 3.12 RT-qPCR analysis of naïve pluripotency markers in various reset cell cultures. Error bars indicate SD of three independent experiments.

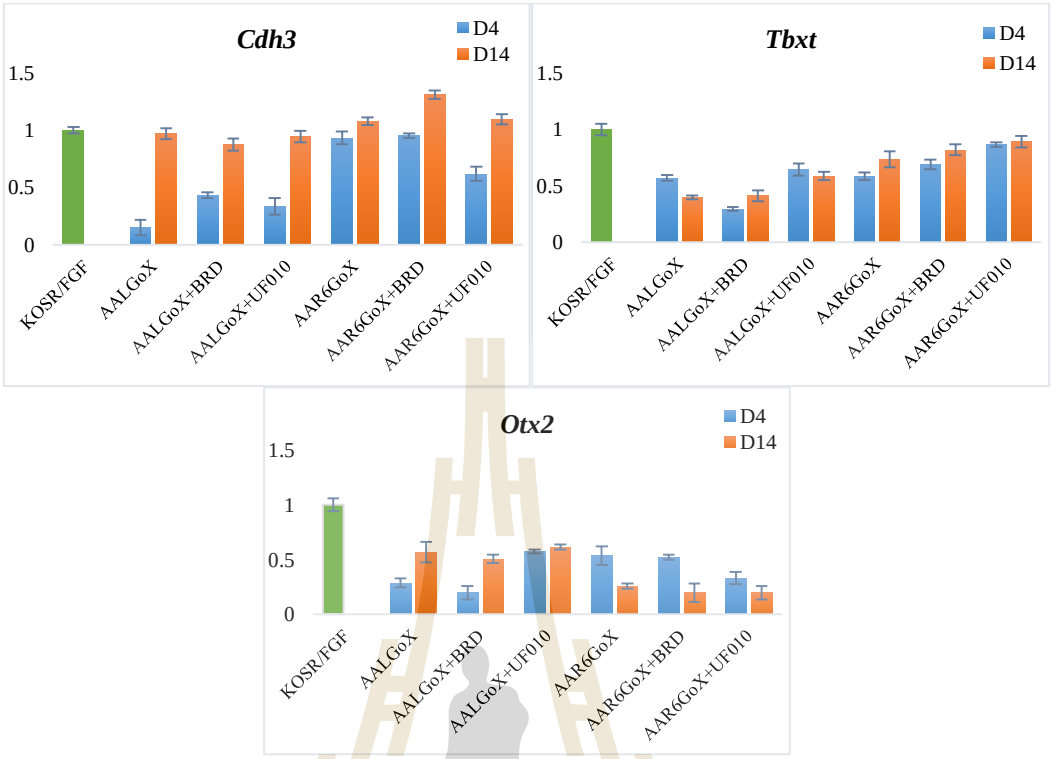

Figure 3.13 RT-qPCR analysis of prime pluripotency markers in various reset cell cultures. Error bars indicate SD of three independent experiments.

Immunostainning of B19-rbiPSCs reprogrammed with AALGÖX medium with HDAC inhibitors showed a strong increase of Sox2 proteins at day 14 compared with B19-rbiPSCs of control group, which was confirmed by imaging and quantification (Fig 3.14 and 3.15). Esrrb, a marker of naïve pluripotency in mice (Dunn et al., 2014) was strongly expressed in AAR6GÖX with or without HDAC inhibitors at day 14 compared with control group.

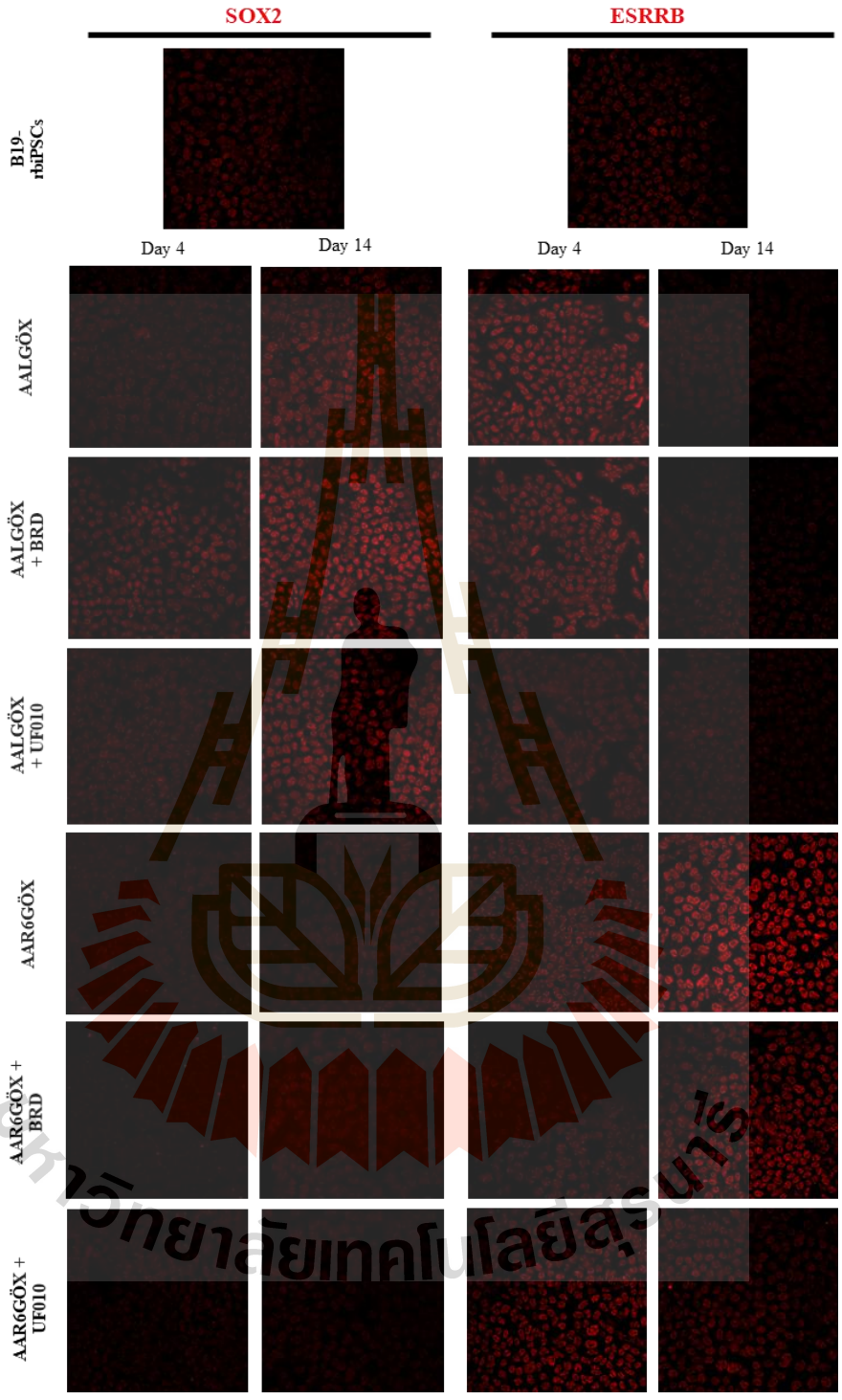

Figure 3.14 Immunostaining for pluripotentcy markers; core: SOX2 and naïve: ESRRB during reprogramming of B19-rbiPSCs with H3K9 methylation inhibitor and HDACi in media containing LIF or R6. Magnification of 400x.

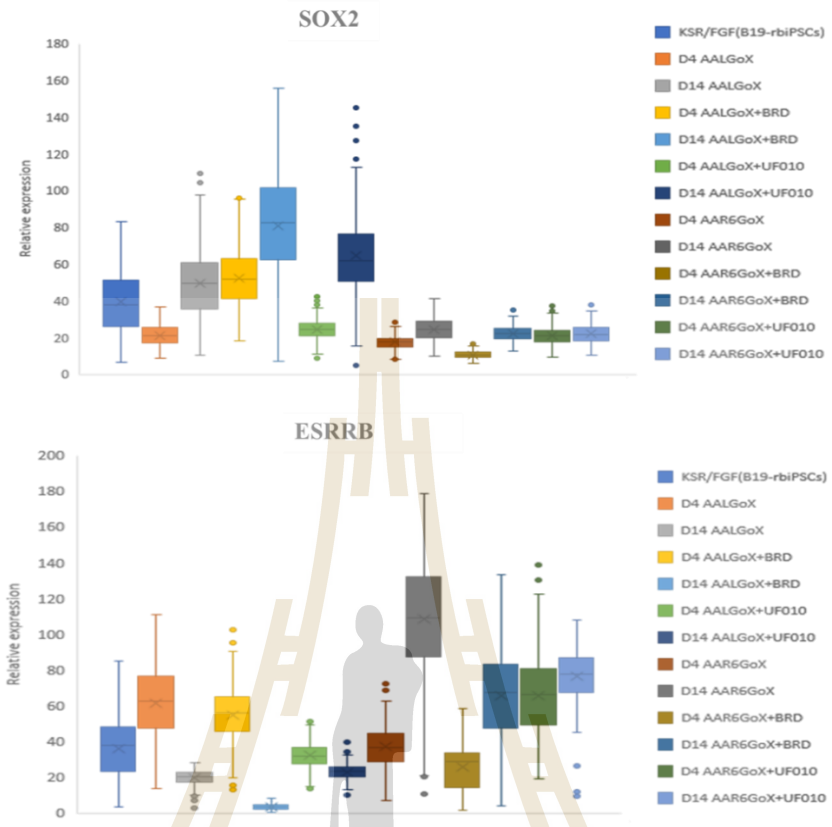

Figure 3.15 Boxplot representation of the fluorescent intensities quantifications for pluripotentcy markers SOX2 and ESRRB during reprogramming of B19-rbiPSCs with H3K9 methylation inhibitor and HDAC inhibitors in media containing LIF or R6.

As a mirror image, we observed the expression of Tbxt and Otx2, a marker of primed pluripotency that expressed in mouse EpiSCs (Kalkan et al., 2019; Kinoshita et al., 2020). We saw the absence or low expression of Otx2 in AALGÖX supplemented with or without HDAC inhibitors compared with control group, which was confirmed by imaging and quantification (Fig 3.16 and 3.17). While Tbxt was weakly expressed in AALGÖX supplemented with HDAC inhibitors at day 14 compared to the strong increase observed in cells of AAR6GÖX groups.

