

Exploring the function of NLRP7 in regulating human maternal imprinting

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Abstract

Genomic imprinting is an epigenetic phenomenon where gene expression is regulated in a parent-of-origin-specific manner. Maternal imprinting, established by de novo DNA methylation during oocyte development, is generally protected from global DNA demethylation post-fertilization. In the context of complete hydatidiform mole (CHM), the loss of maternal imprinting in cells from molar tissue with defective NLRP7 suggests that NLRP7 may play a role in maintaining maternal imprinting. NLRP7, a human maternal effect protein, is highly expressed in developing oocytes and preimplantation human embryos. However, its specific function in regulating maternal imprinting remains unclear. In this project, human embryonic stem cells are utilised as a cell model to investigate the role of NLRP7 in maternal imprinting during this conversion process and to explore the molecular mechanisms involved.

Single nucleotide polymorphisms (SNPs) were identified in hESCs, enabling the analysis of their allelic expression patterns in imprinting studies. NLRP7 expression was found to be very low in primed hESCs but increased at the late stages of naïve conversion. This suggests that the initial low level of NLRP7 might contribute to the loss of maternal imprinting. Subsequently, NLRP7 was overexpressed in hESCs, which were then reprogrammed to a naïve state. Maternal imprinting was partially retained in these naïve NLRP7-overexpressing hESCs, whereas paternally imprinted genes maintained. This indicates that NLRP7 specifically protects maternal imprinting from global demethylation. Moreover, NLRP7 was predominantly localised in cytoplasm, with a small amount of NLRP7 present in the nucleus, and it co-localized with spindle fibres during cell division. Consequently, my findings demonstrate NLRP7 can partially rescue the loss of maternal imprinting during global DNA demethylation process. More importantly, this is the first time showing that NLRP7 can be in closely contact with nuclear DNA during cell division. This localization suggests a possible mechanism by which NLRP7 regulates maternally imprinted genes.

Statement of Originality

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Abbreviations

-CH ₃	Methyl group
2i/L	Cocktail of MEKi, GSK3i and LIF
4CL	Cocktail of MEKi, HDACi, tankyrase inhibitor, EZH2i and hLIF
5acC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5iLAF	Cocktail of MEKi, GSK3i, SRCi, bRAF _i , ROCK _i with LIF, Activin and bFGF
5mC	5-Methylcytosine
αPKC	atypical PKC
APS	Ammonium Persulfate
BCA	Bicinchoninic Acid
BER	Base excision repair
bFGF	Basic Fibroblast Growth Factor
BiCHM	Biparental CHM
BMP4	Bone morphogenetic protein 4
BSA	Bovine Serum Albumin
BWS	Beckwith-Wiedemann syndrome
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase activation and recruitment domain
cDNA	Complementary DNA
CGI	CpG island
CHM	Complete hydatidiform mole
CpG	5'—C—phosphate—G—3'
Cy	Cynomolgus monkeys
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMR	Differentially methylated region
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1 _o	Oocyte-specific isoform of DNMT1
dNTP	deoxy-ribonucleoside triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
DSBH	Double-stranded β-helix

EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced green fluorescent protein
EPI	Epiblast
EpiSC	Epiblast stem cell
ESC	Embryonic stem cell
FBS	Fetal Bovine Serum
FGF2	Basic fibroblast growth factor
FIIND	Domain with function to find
gDNA	Genomic DNA
GFP	Green fluorescent protein
HDAC2	Histone deacetylase 2
HEK293T	Human embryonic kidney cell line
hESC	Human embryonic stem cell
hLIF	Human Leukemia Inhibitory Factor
HM	Hydatidiform mole
HP1	Heterochromatin protein 1
hPESC	Human parthenogenetic embryonic stem cells
ICM	Inner cell mass
ICR	Imprinted control region
Igf2r	Insulin-like growth factor 2 receptor
iMEF	Irradiated mouse embryonic fibroblast
iPSC	induced pluripotent stem cells
JAK–STAT3	Janus kinase–signal transducer and activator of transcription 3
KSR	Knockout Serum Replacement
LB	Lysogeny Broth
LG	L-Glutamine
LIF	Leukaemia inhibitory factor
lncRNA	Long noncoding RNA
LoM	Loss of methylation
LRR	Leucine-rich repeat
MAF	Minor allele frequency
MEF	Mouse embryonic fibroblast
MEF-CM	iMEF-Conditioned Medium
mEpiSCs	mouse EpiSCs
mESC	mouse ESC
Min	Minutes
MLID	Multi-locus imprinting disturbance

NACHT	Nucleotide-binding oligomerization domain
NaCl	Sodium Chloride
NAD	NACHT-associated domain
NEAA	Non-Essential Amino Acids
NHSM	Naïve Human embryonic Stem cell Media
NLR	NOD-like receptors
NLRP	NACHT, LRR and PYD domains-containing protein
NP-40	Nonidet P-40
PAC	Puromycin acetyltransferase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin-Streptomycin
PGC	Primordial germ cell
PGCLC	Primordial germ cell like cell
PMSF	Phenylmethanesulfonyl Fluoride
PSC	Pluripotent stem cell
PYD	Pyrin domain
PVDF	Polyvinylidene Fluoride
PXGL	Cocktail of WNTi, HDACi, MEKi and LIF
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIPA	Radio Immunoprecipitation Assay
RNA	Ribonucleic acid
rNLRP	Reproductive NLRP
SCMC	Subcortical maternal complex
SDS	Sodium Dodecyl Sulfate
Sec	Second
SNP	Single-nucleotide polymorphism
SRS	Silver–Russell syndrome
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TBS-T	Tris Buffered Saline with Tween-20
TDG	Thymine DNA glycosylase
TEMED	Tetramethylethylenediamine
TET	Ten-eleven translocation methylcytosine dioxygenase enzymes
TGFβ	Transforming growth factor β
TKO	Triple knockout

Tme	T-maternal effect
UPD	Uniparental disomy
YY1	Yin and Yang 1
ZFP57	Zinc Finger Protein 57
ZGA	Zygotic genome activation

Chapter 1

Introduction

1.1. GENOMIC IMPRINTING

Genomic imprinting is a hereditary epigenetic phenomenon that controls gene expression based on the parent of origin, without reliance on the DNA sequence itself (Ferguson-Smith, 2011). In most of the mammalian genome, genes are typically expressed from both parental alleles, a pattern known as biallelic expression, and regulated by transcription factors and other regulatory elements binding to gene enhancers and promoters (Buckland, 2004). By contrast, at an imprinted locus, parental imprinted genes are expressed mono-allelically with either maternally or paternally inherited allele being transcriptionally silenced through distinct epigenetic modifications, predominantly DNA methylation (Tucci et al., 2019). These processes significantly impact embryonic and placental development and allow imprinted genes to fulfil distinct roles in various cell types throughout the life (Tucci et al., 2019). Hence, genomic imprinting represents a crucial aspect of non-genetic inheritance, offering a rich field for exploring genomic regulation.