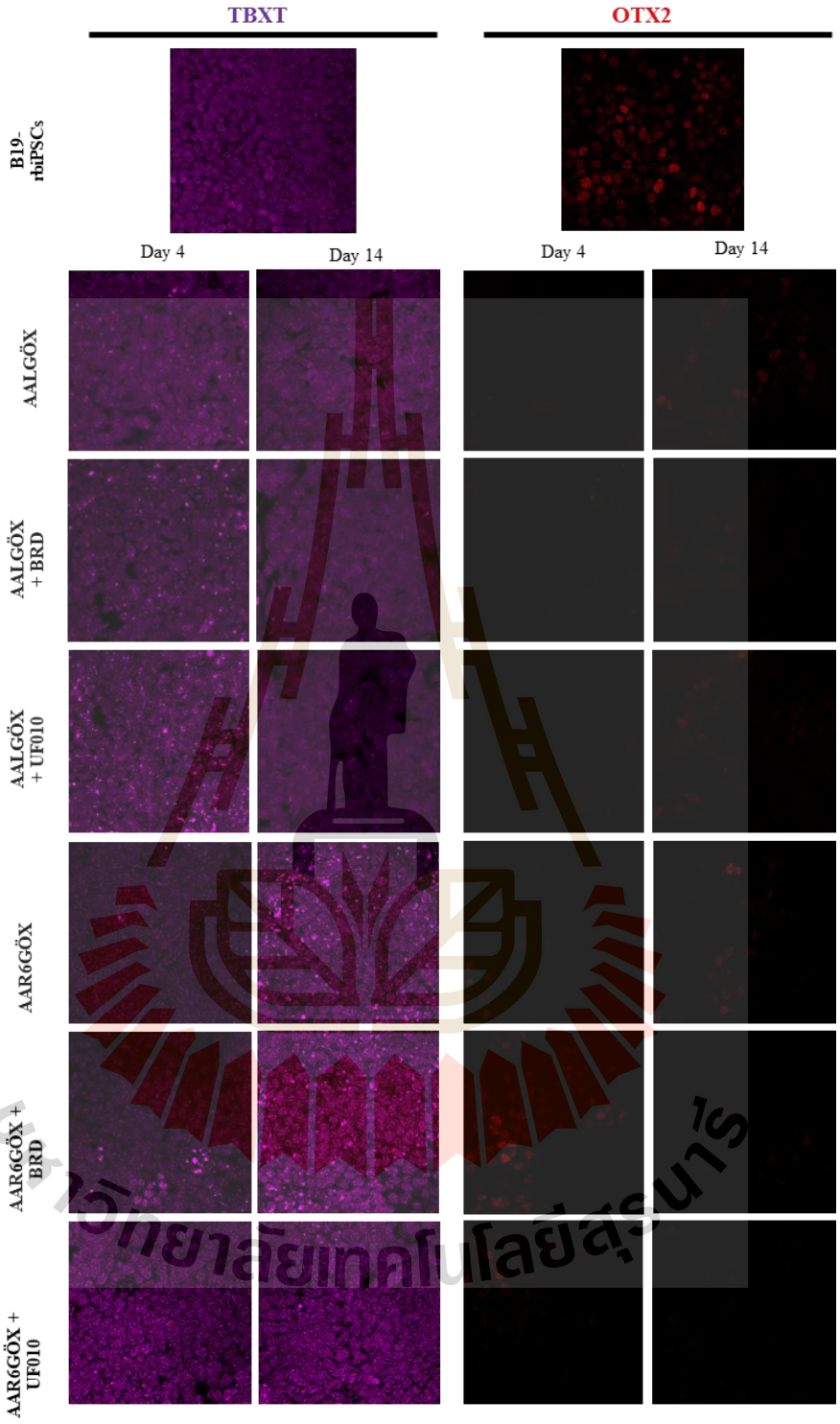

Figure 3.16 Immunostaining for primed pluripotentcy markers; TBXT, OTX2 during reprogramming of B19-rbiPSCs at day 4 and day 14. Magnification of 400x.

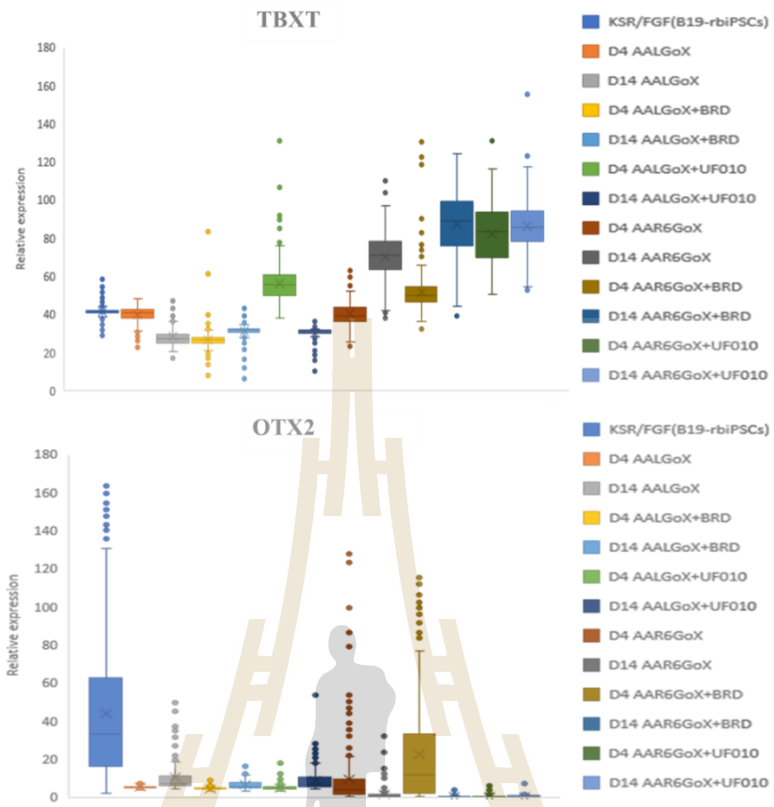

Figure 3.17 Boxplot representation of the fluorescent intensities quantifications for primed pluripotentcy markers; TBXT, OTX2 during reprogramming of B19-rbiPSCs at day 4 and day 14.

We then examined two different epigenetic marks, permissive (H3K14 acetylation : H3K14ac) and repressive (H3K9 trimethylation : H3K9me3), in B19-rbiPSCs cultured in AALGÖX or AAR6GÖX with or without HDAC inhibitors and compared them with B19-rbiPSCs cultured with KOSR/FGF to evaluate their epigenetic reconfiguration upon reprogramming. All two epigenetic markers could be detected with the expected nuclear distribution (Fig. 3.18). We observed a strong increase in H3K14ac in all experimental groups with AALGÖX compared to the control B19-rbiPSCs, which was confirmed by imaging and quantification (Fig 3.19).

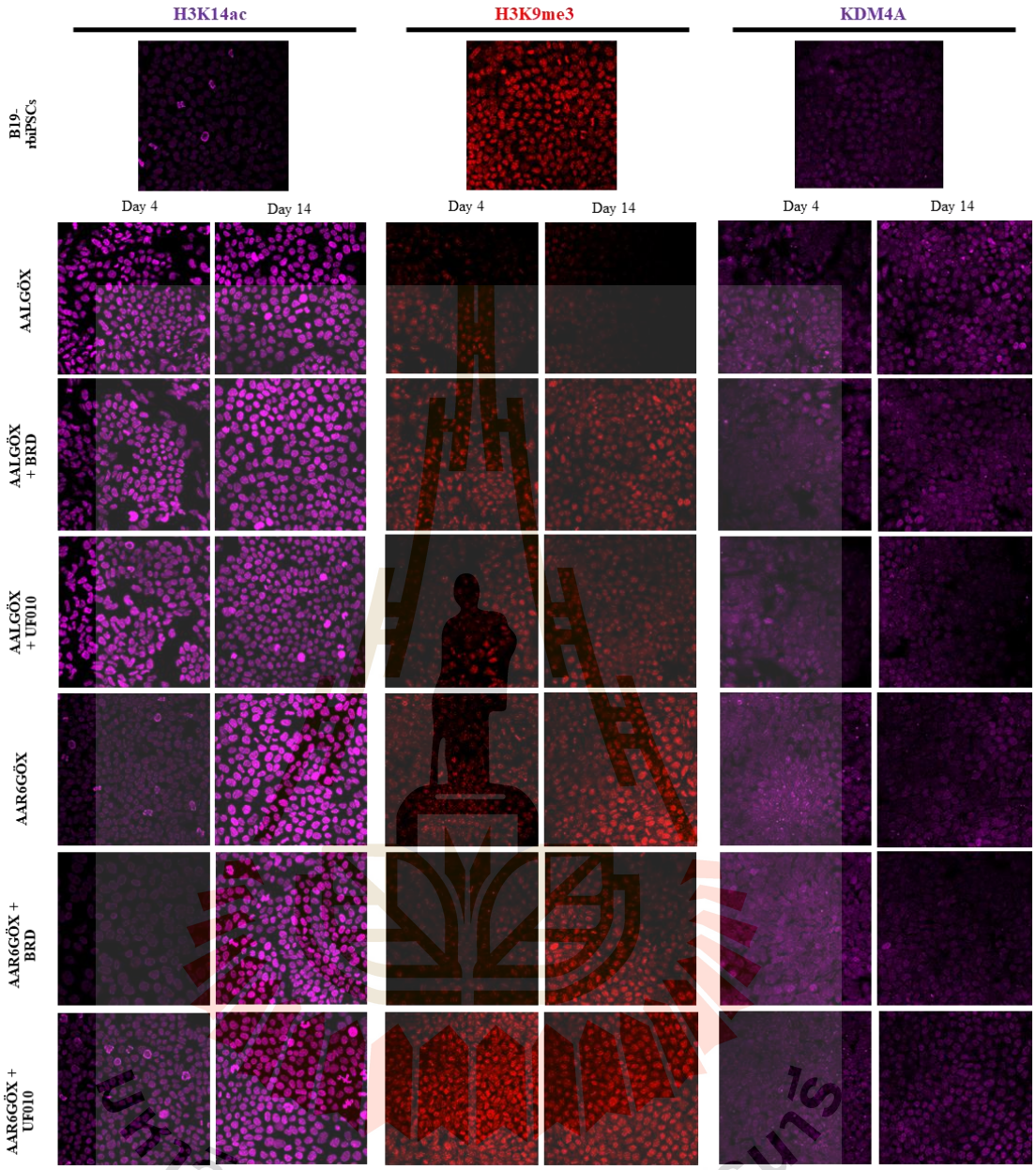

Figure 3.18 Immunostaining of B19-rbiPSCs and reprogrammed cells with various epigenetic markers, permissive (H3K14ac: purple) or repressive (H3K9me3: red) and KDM4A (violet). Magnification of 400x.

H3K9me3 staining, that accumulated on heterochromatin clusters, dropped in all experimental groups in AALGÖX compared with control B19-rbiPSCs. This suggests that repressive epigenetic marks tend to decrease and are replaced by permissive ones upon reprogramming. The B19-rbiPSCs cultured in AALGÖX with

HDAC inhibitors adopted a more open chromatin configuration, characteristic of naïve cells (Smith et al., 2016). Kdm4a is a histone demethylase that plays important roles in a variety of biological processes, including transcription and the response of cells to DNA damage, through the regulation of methylation levels at histone H3 lysines 9 and 36. As cells cultured in ALGÖX supplemented with H3K9 methyltransferase inhibitor (A366) at day 14 strongly expressed Kdm4a at least more than B19-rbiPSCs control group and another experimental groups. These results are well correlated to the level of H3K9 methylation. Cells cultures in AALGÖX with or without HDAC inhibitors at day 14 showed significantly higher expression of Kdm4a gene comparing with cells cultures in AAR6GÖX with or without HDAC inhibitors and control group.

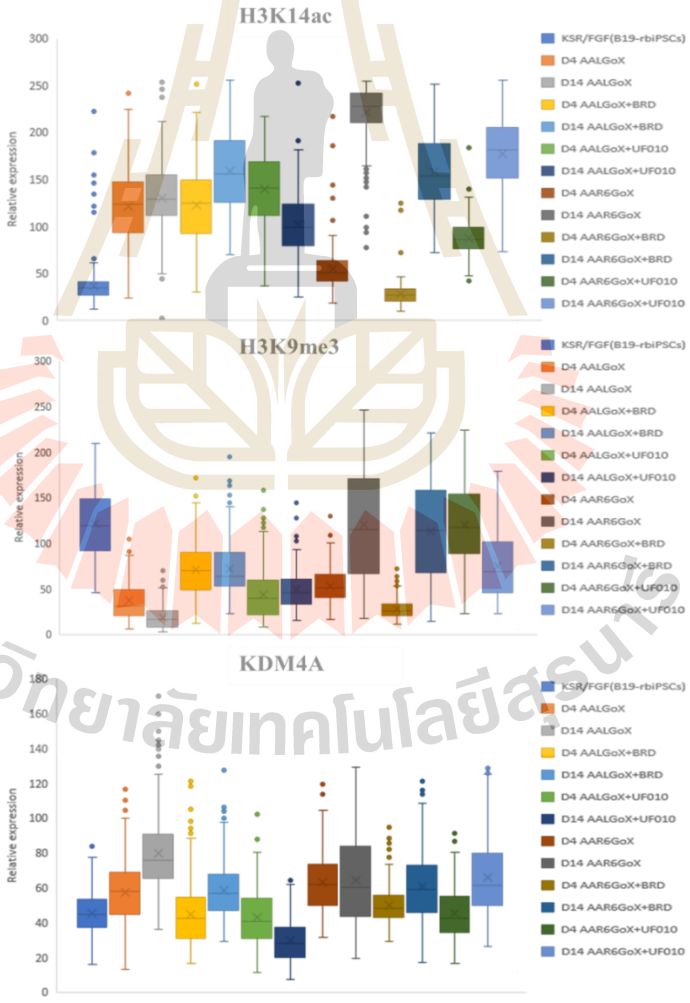

Figure 3.19 Boxplot representation of the fluorescent intensities quantifications for epigenetic reconfiguration.

Female naïve cells are expected to have two active X chromosomes in rabbit, as in mouse. Through the study of X chromosome coating by XIST RNA, it was shown that X inactivation take place also in ICM cells of rabbit blastocyst (Okamoto et al., 2011). We analyzed H2AK119ub marks, a post-translational modification of histones associated with X chromosome inactivation in mice (Chaumeil et al., 2011).

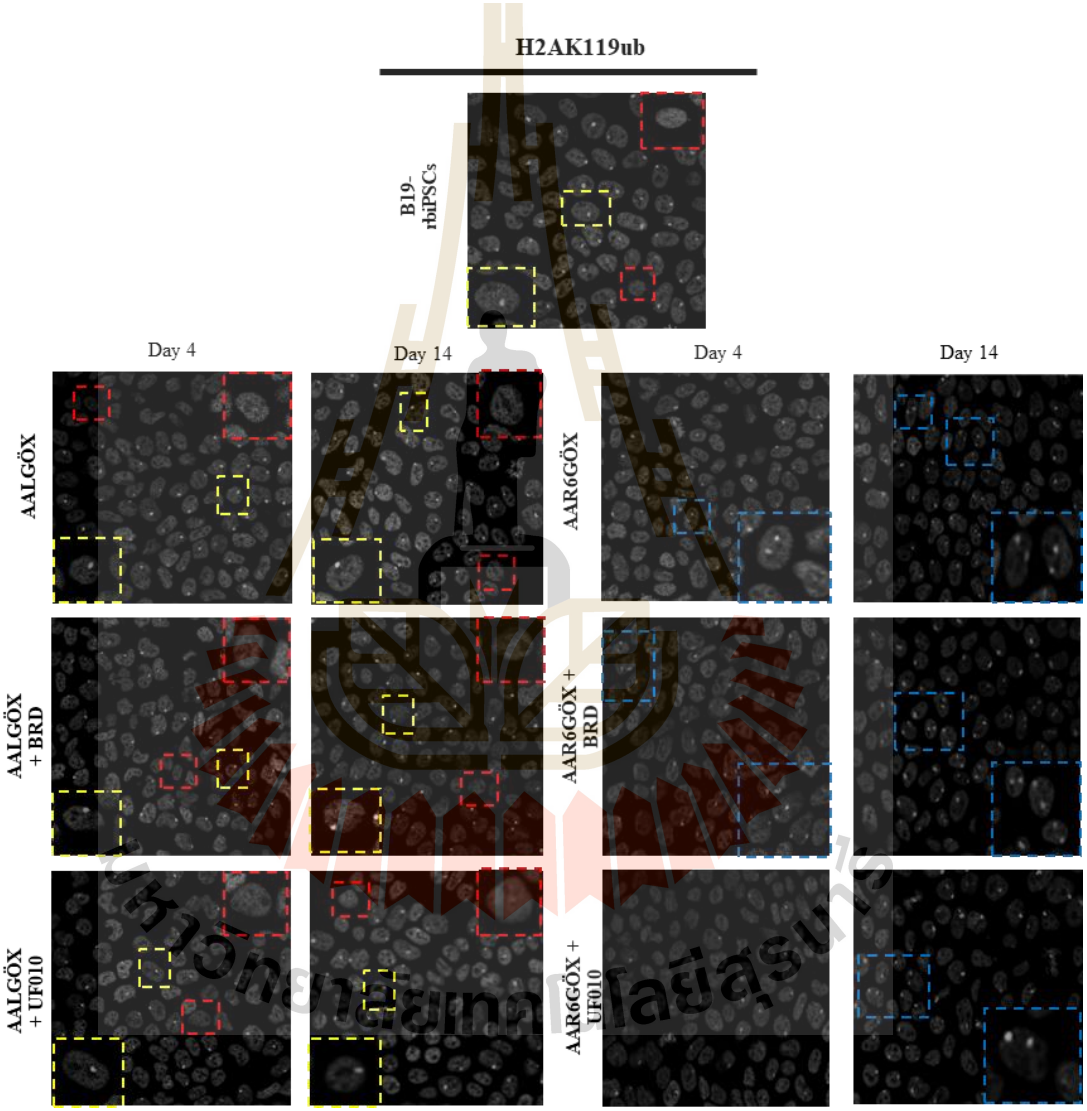

Figure 3.20 X-chromosome status. Immunostaining of histone 2A lysine119 ubiquitinated (H2AK119ubi) marks in reprogrammed rbiPSCs at day 4 and day 14; Yellow: X inactive, Red: X active, Blue: Diploid X inactive (mutated cells). Magnification of 400x.

In primed female B19-rbiPSCs, H2AK119ub immunostainning appears as diffuse spots (Fig 3.20). Similar pattern of X inactivation was reported for primed female hPSCs and usually features inactive X chromosome (Mekhoubad et al., 2012; Silva et al., 2008). The cells in all experimental groups of ALGÖX medium showed single nuclear foci as cells in the control group. Whereas the cells in experimental groups of AR6GÖX medium supplemented with epigenetic modifiers showed 2 inactive X chromosomes, suggesting the presence of mutation.

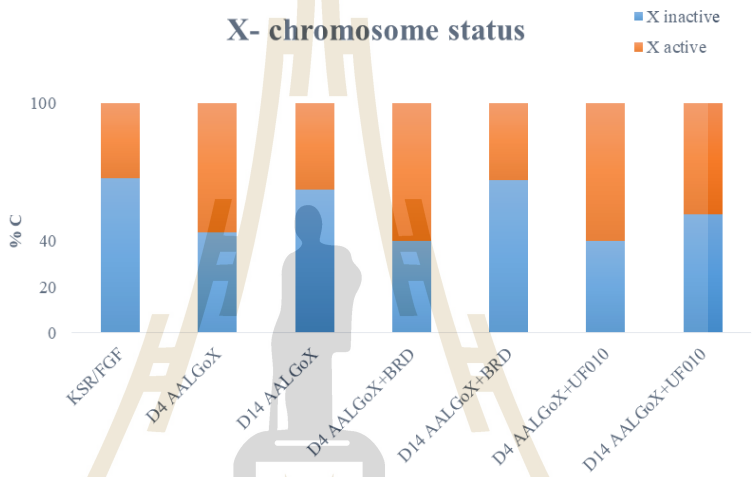

Figure 3.21 X-chromosome status of B19-rbiPSCs and reprogrammed cells with various epigenetic modifiers. Bar charts show quantification of X-chromosome activation status based on H2AK119ub signals from samples of at least 100 cells.

We also examined X-chromosome status after reprogramming, cells had been reverted to a naïve-like PSC state by culture in ALGÖX medium with H3K9me inhibitor (A366), A366+ HDAC inhibitor (BRD4354), and A366+ HDAC inhibitor (UF010) for 4 days. We found that the percentages of cells (Fig 3.21) with a single nuclear focus were decreased from 67.35% in control group (B19-rbiPSCs cultured in KOSR/FGF) to 43.59%, 40.20% and 40.07% in ALGÖX medium with A366, A366+BRD4354, and A366+UF010, respectively. But after 14 days of culture, these percentages of cells with X inactivation were increased to 62.34%, 66.67% and 51.67% absence of A366, A366+BRD4354, and A366+UF010. However these

percentage of cells with X inactivation in all experimental groups stayed lower than that in control group.

3.5 Discussion

Our findings demonstrate that reprogrammed cell populations exhibiting a range of properties consistent with naïve pluripotency can readily be generated from primed rabbit iPSCs by epigenetic modification including DNA demethylation, H3K9 methyltransferase (H3K9me) inhibition, and histone deacethylase (HDAC) inhibition.

Reprogrammed cells with VALGÖX medium with HDAC inhibitor (VPA) express naïve pluripotency factors *Klf4* and *Stat3* that is correlated with the presence of these same naïve pluripotency markers in XAV-supplemented cultures of mouse cells (Guo et al., 2017). XAV inhibits facilitate the propagation of pluripotent cells in alternative states (Kim et al., 2013; Zimmerlin et al., 2016). In contrast, two markers of core pluripotency *Oct4* and *Nanog* were lower expressed in CRM1 and VALGÖX supplement with HDAC inhibitor (VPA) compared with B19-rbiPSCs cultured in KOSR/FGF medium. We also observed that using CRM1, cells more differentiation than using directly VALGÖX with HDAC inhibitor (VPA). XAV inhibitor reduced numbers of differentiating or dying cells (Guo et al., 2017). The JAK-STAT, NOTCH, WNT, MAPK, and TGF-ß signaling pathways are associated with the regulation and maintenance of pluripotency in rodents and primates (Boroviak et al., 2015; Boroviak et al., 2018; Mohammed et al., 2017; Nakamura et al., 2016). Consistent with naïve pluripotency data in mice (Mohammed et al., 2017). GÖ6983 is a broad specificity PKC inhibitor that facilitates mouse ESC self-renewal (Dutta et al., 2011), and probably help reprogramming of rabbit primed cells.

Naïve PSCs such as mouse embryonic stem cells (mESCs) derived from the inner cell mass (ICM) of pre-implantation blastocysts represent a developmental ground state in response to the cytokine LIF and the inhibition of kinases including GSK3 and MAPK/ERK (Yu and Cui., 2016). The chemical inhibitors of various kinases (BRAF, $p38^{MAPK}$, GSK3, MEK1/2, SRC, and JNK) were used to facilitates reprogramming of primed human PSCs (Takashima et al., 2014). We adapted these protocols by using inhibition of MAPK : BRAF with SB590885, p38 with SB203580, and JNK with SP600125, to improve the reprogramming of rabbit primed toward a naïve-like state. B19-rbiPSCs cultured in the presence of HDAC inhibitor VPA following by addition of JNK inhibitor

(SP600125) in VALGÖX medium for 6 days express transcription regulators and other genes that are found in human pre-implantation epiblast and human ESCs (Guo et al., 2017), mouse ESCs (Dunn et al., 2014), ICM and early EPI in mouse and cynomolgus macaque embryos (Messmer et al., 2019; Mohammed et al., 2017; Nakamura et al., 2016) but are low or absent in primed PSCs. These include the characterised core pluripotency *Nanog* and three markers of naïve pluripotency *Stat3, Klf4* and *Ooep* that were more highly expressed in these cells compared to B19-rbiPSCs cultured with KOSR/FGF. In contrast, markers of core pluripotency *Oct4* was highly expressed in cells reprogrammed with same condition using SP600125 compared to cells cultured with BRAF inhibitor (SB590885) or p38 inhibitor (SB203580) but stayed nethertheless lower expressed than in the B19-rbiPSCs control group.

HDAC inhibitors are global epigenetic destabilisers that have been used to facilitate nuclear transfer (Ogura et al., 2013), somatic cell reprogramming (Huangfu et al., 2008), mouse EpiSC resetting (Ware et al., 2009), and human PSCs resetting (Takashima et al., 2014; Guo et al., 2017). Chemically reprogramming rabbit cells with HDAC inhibitors (BRD4354 and UF010) and JNK inhibitors (SP600125) can improve the efficiency of conversion to a more naïve state that can subsequently be propagated in VALGÖX medium without feeders. This result is correlated with monkey reset cells that can be cultured on pre-coated plates without feeders (Takashima et al., 2014) and human PSCs reprogrammed in tt2iLGÖ that showed consistent feeder-free expansion with typical naïve morphology, growth and marker profiles (Guo et al., 2017). The markers of core pluripotency *Oct4* and *Nanog,* as well as naïve pluripotency *Klf4* were highly expressed in cells cultured in VALGÖX with HDAC inhibitor (BRD4354) at passage 1 compared the cells treated with HDAC inhibitors (UF010 or VPA) and B19-rbiPSCs control group.