1.1.1. Importance of Genomic Imprinting

Genomic imprinting plays an important role in both embryonic and postnatal development. Mouse embryos with either two maternal (diploid gynogenetic) or two paternal (androgenetic) gene copies showed abnormal development, highlighting the necessity of imprinting regulation for normal embryonic development, beyond the presence of a complete chromosome set consisting of paternal and maternal chromosomes (McGrath and Solter, 1984). The discovery of the first imprinted locus in mice, *T-maternal effect* (*Tme*), revealed that imprinting was restricted to some regions of whole genome. The deletion of this region in mice leads to different outcomes based on whether the maternal or paternal allele is affected. (Johnson, 1974). Specifically, maternal deletion of the *Tme* locus results in lethality, whereas paternal deletion has no apparent effect (Barlow et al., 1991). So far, the well-known

hypothesis that imprinting arose in placental mammals is the ‘Parental conflict hypothesis’: a conflict arises between the maternal and paternal genomes in the growth and development of offsprings during pregnancy, in which maternally imprinted genes restrain foetal growth, while paternally imprinted genes promote the growth and fitness (Moore and Haig, 1991).

In humans, genomic imprinting significantly influences embryonic development, and its dysregulation poses considerable risks to normal development and health, leading to developmental abnormalities, diseases and infertility. The majority of human imprinting disorders are linked to uniparental disomy (UPD) (Table 1.1), where both copies of a chromosome pair or part of the chromosome come from a single parent (Monk et al., 2019, Eggermann et al., 2018). For example, maternal Chr 7 UPD leads to Silver-Russell syndrome (SRS), a congenital growth disorder with severe growth retardation; while paternal Chr11p15 UPD results in Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth disorder (Bartholdi et al., 2009).

Table 1.1 Summary of Imprinting disorders associated with UPD regions.

Disorder	UPD	Imprinted gene affected
Transient Neonatal Diabetes Mellitus	UPD(6q24)Pat	<i>KCNJ11 or ABCC8</i>
Silver-Russell Syndrome	UPD(7)Mat	<i>IGF2 or CDKN1C</i>
Beckwith-Wiedemann Syndrome	UPD(11p15)Pat	<i>CDKN1C</i>
Kagami-Ogata syndrome	UPD(14)Pat	<i>RTL1</i>
Temple syndrome	UPD(14)Mat	<i>DLK1</i>
Angelman Syndrome	UPD(15q12)Pat	<i>UBE3A</i>
Prader-Willi Syndrome	UPD(15q12)Mat	<i>SNRPN or NDN</i>
Pseudohypoparathyroidism	UPD(20)Pat	<i>GNAS</i>
Mulchandani-Bhoj-Conlin syndrome	UPD(20)Mat	-

In addition, loss of methylation (LoM) at the imprinting ICR can also cause developmental abnormalities. SRS often occurs in patients with hypomethylation at paternal chr11p15 *H19/IGF2* locus IG-DMR (60% cases), leading to paternal imprinting defect on the locus with reduced IGF2 expression in the paternal allele, hampering growth and leading to growth retardation (Bartholdi et al., 2009). Interestingly, loss of methylation in the same chromosomal region but affecting different alleles can lead to distinct syndromes. For example, loss of methylation on maternal chr11p15 KvDMR causes Beckwith-Wiedemann syndrome (BWS) with the overgrowth and cancer predisposition (Poole et al., 2012) (Figure 1.1).

Moreover, some of the imprinting-associated disorders resulted from loss of function effect. Prader-Willi syndrome showing muscle weakness arises from mutations in paternally 15q11-13 region containing maternally imprinted genes *SNRPN* and *NDN*, leading to loss of expression and function of these genes. Whereas Angelman syndrome, a genetic disorder affecting central nerve system, is caused by the loss of *UBE3A* gene in the maternal 15q11-13 (Buiting, 2010). Since *UBE3A* gene is paternally imprinted in the brain, loss of it in maternal allele leads to loss of its expression and function. Beyond these rare syndromes, imprinting defects are also implicated in other diseases. Transient neonatal diabetes mellitus, a type of neonatal diabetes at birth but not permanent, is associated with paternal UPD on 6q24 which induces the overexpression of *PLAGL1* gene (Ma et al., 2004). In addition, Wilm's tumour (nephroblastoma), a paediatric kidney cancer found in children linked to *IGF2* overexpression and reduced *H19* expression from the maternal allele (Dome and Coppes, 2002). Given the complexities and the critical role of genomic imprinting, further research in this field is imperative to advance our understanding and develop effective treatments for related conditions.

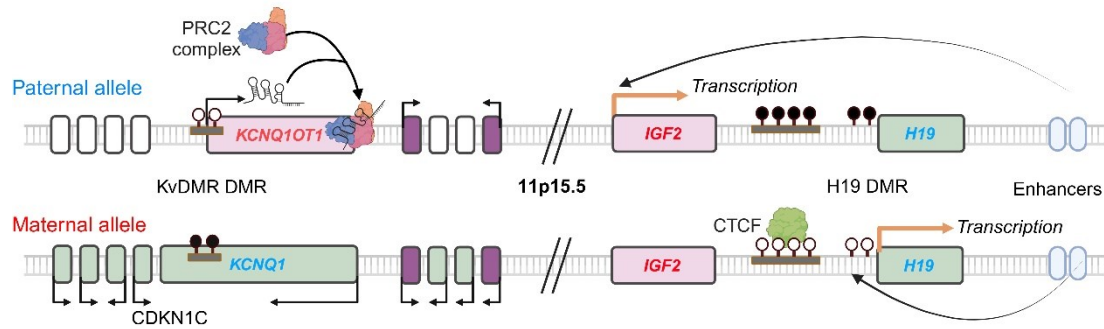


Figure 1.1 The Imprinting Control Regions at 11p15.