B19-rbiPSCs cultured in the presence of H3K9me inhibitor (A366) and HDAC inhibitors showed expression of transcription regulators and naïve pluripotentcy markers including *Oct4, Nanog, Klf4, Stat3* and *Ooep* in higher extent than B19-rbiPSCs cultured in the presence of DNA demethylase Vitamin C and HDAC inhibitor or control group. These results indicate that vitamin C supplementation is less efficient for resetting, as it was shown on human cells expressing similar level of naïve markers with or without exposure to vitamin C (Guo et al., 2017). The

mechanism by which H3K9me inhibition and HDAC inhibition promotes reprogramming seems likely to involve the generation of a more open chromatin environment. We still need to verify the global expression profile of our reprogrammed cells which should dramatically differ from those of primed PSCs and resemble to those of previously described human naïve-like cells generated by inducible or transient transgene expression (Takashima et al., 2014) or by adaptation to culture in 5i/L/A/(F) (Theunissen et al., 2014).

Propagation of cells in AALGÖX medium and HDAC inhibitors (BRD4354) resulted in increased mRNA expression of core (*Oct4, Sox2*), and naïve (*Klf4, Esrrb, Kdm4a*) pluripotency markers described in mice (Dunn et al., 2014), and decreased expression of primed markers *Cdh3, Tbxt, Otx2* (Kalkan et al., 2019; Messmer et al., 2019). Moreover the presence of H3K9me inhibitor and HDAC inhibitors increased the expression of naïve marker *Dppa5* (Tanaka et al., 2006) in the cells, while this marker expression was decreased in the absence of these inhibitors. The cells cultured in medium supplemented with LIF expressed higher levels of naïve marker *Stat3* than cells cultured with IL6+IL6R, even if these two types of cytokine should induce the same signaling pathways downstream of gp130. Immunostainning of reprogrammed B19-rbiPSCs in presence of LIF or IL6+IL6R with or without HDAC inhibitors showed increase of different marker expression: Sox2 with LIF and Esrrb with IL6+IL6R at day 14 confirming a slightly different effect on GP130 signaling pathways. Finally, absence or low expression of primed markers as Tbxt and Otx2, in cells cultured in AALGÖX with or without HDAC inhibitors confirmed the efficiency of reprogramming process in presence of LIF. Morever, media with IL6+IL6R induced X chromosome triplicate by unknown mechanism, probably by inducing to high selection pressure on cells.

During reprogramming of human primed cells, DNA methylation is globally reduced to a level similar to that reported for human ICM (Guo et al., 2014). This is regarded as a key process for erasure of epigenetic memory in the naïve phase of pluripotency (Lee et al., 2014). We also observed a strong increase of H3K14 acetylation in all experimental groups in ALGÖX supplemented with H3K9me inhibitor (A366). These results indicate that H3K9me inhibitor had induced upregulation of active marks as H3K14ac and downregulation of negative marks like H3K9me3, since this mark is associated with gene silencing (Takashima et al., 2014).

Naïve pluripotency is associated with low DNA methylation (Leitch et al., 2013; Leitch et al., 2016), and enrichment of active histone modifications at promoter regions of developmental genes (Hayashi et al., 2008). As cells cultured in ALGÖX supplemented with H3K9me inhibitor (A366) showed stronger expression of Kdm4a than B19-rbiPSCs control group, confirming the importance of this mechanism in the rabbit. Reversible methylation of lysine residues has emerged as a central mechanism for epigenetic regulation. Kdm4a is a histone demethylase that targets tri- and dimethylation marks on histone H3 lysines 9 and 36. Changes in Kdm4a abundance correlate with alterations in histone H3 lysine 9 and 36 methylation levels, and transcription of a Kdm4a target gene (Tan et al., 2011).

A hallmark of the transient phase of naïve pluripotency in both rodent and human ICM cells is the presence of two active X chromosomes in females (Okamoto et al., 2011; Petropoulos et al., 2016; Sahakyan et al., 2017; Vallot et al., 2017). Primed female B19-rbiPSCs presented diffuse spots of H2AK119ubi marks that was shown to correspond to X inactivation in primed female hPSCs (Mekhoubad et al., 2012; Silva et al., 2008). Nevertheless, the cells in all experimental groups of ALGÖX medium showed higher proportion of cells presenting only one nuclear focus with no differences between added epigenetic modifiers. X-chromosome status had been reverted to a naïve-like pluripotency state by culture rabbit cells in ALGÖX medium with H3K9me inhibitor (A366), A366+HDAC inhibitors (BRD4354), and A366+HDAC inhibitors (UF010). These results indicated that H3K9me inhibitor and HDAC inhibitors can reactivate the silent X chromosome. Demethylation also extends to imprinted loci, as noted for other human naïve-like stem cells (Pastor et al., 2016; Theunissen et al., 2016). Loss of imprints is observed in conventional hPSCs (Nazor et al., 2012) and in mouse ESCs (Dean et al., 1998; Greenberg and Bourc'his, 2015; Walter et al., 2016). However, we did not have the possibility to test the status of rabbit imprinting genes.

3.6 Conclusions

The small molecules TGF-β (Activin A), PKC inhibitors (GÖ6983) and tankyrase inhibitors (XAV939) showed high efficiency to develop reprogramming base-medium on feeder-free system. The inhibition of histone deacetylase and inhibition of histone 3 lysine 9 methyltransferase had enhanced the reprogramming of primed rbiPSCs to

more naïve status. This new culturing protocol resulted in transcriptional and epigenetic reconfiguration, as substantiated by the expression of naïve markers including *Oct4, Sox2, Klf4, Stat3, Esrrb, Dppa5, Kdm4a* and the presence of histone modifications associated with naïve pluripotency. Furthermore, H3K9me inhibition and HDAC inhibition promoted reprogramming by downregurating H3K9me3 and upregurating H3K14ac. The reprogrammed cells showed decreased percentage of cells with X inactivation, demonstrating the role of these inhibitors in reactivation of the silent X chromosome. All together, these data have shown that the best reprogramming media are AALGÖX with UF010 or BRD4354. These preliminary results are now completed in the rabbit team of SBRI in order to be published.

3.7 References

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CHAPTER IV

REPROGRAMMING RABBIT FIBROBLASTS INTO INDUCED PLURIPOTENT STEM CELLS USING SENDAI VIRUS AND EPIGENETIC MODIFIERS

4.1 Abstract

Takahashi and Yamanaka demonstrated 16 years ago that fibroblasts could be reprogrammed into bona fide pluripotent stem cells by overexpressing four transcription factors *Oct4, Sox2, Klf4,* and *c-Myc* using retroviral integrating. The experimental conversion of differentiated cells into the pluripotent state could now be obtained with techniques using no integrating vectors. Here we present attempts to reprogram rabbit fibroblasts and rabbit mesenchymal stem cells using Sendai virus or mRNA transfection and epigenetic modifiers. The rbFs showed higher transduction and transfection efficiency than rbMSCs. The rbFs reprogramming process with Sendai virus expressing *hKlf4, hOct3/4, hSox2* and *c-Myc* and using medium supplemented with H3K9 methyltransferase inhibitor (A366) and HDAC inhibitor (UF010), generated iPSCs-like clones that expressed rabbit pluripotent transcription factors *Oct4* and *Nanog*. The expression of *Sox2* was not different to that of B19-rbiPSCs. Moreover, feeder-free culture medium simplifies the selection and the amplification of reprogrammed clones.

4.2 Introduction

Pluripotent stem cells (PSCs) can exist in two morphologically, molecularly and functionally distinct pluripotent states, designated as the naïve and primed states (Nichols and Smith, 2009). In 2006, Takahashi and Yamanaka demonstrated that fibroblasts could be reprogrammed into bona fide pluripotent stem cells by overexpressing four transcription factors, namely *Oct4, Sox2, Klf4,* and *c-Myc* (Takahashi and Yamanaka., 2006). The resulting “induced pluripotent stem cells,” or iPSCs, displayed the cardinal features of their embryonic counterpart, the embryonic

stem (ESCs). This major discovery was made in mice, and was soon applied to human with a similar outcome (Takahashi et al., 2007; Yu et al., 2007). Since these pioneering studies, the iPSCs technology was implemented in several other species, including rhesus macaque (Liu et al., 2008), pig (Ezashi et al., 2009), and rabbits (Honda et al., 2010; Tancos et al., 2012; Osteil et al., 2013). The published rabbit iPSCs lines were produced from New Zealand White rabbit embryonic cells or adult fibroblasts using retroviral vectors expressing the classical four genes demonstrated (Osteil et al., 2013; Honda et al., 2010; Afanassieff et al., 2014; Tancos et al., 2017; Phakdeedindan et al., 2019). In this study, we tested the capacities of rabbit adult fibroblasts and rabbit mesenchymal stem cells to be reprogrammed with non-integrating methods as Sendai virus transduction and mRNA transfection using epigenetic modifiers.

4.3 Materials and Methods

4.3.1 Adult rabbit fibroblasts (rbFs) and rabbit mesenchymal stem cells (rbMSCs).

Adult rabbit fibroblasts (rbFs) and rabbit mesenchymal stem cells (rbMSCs) were obtained from the rabbit team at the Stem cell and Brain Research Institute. rbFs and rbMSCs were cultured as described elsewhere (Afanassieff et al., 2014).

4.3.2 Feeder preparation

Feeder preparation was described previously, in chapter 3.3.3

4.3.3 Analysis of the properties of rabbit fibroblasts and rabbit mesenchymal stem cells.

4.3.3.1 Mycoplasma testing

Cells were plated at density about $1x10^5$ cells/ well of 6 well-plate and cultured in rabbit fibroblast medium without antibiotics (DMEM medium supplemented with 10% fetal bovine serum, 1% NEAA, and 10 mM β-mercaptoethanol. Cells were cultured under humidified 5% CO_2 at 37°C for 5 days. Then 1 mL of cell supernatant samples were collected and heated at 95°C for 5 min. After centrifugation for 5 sec at maximum speed, the sample supernatants were analyzed for mycoplasma by conventional PCR using a Venor™ GeM Mycoplasma Detection Kit (MB Minerva biolabs 11-8100, Berlin, Germany).

4.3.3.2 Growth rate

To study the growth rate and proliferation velocity of fibroblasts and mesenchymal stem cells, the growth curves for these cells were plotted. The cells of each group were seeded and cultured in rabbit fibroblast medium. Every two days, the cells were harvested, dissociated with trypsin/EDTA, treated with diluted trypan blue and counted with Countess™ 3 Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA) until day 10.

4.3.4 Reprogramming of rbFs and rbMSCs with Sendai virus.

4.3.4.1 Transduction rate of EmGFP Sendai fluorescence reporter.

CytoTune®-EmGFP Sendai Fluorescence Reporter used for transducing EmGFP was purchased from Thermo Fisher Scientific. $1x10^5$ rbFs or rbMSCs were infected with EmGFP Sendai Fluorescence Reporter with multiplicity of infection (m.o.i.) 5 or 15, in the presence of 4 ug/ml polybrene (Sigma, St. Louis, Missouri, USA). Three days after the infection, cells were dissociated using trypsin into single cells, counted and resuspended at $5x10^5$ cells/500 ul of PBS and keep on ice until analyzed using FACS canto II cytometer and FACSDiva software (Beckton Dickinson, New Jersey, USA).

4.3.4.2 Reprogramming of rbMSCs with Sendai virus on feeder or feeder-free system.

CytoTune™-iPS 2.0 Sendai Reprogramming Kit used for reprogramming rbFs and rbMSCs was purchased from Thermo Fisher Scientific. To generate rbiPSCs, $1x10^5$ rbMSCs were infected with a mix of Sendai Viruses expressing *hKlf4 + hOct4 + hSox2, hKlf4* alone and *hc-Myc* alone at a multiplicity of infection (m.o.i.)15: 15: 9, respectively, in the presence of 4 ug/ml polybrene (Sigma) and MEF medium as prescribed by manufacturer. Four days after the infection, rbMSCs were trypsinized into single cells and replated at low density ($6x10^2$ cells/cm^2) onto inactivated MEFs in rbiPSC medium or on Matrigel in VALGÖX medium. The medium was changed every other day for 2 to 3 weeks until iPSC-like colonies appeared.

4.3.4.3 Reprogramming of rbFs and rbMSCs with Sendai virus and epigenetic modifiers.

$1x10^5$ rbFs and rbMSCs were infected once or twice with the same virus cocktail than before, in the presence of 4 ug/ml polybrene (Sigma) and

AALGÖX medium + UF010. Four days after the infection, rbFs and rbMSCs were trypsinized into single cells and replated at low density ($6x10^2$ cells/cm^2) onto Matrigel in ALGÖX medium. The medium was changed every other day for 2 to 3 weeks until iPSC-like colonies appeared.

4.3.5 Reprogramming of rbFs and rbMSCs with mRNA transfection.

4.3.5.1 Reprogramming of rbFs and rbMSCs with mRNA transfection and epigenetic modifiers.

Stemgent® StemRNA™ 3rd Gen Reprogramming Kit used for reprogramming rbFs and rbMSCs were purchased from Reprocell. To generate rbiPSCs, $0.5x10^5$ rbFs and rbMSCs were infected four times with a mix of the totality of the kit components, namely mRNA cocktails expressing OSKMNL (*Oct4, Sox2, Klf4, cMyc, Nanog, Lin28* reprogramming factor RNAs), ± EKB (E3, K3, B18 immune evasion factors) and ± microRNAs (miRNA), in MEF medium as prescribed by manufacturer. To compare the transfection efficiency, the components of the kit were also used separately: OSKMNL and miRNA, OSKMNL and EKB, OSKMNL alone, respectively. Four days after the last infection, cells were cultured in AALGÖX medium supplemented with UF010 during 4 days. After the epigenetic modifiers were withdrawn from the culture medium. The medium was changed every other day for 2 to 3 weeks until iPSC-like colonies appeared.

4.3.6 Analysis of the expression of pluripotency gene by RT-qPCR.

RT-qPCR analysis were described previously in the chapter 3.3.5

4.3.7 Statistical analysis

Data are represented as arithmetic mean±SD. *P*. values only less than 0.05 ($P<0.05$) were considered significant. Error bars indicate standard errors of the means.

4.4 Results

4.4.1 Growth properties of rbFs and rbMSCs

4.4.1.1 Mycoplasma testing of rbFs and rbMSCs

Several strains of mycoplasma frequently occur as latent contaminants of human and animal cell lines in research laboratories (Drexler and Uphoff., 2002). The mycoplasma contamination in rbFs and rbMSCs culture

supernatants was analyzed by polymerase chain reaction using mycoplasma-specific primer sequences. The results showed that both of rbFs and rbMSCs were not contaminated by mycoplasma since no amplification could be detected after PCR on agarose gel (Fig 4.1).

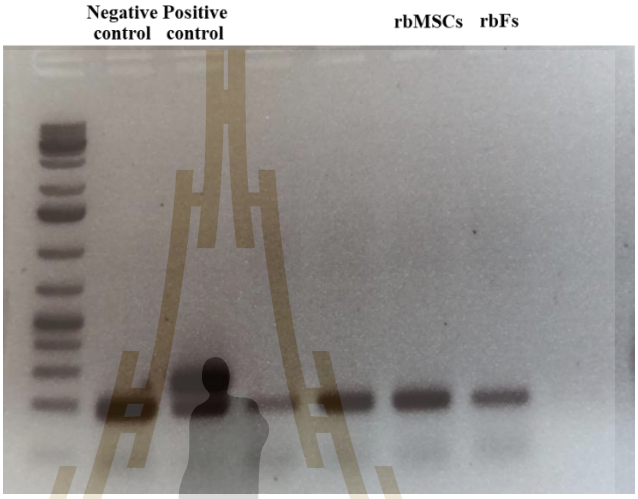

Figure 4.1 Mycoplasma testing of rbFs and rbMSCs.

4.4.1.2 Growth rate of rbFs and rbMSCs

The cell growth rate was an important factor in selecting cells for reprogramming because cells need to proliferate in order to be reprogrammed, but a too high growth rate induce difficulties to detect and select the reprogrammed cell clones. As shown in Figure 4.2 rbFs had the highest rate of growth and proliferation, whereas the lowest growth belonged to rbMSCs.

4.4.2 Reprogramming with Sendai visuses

4.4.2.1 Transduction rate of EmGFP Sendai fluorescence reporter

Fluorescent proteins like Green Fluorescent Protein (GFP) are often used as such reporters during transduction efficiency experiments. The success of a transduction experiment is defined by the ratio of cells expressing the used reporter. This ratio is also known as transduction efficiency (Gubin et al., 1999).

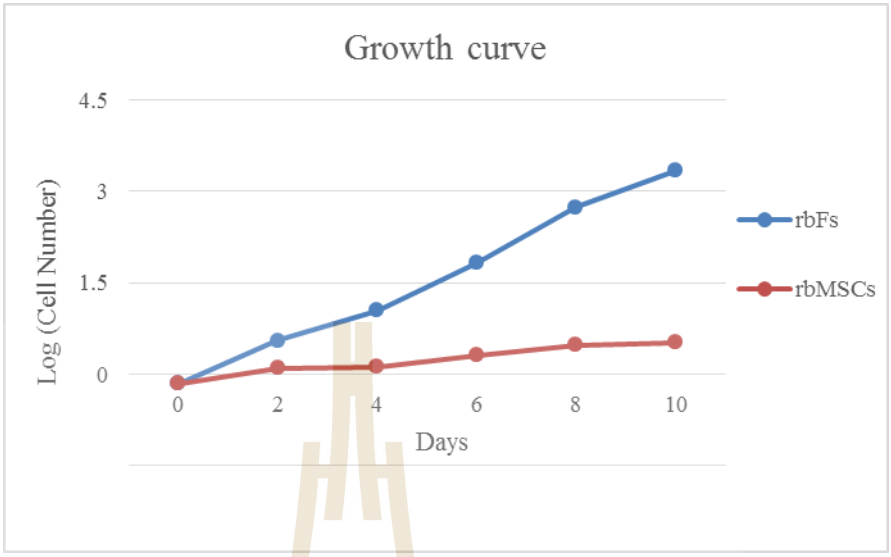

Figure 4.2 Growth curve for rbFs and rbMSCs cultured during 10 days in rabbit fibroblast medium.

Table 4.1 Transfection rate of EmGFP Sendai fluorescence reporter.

Cells	Transfection rate of EmGFP sendai fluorescence reporter	
	MOI: 5	MOI: 15
rbFs	64.0 %	73.3 %
rbMSCs	30.2 %	33.6 %

In four separate experiments, 64% and 73.3% of transduced rbFs expressed GFP with a MOI of 5 and 15, respectively and only 30.2% and 33.6% of transduced rbMSCs expressed GFP with a MOI of 5 and 15, respectively (Table 4.1 and Fig 4.3).

4.4.2.2 Reprogramming of rbMSCs with Sendai virus with or without feeder cells.

rbMSCs were transducted with a mixture of 3 Sendai vectors that expressed human *hKlf4, hOct3/4, hSox2* and *hc-Myc* transcription factors. Four days after the transduction, the rbMSCs were dissociated with trypsine and replated on feeder cells in the rbiPSCs medium supplemented with 10 ng/ml FGF2 and 20% KOSR or replated on matrigel in VALGÖX medium (N2B27 conditioning medium supplement with DNA demethylase (vitamin C), TGFB (Activin A), LIF, Protein kinase C inhibitor (GÖ6983), and Tankyrase inhibitor (XAV939). The transducted cells were

grown on feeder or matrigel for 2-3 weeks, but we can't observe colonies with compact morphologies a specific feature of iPSCs (Fig 4.4). Moreover, the transducted cells cultured on feeder were difficult to localized since morphologies of feeders and MSCs are closed.

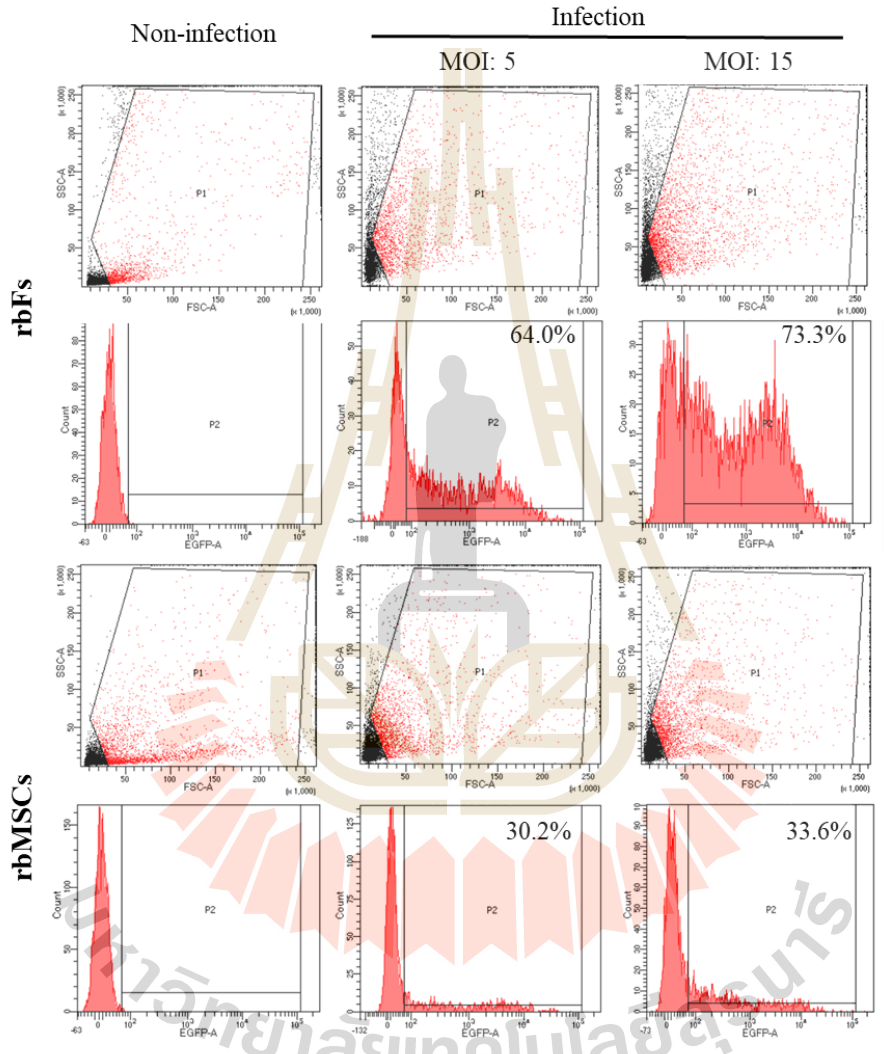

Figure 4.3 Flow cytometry analysis of EmGFP expression.