KvDMR DMR: On the paternal allele, the unmethylated ICR allow the expression of maternally imprinted gene *KCNQ1OT1* and lncRNA *KCNQ1OT1* that cooperate with PRC2 complex to inhibit the expression of paternally imprinted gene *KCNQ1* and other surrounding genes; whereas paternally imprinted genes (eg: *CDKN1C* and *KCNQ1*) are expressed on maternal allele. Meanwhile, some genes were biallelically expressed on both alleles. **H19 DMR:** On the maternal allele, the unmethylated ICR is bound by the CTCF protein which block the interaction between transcription-bound enhancer and promoter of maternally imprinted gene (*IGF2*); allowing the enhancer to facilitate the expression of paternally imprinted gene (*H19*). Whereas on the paternal allele, the methylated ICR inhibit CTCF binding to the ICR and distal enhancer acting onto *H19* promoter, permitting the interaction between distal enhancer and *IGF2* promoter and activating the expression of *IGF2*. The blue and red colours represent paternal and maternal alleles, respectively; purple colour represents biallelic expression. Open and closed circles represent unmethylated and methylated CpGs, respectively.

1.1.2. Molecular Regulation of Genomic Imprinting

1.1.2.1. Allele-specific DNA methylation controls genomic imprinting

Differential DNA methylation on regions in maternal and paternal strands is considered a predominant regulating mechanism for imprinting and these regions are termed imprinting control region (ICR) (Fig 1.1). Asymmetrically Inherited DNA methylation at the ICRs serves as a biological memory of the gene's parental origin, regulating the expression of imprinted genes in an allele-specific manner (Kelsey and Feil, 2013). The imprinted genes are often clustered throughout the mammalian genome with each cluster containing several imprinted genes that are regulated by one single ICR. This ICR exhibits distinct methylation patterns between maternal and paternal alleles, thus also known as differentially methylated region (DMR) (Kelsey and Feil, 2013, Tucci et al., 2019).

Imprinted genes are typically expressed from only one allele in most of the cell types, although the imprinted expression of certain genes may be limited to specific tissues or at certain developmental stages. When maternal imprinting take place, maternally imprinted genes are silenced on maternal allele but are expressed on paternal allele; conversely, paternally imprinted genes are only expressed from the maternally inherited allele but are silenced on their paternal allele (Hanin and Ferguson-Smith, 2020). The methylation of DMRs can suppress the expression of imprinted genes by blocking the binding of transcription factors (Moore et al., 2013). To maintain this methylated state, the methylated DMRs attract transcription repressors, such as ZFP57, to themselves. Conversely, transcription factors like CTCF, POU5F1, and SOX2 bind to unmethylated DMRs, maintaining their unmethylated condition (Monk et al., 2019).

Moreover, within each gene cluster, an imprinted gene can produce long noncoding RNA (lncRNA) that regulates adjacent imprinted genes through a cis-acting mechanism (MacDonald and Mann, 2020). For example, the maternally imprinted gene *KCNQ1OT1* was identified to produce a lncRNA in mammals, which controls the expression of multiple imprinted genes within the same locus, e.g. *CDKN1C*. The *KCNQ1OT1* is actively expressed on the paternal allele and the resulting lncRNA binds to the promoters of the surrounding genes *in cis*, recruiting the PRC1/2 complex and histone methyltransferase G9a to the promoters, condensing and silencing those protein-coding genes. Conversely, on the maternal allele, the expression of *KCNQ1OT1* lncRNA is inhibited by the methylated maternal DMR, hence the surrounding genes are permitted to be expressed (Figure 1.1) (Zhou et al., 2024, Pauler et al., 2012).

Intriguingly, numerous maternally methylated DMRs are intragenic and are often correlated with promoters of lncRNA, whereas methylated DMRs on paternal

chromosomes are intergenic and act as insulators or enhancers (Kota et al., 2014, Barlow and Bartolomei, 2014).

1.1.2.2. DNA methylation by DNA methyltransferases

DNA methylation is a heritable epigenetic modification occurring primarily at CpG dinucleotides (Tucci et al., 2019). These dinucleotides are often distributed to CpG islands (also called CG islands, CGI) that are short DNA regions being CG- or CpG-rich (>50%) (Deaton and Bird, 2011). The CpG islands are widespread throughout the human genome, but approximately 60% of these islands are associated with gene promoters or enhancers and play crucial roles in gene regulation (Jeziorska et al., 2017, Li and Zhang, 2014). DNA methylation is established by DNA methyltransferases (DNMTs) that covalently transfer the methyl group to unmethylated cytosine via their C-terminal catalytic domain (Goll and Bestor, 2005, Chen and Zhang, 2020). There are 4 DNMTs identified in mammals, DNMT1, DNMT3A, DNMT3B, and DNMT3L, in which DNMT3L does not contain an active catalytic domain and thus cannot perform methylation itself (Li and Zhang, 2014). The other three DNMTs are categorised into two groups based on their functions: *de novo* DNA methylation and DNA methylation maintenance (Fig 1.2).

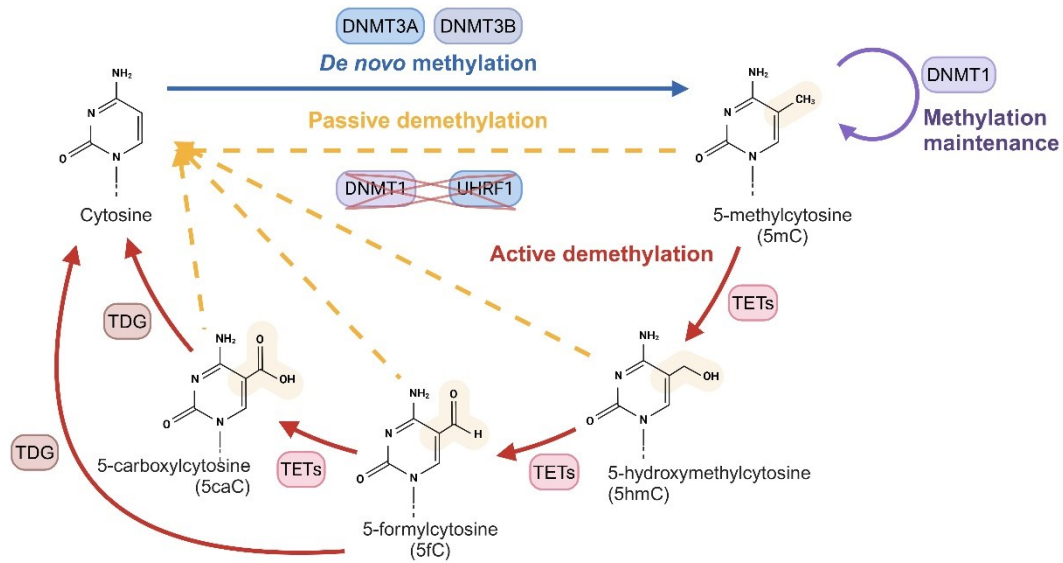


Figure 1.1 The summary of DNA methylation and DNA demethylation.

Unmodified cytosine can be methylated to 5mC by DNMT3A/3B (*De novo* methylation; blue arrow) and 5mC is maintained by DNMT1 (Methylation maintenance; purple arrow); 5mC can be oxidized to different forms (5hmC, 5fC and 5caC) via TETs and can be converted back to unmodified cytosine via replication-dependent dilution (Passive demethylation, yellow dash arrow). The 5fC and 5caC can be directly eliminated and repaired via TDG-BER method (Active demethylation, red arrow).

i. DNMT1

DNMT1 is crucial for maintaining methylation during DNA replication, recognizing the hemimethylated CpG sites on newly replicated strands and adding methyl groups to the sites to maintain the conserved CpG methylation pattern (Li et al., 1992). This process requires E3 ubiquitin ligase UHRF1 that recognizes hemimethylated DNA and recruits DNMT1 to the replication foci during the S phase (Avvakumov et al., 2008, Chen and Zhang, 2020). Deficient UHRF1 in cells shows a diffused nucleoplasmic localization of DNMT1 and undergoes global loss of DNA methylation with cell divisions (Sharif et al., 2007, Bostick et al., 2007). This function of UHRF1 is supported by the *Uhrf1*-knockout mice that exhibit a similar phenotype as *Dnmt1*-knockout mice with embryonic lethality after gastrulation (Sharif et al., 2007). Notably, DNMT1 has an oocyte-specific isoform (DNMT1o), only being present in oocytes and early embryos and its main function is to maintain parental-specific DNA methylation at imprinted loci

(Howell et al., 2001). The mouse embryo derived from *Dnmt1o* deficient oocyte, with functional somatic *Dnmt1*, shows partial loss of imprinting and embryos die before birth. Moreover, the embryo with double knockout of both isoforms of *Dnmt1* displays a complete loss of imprinting at the blastocyst stage (Howell et al., 2001, Hirasawa et al., 2008). Additionally, DNMT1o is also found in human embryos, and disruptions in DNMT1o function are associated with mosaic DNA hypomethylation at imprinted loci (Petrussa et al., 2014). Thus, DNMT1o emerges as a crucial factor in preserving methylation patterns at imprinted loci during the initial stages of embryo development.

ii. DNMT3A and 3B

DNMT3A and 3B are responsible for *de novo* DNA methylation. They are expressed during gametogenesis and early embryonic development and mainly recognise the unmethylated cytosine in CGI to establish new methylation patterns (Okano et al., 1999, Kato et al., 2007). Although the function of both DNMT3A and 3B is similar for *de novo* DNA methylation, the individual *Dnmt3a* or *3b* knockout mice show different phenotypes. The *Dnmt3a*-null mice are normal at birth but show abnormal postnatal development, leading to their death at 4 weeks; while the *Dnmt3b* knockout mice are embryonic lethal at E9.5 but develop normally before that (Kaneda et al., 2004, Okano et al., 1999). This difference likely indicates that the functions of *Dnmt3a* and *Dnmt3b* are slightly different, resulting in diverse phenotypes. Indeed, research found that *Dnmt3a* specifically methylates imprinted genes in germ cells and the *XIST* gene on the X chromosome, whereas *Dnmt3b* is capable of methylating centromeric satellite repeats (Li et al., 2007, Sergeeva et al., 2023). Notably, recent research found that *Dnmt3a* and *3b* have the same importance as *Dnmt1* in maintaining DNA methylation at all repeats, especially DMR and other genomic regions in mESCs regardless of methylation level (Liu et al., 2022). Hence, both methyltransferases have the capacity to methylate almost all DNA loci and also cooperate with *Dnmt1* in maintaining DNA methylation.

The activation of DNMT3A and DNMT3B requires unmethylated H3K4 to release their catalytic domain, whereas the modified H3K4me3 can bind to unmethylated CpG to block the DNMT3A/3B (Cedar and Bergman, 2009, Li and Zhang, 2014). Additionally, changes in chromatin conformation serve as triggers for DNA methylation. Specifically, on maternal ICRs, the removal of H3K4me2 at intragenic CGI by the demethylase KDM1B is a permissive signal for H3K36me3 deposition over transcribed regions by the methyltransferase SETD2. The H3K36me3 further recruits DNMTs on the ICR region for DNA methylation (Stewart et al., 2015, Xu et al., 2019). This information reveals the establishment of DNA methylation by DNMT3A/3B is guided by histone modification.