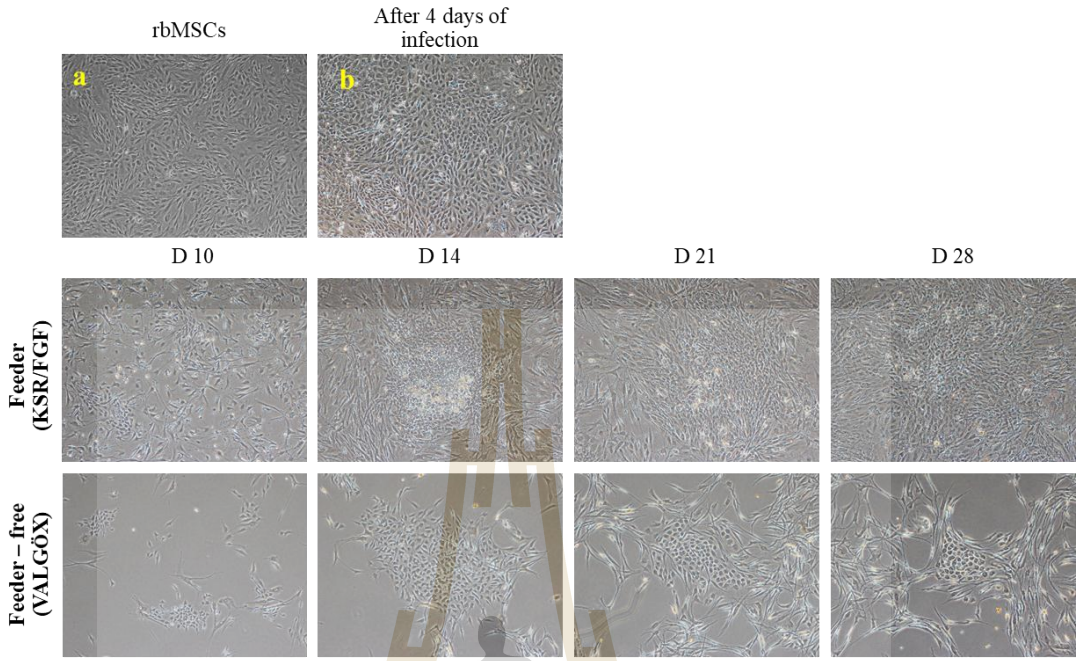

Figure 4.4 Phase-contrast images of rbMSCs before (a) and after (b) infection with Sendai virus on feeder or feeder-free system. Magnification of 40x.

4.4.2.3 Reprogramming of rbFs and rbMSCs with Sendai virus and epigenetic modifiers

Using the same protocol as before, rbFs and rbMSCs were transducted once or twice with the cocktail of 3 Sendai viruses. During transduction with Sendai virus cells were grown in AALGÖX + UF010 medium containing H3K9me inhibitor (A366) and HDAC inhibitor (UF010) for enhancing the reprogramming process. Four days after the transduction, the rbFs and rbMSCs were dissociated and replated on matrigel in ALGÖX medium. The culture medium was changed every day thereafter until some colonies with compact morphologies appeared (Fig. 4.5). In these conditions, no reprogrammed rbMSCs colonies appeared (Fig. 4.6).

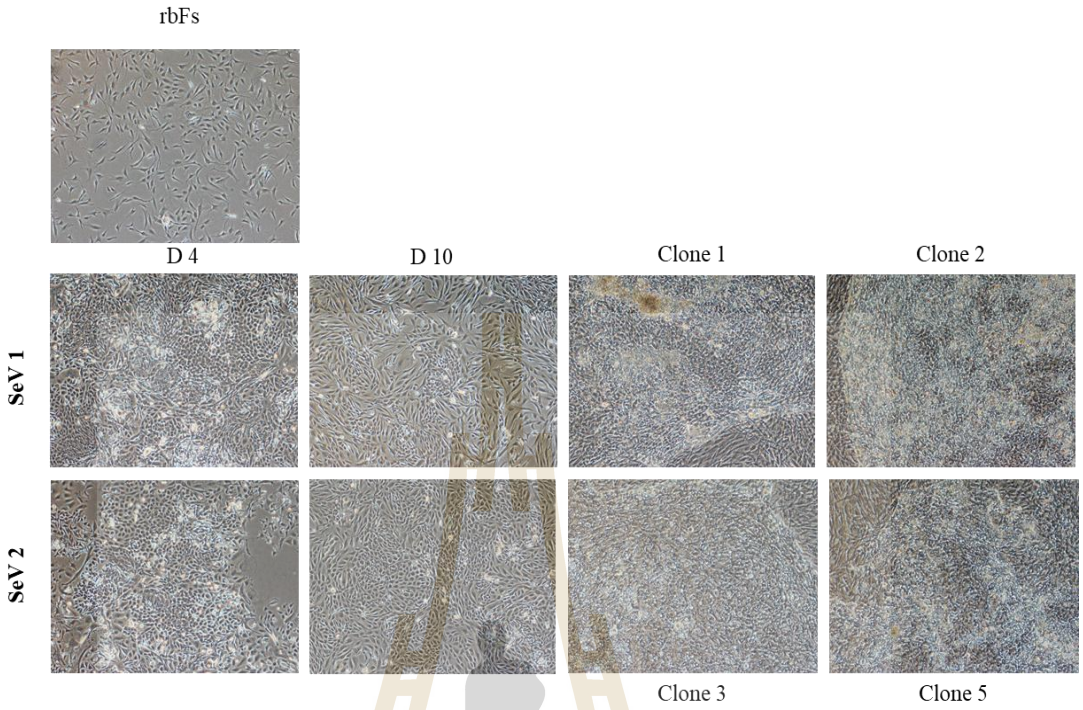

Figure 4.5 Phase-contrast images of rbFs before and after infection with Sendai virus and epigenetic modifiers. Sev1 = one transduction; Sev2 = two consecutive transductions. Magnification of 40x.

However, in the same conditions, twenty and seven clones were selected between the 17th and 28th day following the fibroblast transduction. These 27 clones were passaged by trypsin dissociation into single-cell suspensions. Four clones can expanded and grown on matrigel in ALGÖX medium. At passage 4 the clone 5 resulted from a double transduction was expressing rabbit *Oct4* and *Nanog* at higher level than the control B19-rbiPSCs and rbFs (Fig. 4.7). The expression of rabbit *Sox2* was not different in this clone than in B19-rbiPSCs control. Finally, all 4 clones amplified until passage 4 showed higher expression of naïve marker *Klf4* compared to that of B19-rbiPSCs. Nevertheless, surprisingly, rbFs presented also higher level of *Klf4* expression, showing that these preliminary results need to be confirmed.

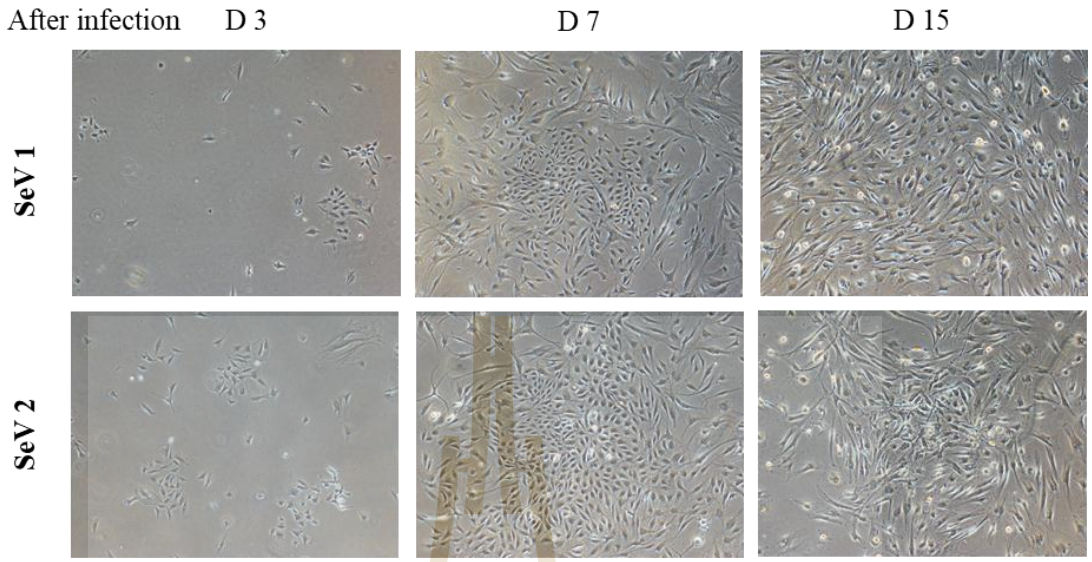

Figure 4.6 Phase-contrast images of rbMSCs before and after infection with Sendai virus and epigenetic modifiers. Sev1 = one transduction; Sev2 = two consecutive transductions. Magnification of 40x.

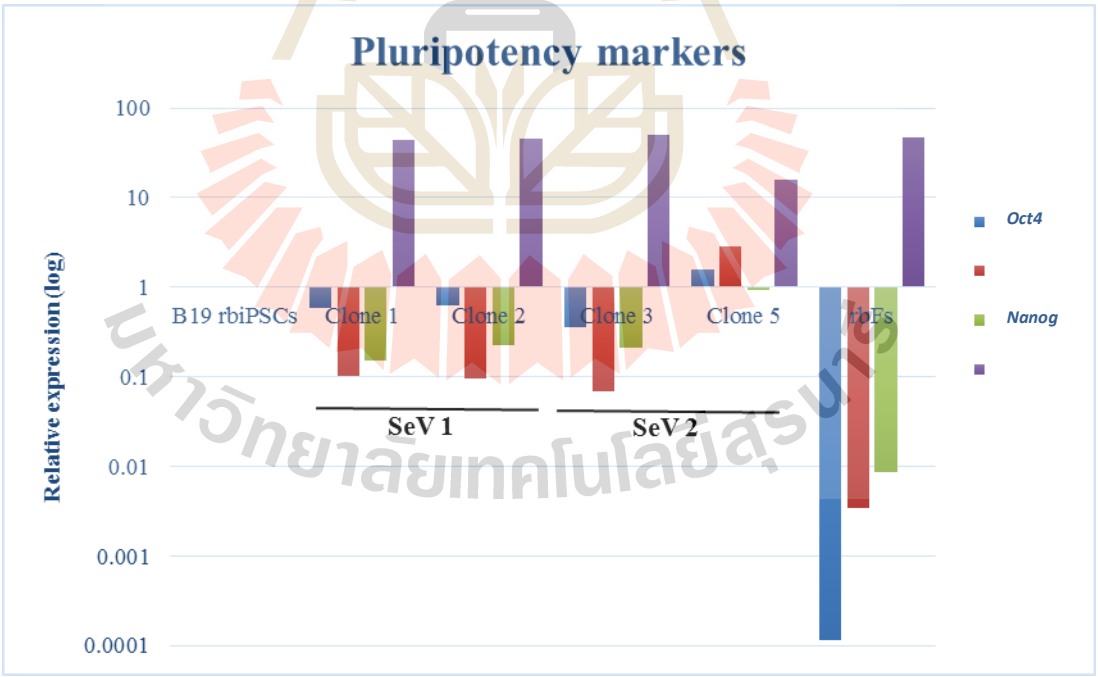

Figure 4.7 RT-qPCR analysis of general and naïve pluripotency markers in rbF reprogammed cells with Sendai virus and epigenetic modifiers. Error bars indicate SD of three independent experiments.