iii. DNMT3L

DNMT3L, despite having an inactive catalytic domain, has been found to act as a crucial cofactor for DNMT3A and DNMT3B in cells, enhancing their methyltransferase activity (Chedin et al., 2002, Bourc'his et al., 2001). It forms a butterfly-shaped tetramer with DNMT3A (DNMT3L-DNMT3A-DNMT3A-DNMT3L), a structure that stabilizes the DNMT3A dimer, allowing it to bind directly to DNA. The disruption of this tetramer can abolish the function of DNMT3A, which might be resulted by eliminating the conformation of DNMT3A dimer (Jia et al., 2007). In mice, *Dnmt3l* was reported to accelerate DNA methylation by maintaining the stability of *Dnmt3a* in mESCs (Veland et al., 2019). *Dnmt3l* knockout mice show normal growth to adults, but it has defects in reproduction or embryonic development regardless of sex but show different phenotypes. The *Dnmt3l-null* male is infertile owing to the restricted differentiation of spermatogonia to mature spermatocytes, while the female null mice can be pregnant, but the embryos die at mid-gestation due to loss of maternal imprinting (Hata et al., 2002). These findings underscore the vital role of *Dnmt3l* in both mouse reproductive and embryonic development through its regulation and establishment of DNA methylation. However, the expression pattern of DNMT3L in humans is different from

that in mice. In mice, *Dnmt3l* is highly expressed in oocytes while in humans it is undetectable in oocytes but sharply upregulated after the 8-cell stage (Yan et al., 2013, Petropoulos et al., 2016). The exact function of DNMT3L in human reproduction and development remains to be identified.

iv. DNA methylation in human

Given the impracticality of studying human embryos, research on human DNA methylation primarily utilizes hESCs that resemble post-implantation epiblasts. To date, hESCs with deficient *DNMT1* exhibit widespread DNA demethylation, leading to rapid cell death (Liao et al., 2015). By contrast, knockout of *DNMT3A* or *3B* individually or combined show no effect on hESC self-renewal and three germ-layer differentiation. Only the *DNMT3A/3B* double knockout exhibits a mild loss of CpG methylation after long-term proliferation (Liao et al., 2015). It is likely that functional and mechanistic differences exist in DNMTs between humans and mice. For instance, DNMT3L, which is present in mouse oocytes, is absent in human oocytes. This suggests that DNMT3A might establish DNA methylation independently in human germ-line cells, without the involvement of DNMT3L (Guo et al., 2014, Okae et al., 2014). Consequently, numerous questions regarding DNMT functions and their mechanisms in humans remain unanswered, highlighting the need for further investigation.

1.1.2.3. DNA demethylation by passive and active mechanisms

During embryonic development, DNA methylation patterns can be reset to erase epigenetic memory inherited from parental chromosomes through passive and active demethylation mechanisms (Fig 1.2) (Rougier et al., 1998). The passive process involves the dilution of DNA methylation markers during DNA replication, primarily due to the absence of DNMT1, which is essential for maintaining methylation on double-stranded DNA (Kagiyada et al., 2013). Conversely, the active demethylation pathway relies on an enzymatic process that removes the methyl group (Wu and Zhang, 2017).

This active process is facilitated by the ten-eleven translocation (TET) methylcytosine dioxygenase enzymes, including TET1, TET2, and TET3. These enzymes share a common cysteine-rich domain that stabilizes the TET-DNA interaction and a C-terminal double-stranded β -helix (DSBH) domain for catalysing the oxidation of methylcytosine (Wu and Zhang, 2017). Unlike TET2, TET1 and TET3 possess a CXXC zinc finger domain at their N-terminal, displaying a high affinity for CpG-rich DNA regions (Xu et al., 2012). All three TET proteins can sequentially oxidize 5-methylcytosine (5mC) into 5-hydroxymethyl cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxyl cytosine (5caC) (Wu and Zhang, 2017). These oxidation derivatives of 5mC can be eliminated by a replication-dependent dilution process due to the less affinity of UHRF1 and DNMT1 at these oxidative products (Hashimoto et al., 2012, Otani et al., 2013). Furthermore, in the absence of DNA replication, the oxidative 5fC or 5caC can be further catalysed by thymine DNA glycosylase (TDG)-mediated excision, resulting in an abasic site. This site is subsequently repaired through the base excision repair (BER) pathway, restoring the unmodified cytosine state (He et al., 2011, Weber et al., 2016).

Tet1 and Tet2 play crucial roles in mESCs since their knockout result in reduced 5hmC levels and altered gene transcription (Ito et al., 2011). Interestingly, Tet1 and Tet2 exhibit distinct targeting preferences; *Tet1* knockout predominantly affects the promoter regions in mESCs, likely due to its CXXC domain, whereas *TET2* preferentially targets the highly active gene bodies and enhancers (Koh et al., 2011, Huang et al., 2014). Remarkably, a triple knockout (TKO) of all three Tet proteins in mESCs abolishes 5hmC completely. Despite this, cells retain a normal morphology and maintain the expression of pluripotent markers, though their ability for differentiation is impaired (Dawlaty et al., 2014). This finding aligns with observations in TKO hESCs, which maintain pluripotency marker expression but show reduced expression of markers for different lineages (Verma et al., 2018). Furthermore, studies have identified a positive correlation between TET expression and demethylation

levels, particularly in the absence of *DNMT3A/3B* in both hESCs and mESCs. This suggests a competitive regulatory interaction between DNMT3A/3B and TETs at somatic regulatory regions (Charlton et al., 2020). In conclusion, TETs are important for DNA demethylation throughout the genome and hypomethylation status may affect ESCs differentiation during embryonic development.

1.1.3. Dynamic DNA methylation in early embryonic development

Genomic DNA methylation undergoes significant changes during gametogenesis and embryonic development in mammals, a critical process that allows cells to reset epigenetic memory and adopt the new cell fate. This process is also important for establishing proper imprinting. This dynamic cycle has been extensively studied in humans and mice and involves two rounds of erasure and establishment of DNA methylation (Fig 1.3).

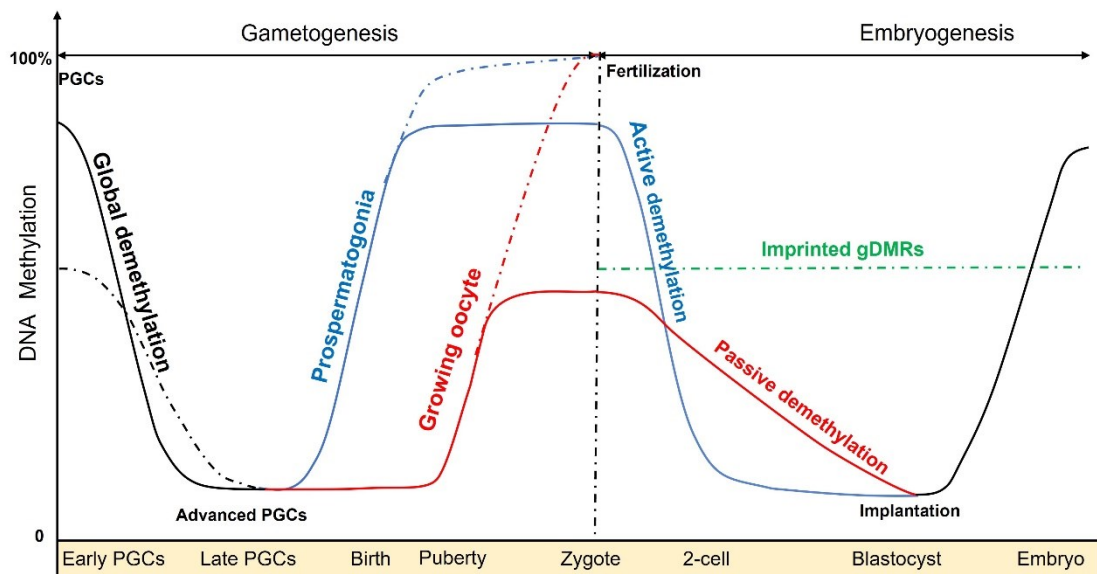


Figure 1.2 Dynamic DNA methylation change during human embryogenesis.

The global methylation level is shown in black during PGC development and post-implantation stage. The dynamic *de novo* methylation and demethylation process between male and female are represented by blue and red respectively. The methylation status of ICRs is shown as dashed lines; the green dashed line represents imprinted gDMRs post-fertilisation.

1.1.3.1. *DNA methylation during gametogenesis*

Gametogenesis starts from the emergence of the primordial germ cells (PGCs) that are differentiated from post-implantation posterior epiblast at the time of gastrulation (Bowles and Koopman, 2010). These PGCs then proliferate, meanwhile migrate to the congenital ridge, the precursor of gonads where they further develop in the gonads to become gametes, either oocytes or sperms depending on the individual sex (Bowles and Koopman, 2010, Sasaki and Matsui, 2008). Recent research on dynamic DNA methylation has shed more light on this process in humans. Studies indicate that the global resetting of the methylome in human PGCs (hPGCs) begins before week 4 of development. By this time, DNA methylation levels are already high in epiblasts as well as in hPGCs. Along with their proliferation and migration to the genital ridge, the methylation levels in hPGCs decrease. By week 7 when they arrive at the genital ridge, DNA methylation is decreased to 19.4% from the initial level of over 80% in hPGCs. Then it reaches the lowest level (< 10%) by week 10 or 11 (Tang et al., 2015, Guo et al., 2015). During this process, the DNA methylation on DMRs of imprinted genes is rapidly erased before week 7 and maintained free of methylation from week 10 to 19 (Guo et al., 2015). TET1 and TET2 enzymes are abundantly present in the hPGCs during their migration. However, only 5hmC is detectable but no downstream oxidative products and TDG. In addition, the DNMTs and UHRF1 are silenced as well (Tang et al., 2015). This suggests that the global DNA demethylation is mainly performed by a passive mechanism with the proliferation in hPGCs (Tang et al., 2015). Nonetheless, the high expression of BER proteins in hPGCs implies that an active mechanism might also play a role in this process (Guo et al., 2015, White et al., 2016).

In the gonads, DNA methylation is re-established in germ cells in a sex-specific DNA methylation pattern, which establishes the distinct methylation patterns between male and female, the foundation of imprinting. In males, *de novo* methylation begins in mitotically arrested prespermatogonia, achieving an approximate methylation level of

85% of that in mature sperm by birth (Guo et al., 2015, Zhu et al., 2018). In contrast, *de novo* DNA methylation starts from puberty in female germ cells when oocytes initiate their growth phase. The DNA methylation of a growing oocyte is increased from a basal level of ~11% to ~60% in a fully grown oocyte and maintained until fertilisation (Yan et al., 2021). During oocyte development, the expression of DNMT1 and DNMT3A is significantly elevated in both growing and mature oocytes, whereas DNMT3B and UHRF1 expression is restricted to mature oocytes. Interestingly, overall DNA methylation levels do not directly correspond with the DNMTs expression levels but are concurrent with the reorganization of chromatin to an open chromatin state, marked by histone modifications like H3K4me3 and H3K27me3 (Yan et al., 2021). This suggests that chromatin remodelling plays a crucial role in facilitating *de novo* methylation in the growing oocyte by making CpG regions more accessible to DNMTs.

1.1.3.2. DNA methylation during early embryogenesis

The matured oocytes and sperm are highly methylated in their genome but with distinct methylation patterns resulting from different regulatory mechanisms in the ovary and testis, respectively. Following fertilization, a subsequent wave of DNA demethylation occurs, which plays an essential role in zygotic genome activation (ZGA) to activate gene expression crucial for early embryonic development in humans (Schulz and Harrison, 2019). This demethylation starts immediately post-fertilization and the overall methylation level declines during early cleavage stages, reaching 30% in the inner cell mass (ICM) by the blastocyst (Fig. 1.3) (Zhu et al., 2018, Guo et al., 2014). Overall, the paternal methylation is rapidly erased before the 2-cell stage likely by an active demethylation mechanism, whereas the DNA methylation in the maternal genome gradually declines possibly involving a passive demethylation mechanism (Zhu et al., 2018, Guo et al., 2014, White et al., 2016). A study in mice suggests that DNA demethylation in the maternal genome results from less accessibility of Dnmt1 and Uhrf1 to the genome during replication, leading to passive DNA demethylation

(Hirasawa et al., 2008). However, another report reveals that an active demethylation could also be involved in the maternal DNA demethylation (Wang et al., 2014). Interestingly, unlike the global demethylation occurring in hPGCs in which methylation at the ICRs is also erased, the differential methylation patterns at ICRs between maternal and paternal genome are retained as that in oocyte and sperm, respectively, during the post-fertilization demethylation (Guo et al., 2014). Taken together, it indicates that global DNA methylation is a dynamic balance between a strong genome-wide demethylation and DNA re-methylation in specific regions (Zhu et al., 2018).