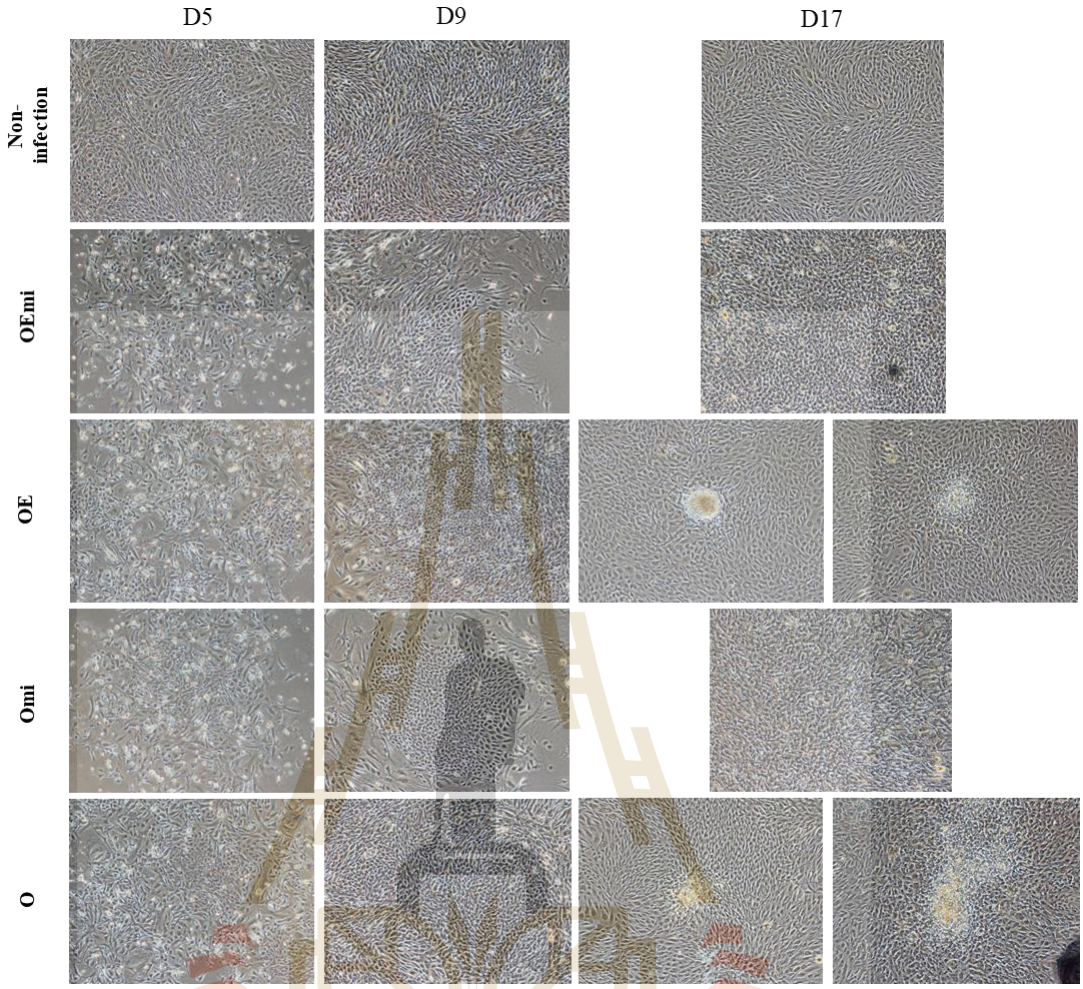

Figure 4.8 Phase-contrast images of rbFs after mRNA transfections in presence of epigenetic modifiers. OEmi = OSKMNL+EKB+microRNA; OE = OSKMNL+EKB; Omi = OSKMNL+microRNA, O = OSKMNL. Magnification 40x.

4.4.3 Reprogramming with mRNA transfection

4.4.3.1 Reprogramming of rbFs and rbMSCs with mRNA transfection and epigenetic modifiers.

rbFs and rbMSCs were transducted four times with mix of mRNA cocktails expressing OSKMNL (*Oct4, Sox2, Klf4, cMyc, Nanog, Lin28* reprogramming factors), +/- EKB (E3, K3, B18 immune evasion factors), and +/- microRNAs (miRNA) in MEF medium. Four days after the transduction with mRNA cocktails, cells were grown in ALGÖX medium containing H3K9 methyltransferase inhibitor (A366) and HDAC inhibitor (UF010) for enhancing the reprogramming process

for 4 day. Then, transducted cells were cultured in ALGÖX medium without epigenetic modifiers. The culture medium was changed every day thereafter until some colonies with compact morphologies appeared in reprogrammed cells (Fig. 4.8) Such colonies appeared only in the rbFs cultures but not in rbMSCs ones (Fig. 4.9). However, we could not amplify any of the rbFs colonies in the ALGÖX medium.

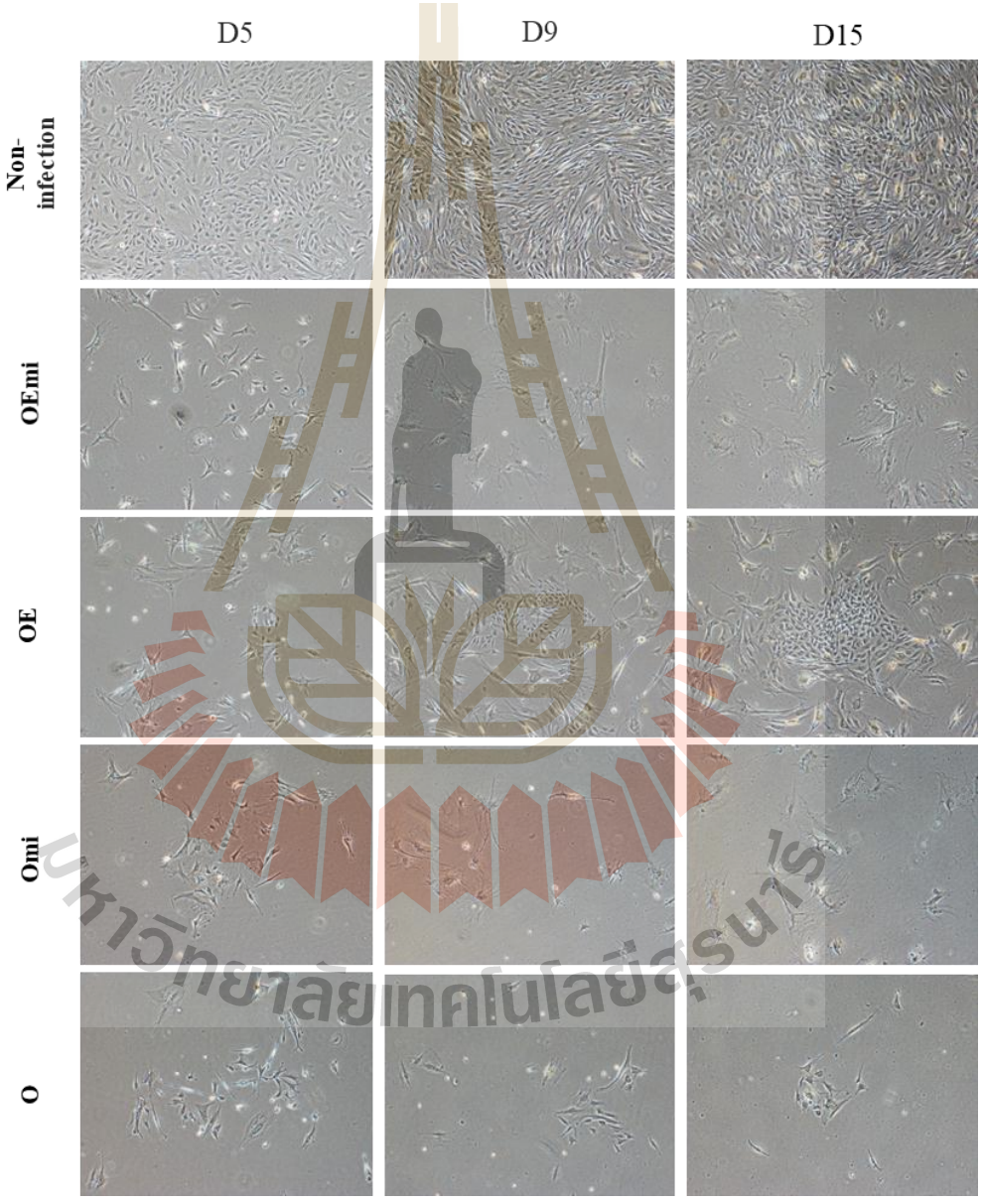

Figure 4.9 Phase-contrast images of rbMSCs after mRNA transfections in presence of epigenetic modifiers. OEmi = OSKMNL+EKB+microRNA; OE = OSKMNL+EKB; Omi = OSKMNL+microRNA, O = OSKMNL. Magnification 40x.

4.5 Conclusions

Mesenchymal stem cells and fibroblasts are present in normal tissues to support tissue homeostasis. Both share common pathways and have a number of common features, such as a spindle-shaped morphology, connective tissue localization, and multipotency (Soundararajan and Kannan., 2018; Ichim et al., 2018).

After verification of the quality of rbF and MSC lines in term of biosecurity (lines free of mycoplasma), and transduction efficiency with Sendai viruses, we applied the reprogramming protocol to rabbit cells by following the recommendations of the manufacturer. We modified the cell supports (feeders or matrigel) and the medium (KOSR/FGF2 primed medium versus ALGÖX naïve medium supplemented with H3K9 methyltransferase inhibitor A366 and HDAC inhibitor UF010).

Only transduced rbFs in naïve medium gave rise to clones showing feature of iPSCs with the reactivation of core pluripotency gene expression. These preliminary results are in line with the used of HDAC inhibitors to facilitate nuclear transfer (Rybouchkin et al., 2006; Kishigami et al., 2007; Dai et al., 2010; Ogura et al., 2013;), reversion of differentiation (Lyssiotis et al., 2007; Durcova-Hills et al., 2008; Tursun et al., 2011), and somatic cell reprogramming (Huangfu et al., 2008; Han et al., 2010; Mali et al., 2010; Liang et al., 2010). It should be noticed that we tried another non-integrative technic to produce rbiPSCs by reprogramming rbFs or rbMSCs with repetitive mRNA transfections. However, after adjustment of several parameters like cell density, mRNA concentration and used media, this technique did not give better results than Sendai vector transduction.

4.6 Conclusions

Attempts to produce rbiPSCs by reprogramming rbFs or rbMSCs with non-integrative methods showed that only naïve medium supplemented with histone methyltransferase inhibitor (A366) and histone deacethylase inhibitor (UF010) allowed the reprogramming of rbFs. Differences in reprogramming cell efficacity between origin of the somatic cells or their proliferation rate were shown in the

mouse. The necessity of erasing epigenetic marks specific of somatic cells is in line with the indispensable role of epigenetic modifiers used in this study.

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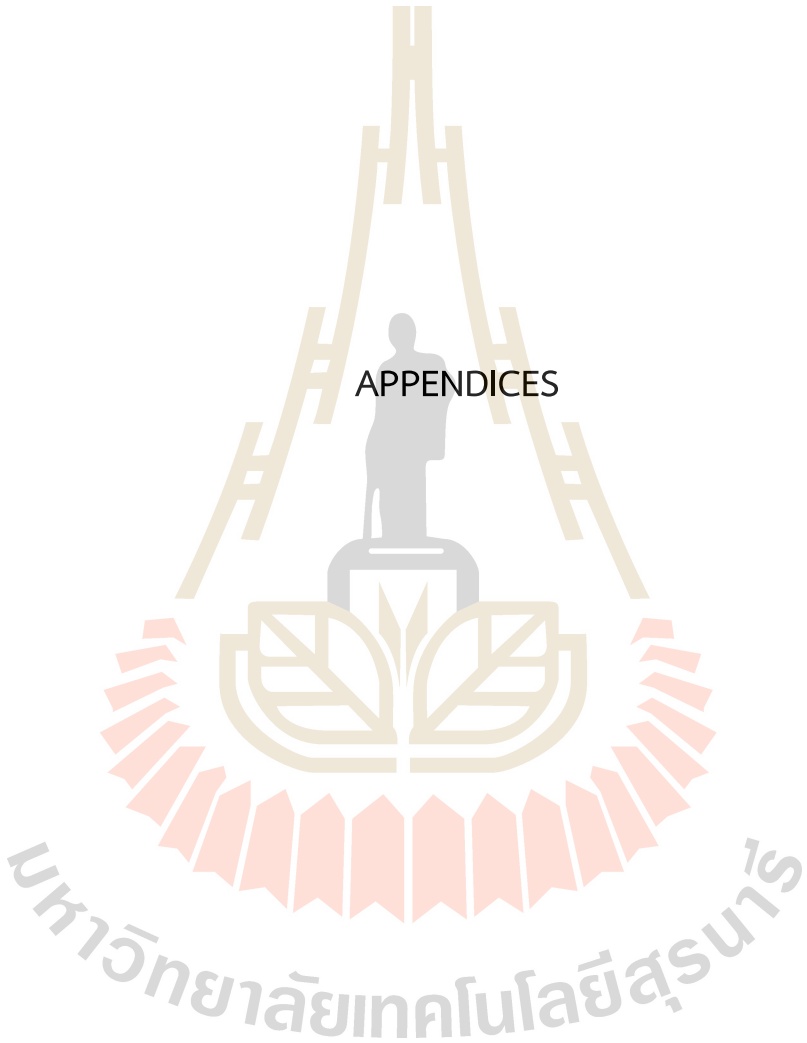
CHAPTER V

OVERALL CONCLUSION

This study provides the requisite technical protocols and resources to facilitate routinely generation and study of candidate rabbit naïve iPSCs. The intersestingly, the same define naïve medium composed of a cocktail of small molecules (Activin A and LIF) and inhibitors of both signaling pathways (PKCi and TNKi) and epigenetic modifiers (HMTi and HDACi) allowed the reprogramming of rbFs into rbiPSCs and the resetting of primed rbiPSCs toward the naïve pluripotency state. These molecules have already showed their efficiency in mouse and primate cell reprogramming, but the ending cocktail used may be different among species. In rabbit, LIF/gp130/Stat3, ActivinA/TGFß and WNT signaling pathways activation appeared to be necessary. In the same way, chromatin opening with HMTi and HDACi are essential to facilitate rbiPSCs resetting and to enhance somatic cell reprogramming. Even if most of results presented in the thesis need to be confirmed and extended, this work established a basic medium indispensable for reprogramming rabbit cells.

APPENDICES



APPENDIX A

SOLUTION PREPARATION

1. Reagent for epigenetic resetting

1.1 L-ascorbic acid (Sigma A8960) 250 mg/ml

Dissolve 2.5 g. of L-ascorbic in *10 ml. of phosphate-buffered saline* (PBS^-). Then *aliquoted* to small eppendorf tube and store at -20°C.

1.2 A366 (MedChem Express HY-12583) 2 mM

Dissolve 5 mg. of A366 in 7.59 ml. of dimethyl sulfoxide (DMSO). Then *aliquoted* to small eppendorf tube and store at -20°C.

1.3 VPA (Sigma 1069-66-5) 1 M

Dissolve 10 g. of valproic acid sodium salt (VPA) in 60.17 ml. of PBS^-. Then *aliquoted* to small eppendorf tube and store at -20°C.

1.4 BRD4354 (BRD, Tocris 6010) 2 mM

Dissolve 5 mg. of BRD4354 in 6.53 ml. of ethanol. Then *aliquoted* to small eppendorf tube and store at -20°C.

1.5 UF010 (UF010, Tocris 5588) 5 mM

Dissolve 5 mg. of UF010 in 3.69 ml. of DMSO. Then *aliquoted* to small eppendorf tube and store at -20°C.

2. Reagent for cell culture

2.1 Rabbit fibroblast medium or mouse embryonic fibroblasts medium

Mix the reagent as follow:

- DMEM (DMEM with high glucose, L-glutamine, phenol red, and sodium pyruvate, Life Technologies, 41966-052) 500 mL
- Fetal Bovine Serum 55 mL
- 100x PSG 5.5 mL
- 100x NEAA (non essential amino acids, 100x, Life Technologies, 11140-035) 5.5 mL

- 50 mM β-mercaptoethanol (Life Technologies, 31350-010). 1.1 mL

2.2 Rabbit induced pluripotent stem cells medium (“A” medium)

Mix the reagent as follow:

- DMEM/F12 (DMEM/ F12 with phenol red, without L-Glutamine, Life Technologies, 21331-020) 500 mL
- KOSR 130 mL
- 100x PSG 6.5 mL
- 100x NEAA (non essential amino acids, 100x, Life Technologies, 11140-035) 6.5 mL
- 50 mM β-mercaptoethanol (Life Technologies, 31350-010). 1.3 mL
- 100 mM sodium pyruvate 6.5 mL

Extemporaneously add FGF2 to a final concentration of 10 ng/mL.

APPENDIX B

THE REPROGRAMMING PROTOCOLS

1. Sendai virus infection

1.1 Reprogramming of rbMSCs with Sendai virus on feeder or feeder-free system.

Day 0 transduce the cells using the CytoTune® 2.0 Sendai reprogramming vectors at the multiplicity of infection (m.o.i.) 15: 15: 9. Dissociate the rbMSCs using 1xtrypsin, count the cells, and prepare a suspension containing $1x10^6$ rbMSCs /mL. Transfer 100 uL of the cell suspension ($1x10^5$) into a 1 well of 6-well plate containing 400 uL of freshly prepared mixture of Sendai viruses and MEF medium in the presence of 4 ug/ml polybrene (Sigma). After centrifugation for 1 h at 2000 rpm, incubate the infected cells at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 24 h.

Day 1 add 2 mL of fresh MEF medium and incubate the infected cells at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 24 h.

Day 2 replace the medium with 2 mL of fresh MEF medium to remove the CytoTune® 2.0 Sendai reprogramming vectors and allow the cells recover at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 24 h.

Day 4 dissociate the infected rbMSCs with 1x trypsin, centrifuge, and replated at low density ($6x10^2$ cells/cm^2) onto inactivated MEFs in rbiPSC medium or on Matrigel in VALGÖX medium. Incubate at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2.

Day 5-21 Replace spent medium every day and monitor the culture vessels for the emergence of iPSC colonies. To avoid acidification, increase the volume of the medium gradually as the cell density increase.

1.2 Reprogramming of rbFs and rbMSCs with Sendai virus and epigenetic modifiers.

Day 0 transduce the cells using the CytoTune® 2.0 Sendai reprogramming vectors at the multiplicity of infection (m.o.i.) 15:15:9. Dissociate the rbFs or rbMSCs using 1xtrypsin, count the cells, and prepare a suspension containing $1x10^6$ rbFs or rbMSCs/mL. Transfer 100 uL of the cell suspension ($1x10^5$) into a 1 well of 6-well plate containing 400 uL of freshly prepared mixture of Sendai viruses and AALGÖX medium + UF010 in the presence of 4 ug/ml polybrene (Sigma). After centrifugation for 1 h at 2000 rpm, incubate the infected cells at 38°C in a humidified atmosphere of 5 % CO_2 and 5% O_2 for 24 h.

Day 1 add 2 mL of fresh AALGÖX medium+UF010 in SeV1 group and incubate the infected cells at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 24 h. For SeV2 group, transduce the cells using mixture of Sendai viruses again.

Day 2 replace the medium with 2 mL of fresh AALGÖX medium+UF010 in SeV1 group and add 2 mL of fresh AALGÖX medium+UF010 in SeV2 group.

Day 3 replace the medium with 2 mL of fresh AALGÖX medium+UF010 in SeV2 group.

Day 4 dissociate the infected rbFs or rbMSCs with 1x trypsin, centrifuge, and replated at low density ($6x10^2$ cells/cm^2) on Matrigel (Beckton Dickinson) in ALGÖX medium. Incubate at 38°C in a humidified atmosphere of 5% CO_2 and 5% O_2.

Day 5-21 Replace spent medium every day and monitor the culture vessels for the emergence of iPSC colonies. To avoid acidification, increase the volume of the medium gradually as the cell density increase.

2. mRNA transfection

Day 0 dissociate the the rbFs or rbMSCs using 1x trypsin, count the cells, and prepare a suspension containing $1x10^6$ rbMSCs/mL. $0.5x10^5$ rbFs and

rbMSCs were plate on Matrigel (Beckton Dickinson) in MEF medium and at 37°C and 5% CO_2.

Prepare NM-RNA reprogramming cocktail to reprogram rbFs or rbMSCs following in a sterile, RNase-free microcentrifuge tube. The components and volume of StemRNA 3rd Gen Reprogramming Kit:

OSKMNL NM-RNA:	32.0 µL
EKB NM-RNA:	24.0 µL
NM-microRNAs:	5.6 µL
Total NM-RNA reprogramming cocktail:	**61.6 µL**

The daily NM-RNA reprogramming cocktail is composed of 0.8 µg OSKMNL NM-RNA±0.6 µg EKB NM-RNA (total mRNA=1.4 µg), and ±0.4 µg NM-microRNAs per transfection per well (6-well plate format). Divide the mixture into single-use aliquots in sterile, RNase-free microcentrifuge tubes following Table A1. Store the aliquots at -80°C for up to three months. Avoid additional freeze thaw cycles.

Table A1 NM-RNA Reprogramming Cocktail in each experimental groups.

Experimental groups	StemRNA 3rd Gen Reprogramming Kit components	Volume/ microcentrifuge tube (uL)
OEmi	OSKMNL +	8
	EKB +	6
	microRNA	1.4
OE	OSKMNL+	8
	EKB	6
Omi	OSKMNL+	8
	microRNA	1.4
O	OSKMNL	8

Day1 Remove the old medium from the wells in the reprogramming plate. Add 2 mL MEF Medium to each well and incubate in hypoxic incubator at 38°C, 5% CO_2, 5% O_2.

Thaw one NM-RNA Reprogramming Cocktail single-use aliquot at room temperature, then immediately place on ice. Label as tube "A (RNA + Opti-MEM)". Label a sterile, RNase-free 1.5 mL microcentrifuge tube "B (RNAiMAX+ Opti-MEM)".

• To tube A, add 234.6 , 236, 240.6, and 242 µL Opti-MEM into tube A that already contains 15.4 µL OEmi, 14 uL OE, 9.4 uL Omi and 8 uL O, respectively.

• To tube B, add 6 µL RNAiMAX transfection reagent to 244 µL Opti-MEM (see Fig A1).

Figure A1 NM-RNA Reprogramming cocktail set-up.

Pipette gently three to five times to mix. Using a pipettor, transfer the entire contents of tube B to tube A drop-wise at meniscus level. Mix by tapping the bottom of the tube. Incubate at room temperature for 15 min. Then, add 500 µL NM-RNA transfection complex solution to the well in the reprogramming plate by tilting the plate and pipetting drop-wise into medium. Mix by rocking in the X- and Y-directions. Return the reprogramming plate to a hypoxic incubator (5% O_2) overnight.

Day 2-4 At the beginning of the day, remove the old medium from the wells in the reprogramming plate. Add 2 mL MEF Medium to each well. Incubate in hypoxic incubator at 38°C, 5% CO_2, 5% O_2 for at least 6 hr. At the end of the day, add 500 µL NM-RNA transfection complex

solution to the well in the reprogramming plate by tilting the plate and pipetting drop-wise into medium. Mix by rocking in the X- and Y-directions. Return the reprogramming plate to a hypoxic incubator (5% O_2) overnight.

Day 5-8 replace the medium with 2 mL of fresh AALGÖX medium+UF010 in all experimental groups.

Day 9-21 Replace the medium with ALGÖX medium every day and monitor the culture vessels for the emergence of iPSC colonies. To avoid acidification, increase the volume of the medium gradually as the cell density increase.

มหาวิทยาลัยเทคโนโลยีสุรนารี

BIOGRAPHY

Worawalan Samruan was born in Angthong, Thailand on December 29th, 1986. She finished her high school from Angthong Pattamaroj Wittayakhom School in Angthong. In 2008, she graduated from Suranaree University of Technology, Nakhonratchasrima, Thailand, with Bachelor's Degree (B.Sc.) of Food Technology. She received a Master Degree (M.Sc.) in Food Technology from Suranaree University of Technology in 2013. Her research topic was "Comparison of biological properties of soybean and fermented soybean extraxcts". In July 2017, she started study Ph.D in Biotechnology at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. Her Ph.D study was supported by MEDEZE Foundation fellowship under supervision of Assoc. Prof. Dr. Rangsun Parnpai. She has been carried out research at Stem cell and Brain Research Institute, INSERM, Bron, France under supervision of Dr. Marielle AFANASSIEFF during 17 May 2019 to 16 March 2020 and during 18 January 2021 to 6 August 2021. Her Ph.D thesis title is "Epigenetic modifiers enhance the reprogramming of rabbit somatic cells and rabbit induced pluripotent stem cells".