The globally hypomethylated blastocyst advances to the implantation. Following implantation, a sharp increase in genomic DNA methylation is detected in both mouse and human embryos (Wang et al., 2014, Guo et al., 2014). Research using mouse models has identified Dnmt3a and Dnmt3b as the primary enzymes responsible for this *de novo* methylation. This process is crucial for gastrulation and the subsequent proper differentiation of cells into the three germ layers (Zeng and Chen, 2019, Dahlet et al., 2020).

1.2. NLRP7

1.2.1. NLRP protein family

In humans, the NLRP family comprises 14 protein members which are mainly clustered on chromosome 11p15 (*NLRP6*, 10 and 14) and 19q13.4 (*NLRP2*, 4, 5, 7, 8, 9, 11, 12, and 13) (Amoushahi et al., 2019). Based on their expression patterns, the NLRP proteins can be categorised into two subfamilies. One subfamily (*NLRP1*, 3, 6, 10 and 12) is highly conserved during evolution from *C. elegans*, rat, and mouse to human and widely expressed across many tissues (Zhang et al., 2008). The NLRP proteins in this subfamily have been found to play an important role in the innate immune system which serves as a defence mechanism against microbial infections and potential threats across almost all species (Zhang et al., 2008). These NLRPs are thought to be responsible for apoptotic and inflammatory signal transduction (Velloso et al., 2019). For example, *NLRP1* was the first NLR family protein studied in the formation of inflammasome complex. It was found to bind the muramyl dipeptide structure, existing in almost all bacteria, to activate the inflammasome assembly (Martinon et al., 2002, Faustin et al., 2007). The deficiency of *NLRP1* in humans can lead to vitiligo-related autoimmune disease (Sehgal and Srivastava, 2006).

The other subfamily of NLRP proteins, including *NLRP2*, 4, 5, 7, 8, 9, 11, 13 and 14, is predominantly expressed in reproductive organs, particularly oocytes, and early embryos; therefore, these proteins are named reproductive NLRPs (rNLRP) (Tian et al., 2009, Martinon et al., 2007). Importantly, rNLRPs show significant variations between species and even in mammals, only human and non-human primates contain all 9 rNLRPs, thus a complete set of 14 NLRP family members. The rNLRPs play crucial roles in mammalian reproduction and early embryonic development (Amoushahi et al., 2019).

1.2.2. The protein structure of NLRPs

Normally, all NLRP proteins in humans contain an N-terminal pyrin domain (PYD), a central nucleotide-binding domain (NACHT) followed by a NACHT-associated domain (NAD) and a C-terminal leucine-rich repeat (LRR) region (Fig. 1.4) (Martinon et al., 2007). However, NLRP1 has two more extra C-terminal domains which are a caspase activation and recruitment domain (CARD) and a domain with a function to find (FIIND), and NLRP10 does not have the LRR region (MacDonald et al., 2013).

The function of each domain in NLRPs has been well-studied in the immune system. The PYD is an effector domain which is crucial to inflammasome assembly and downstream signal transduction. The PYD domain exhibits a highly conserved six-helical bundle structural fold, but some structural variations exist among different NLRPs, mainly on the length of α -helices and on the surface charges that affect the binding interface with different proteins (Bae and Park, 2011, Eibl et al., 2012). The NACHT domain is mainly responsible for ATP binding and hydrolysis which is essential for oligomerization of NLRP7 protein and further release the functional PYD and LRR domains to activate non-functional NLRP7 (Singer et al., 2014). The LRR region contributes to autoregulation, protein-protein interaction and PAMP sensing in the innate immune system, which provides a binding scaffold for molecules including proteins and lipopeptides (Kobe and Kajava, 2001, Ye and Ting, 2008). Moreover, the individual LRR region shows a high affinity for the nucleotide acids in NLRP1 but a higher affinity with the cooperation of NACHT (Bauernfried et al., 2021). The main difference in protein structure in NLRPs is the repeat number of LRR motifs which varies from 2 to 42 in an LRR domain among different NLRPs (Enkhbayar et al., 2004). The different structural conformation of LRR in NLRPs may be responsible for the functional divergence of the NLRP protein family.

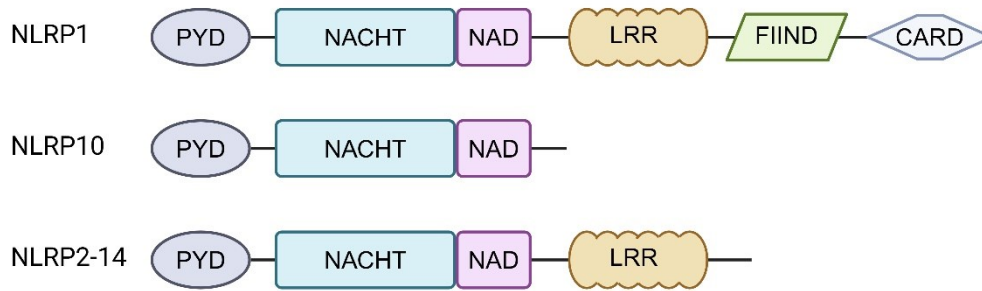


Figure 1.3 Protein structures of NLRP protein family in human

Every NLRP protein features PYD domain, a NACHT domain followed by a NAD domain, and an LRR region at the end. Except for NLRP10 and NLRP1, NLRP10 does not have the LRR region, and NLRP1 equipped with an additional FIIND and CARD domain.

1.2.3. The rNLRPs

In contrast to the ubiquitously expressed NLRPs that are conserved across species and important for innate immunity, rNLRPs evolved and are diverged in function during evolution, and even in mammals, they are different according to different mammalian species. Humans and rhesus macaque monkeys have all 9 rNLRP genes (*NLRP2*, 4, 5, 7, 8, 9, 11, 13 and 14) in their genomes with a high level of conservation, sharing 90% identity between them (McDaniel and Wu, 2009). Whereas rodent species such as mice do not contain *NLRP* 7, 8, 11, and 13 even though they do have rNLRP gene clusters in the genome. In addition, compared to human rNLRPs, mouse rNLRPs are not located closely in the cluster, but are interrupted by other genes (Fig 1.5) (Tian et al., 2009). Some human rNLRP genes evolved from the duplication of existing rNLRP genes and show divergent functions. For example, human *NLRP7* is thought to be evolved from the duplication of *NLRP2* (Fig 1.5). However, the function of *NLRP7* in humans is distinct from the function of *Nlrp2* in mice. Deficiency of *Nlrp2* in mice leads to embryo arrest at the 2-cell stage, while that in humans does not result in a clear phenotype during embryonic development (Peng et al., 2012, Murdoch et al., 2006). However, it is reported that truncated *NLRP2* was associated with Beckwith-Wiedemann syndrome in a single familial case, which is characterized by abnormal

methylation at various loci, such as *KvDMR1* and *MEST* DMRs (Meyer et al., 2009). On the other hand, the recessive mutation *NLRP7* in women leads to complete hydatidiform mole (CHM) due to loss of maternal imprinting in the embryos (Sanchez-Delgado et al., 2015, more on section 1.2.5.3). Thus, *NLRP2* in humans may have a relatively milder function in controlling maternal imprinting than *NLRP7*. Together, it indicates that duplication and functional divergence of rNLRPs may give mammals some advantages in the reproductive system during evolution.

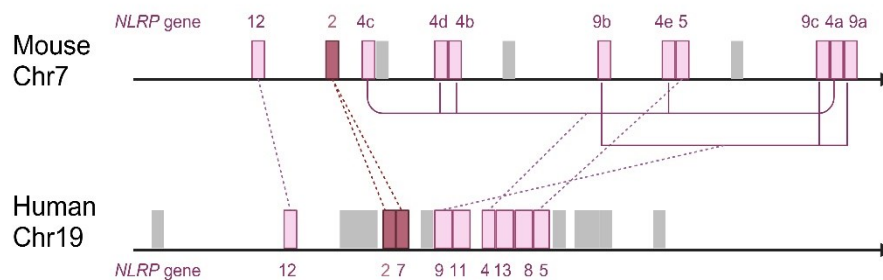


Figure 1.4 Syntenic comparison of NLRP genes in mouse (Chromosome 7) and human (Chromosome 19).

Most *NLRP* genes are clustered on mouse chromosome 7 and human chromosome 19. The coloured bars represent *NLRP* genes, and the orthologues are indicated by dashed lines. For example, human *NLRP7* is originated from a duplication of the *NLRP2/7*.

1.2.4. The function of rNLRPs

rNLRPs are highly expressed in mammalian gonads, particularly the ovary and some are also expressed in preimplantation embryos, having important functions in reproduction and embryogenesis (McDaniel and Wu, 2009). The exact functions of these rNLRPs are not fully understood but maternal deficiency of some *rNLRPs* is reported to result in embryo lethality in mammals. For example, female mice lacking functional *Nlrp5* can have normal oogenesis and fertilization; however, no pups are born as the embryo development is arrested at the 2-cell stage due to reduced transcription in the embryo (Tong et al., 2000). This phenomenon is known as a maternal effect, where the phenotype of the offspring is determined by the maternal genotype but not affected by the paternal genotype (Hager et al., 2008). The maternal-

effect is common among *rNLRPs*. When knockdown any of *Nlrp2*, *Nlrp4* and *Nlrp14* gene expression in mouse oocytes individually, it does not affect fertilisation but embryonic development is severely disrupted, and the embryos are arrested between the 2-to-8-cell stage (Peng et al., 2012, Peng et al., 2014, Hamatani et al., 2004).

Deficiency of *rNLRPs* can also lead to some embryonic developmental issues in humans. Mutations in different functional regions of *NLRP5* and *NLRP2* have been linked to female infertility in humans, due to early embryonic arrest (Mu et al., 2019). However, in some cases, patients with mutations in *NLRP5* or *NLRP2* can give birth, but children from these patients have multi-locus imprinting disturbance (MLID) which leads to imprinting disorders. MLID refers to a subgroup of patients with imprinting disorders, having changes in DNA methylation at multiple imprinted loci. It has been found that MLID is associated with maternal-effect variants altering the maintenance of methylation at gDMRs in early embryogenesis (Sanchez-Delgado et al., 2016). As mentioned in the previous section, a mother with truncated *NLRP2*, lacking the LRR region, gave birth to two children but results in a phenotype like MLID presenting as methylation defect at multiple ICRs. Additionally, these two children suffered from BWS due to the loss of methylation at the imprinted *KvDMR1* CGI (Meyer et al., 2009). Offsprings of mothers with mutations in *NLRP5* suffer from MLID, including BWS and SRS (Silver–Russell syndrome) (Docherty et al., 2015). Notably, biallelic *NLRP7* mutations in women can lead to CHM (Murdoch et al., 2006).

1.2.5. NLRP7 and hydatidiform moles

1.2.5.1. Biparental complete hydatidiform mole (BiCHM)

Hydatidiform mole (HM) is an abnormal human pregnancy in which a gestational trophoblast mass overgrows, while the embryo properly undergrows in the uterus (Candelier, 2016). The most severe type is called CHM, which only has trophoblast tissues without a proper embryo in the uterus (Slim and Wallace, 2013). The incidence

of HM is geographically distributed widely, occurring at 1 in every 600 pregnancies in western countries but 2-10 times higher in other places (Savage et al., 2010, Grimes, 1984, Bracken, 1987). The common form (80%-90%) of CHM is sporadic and androgenetic in origin with a diploid paternal genome which results from the fertilisation of an empty oocyte without its maternal chromosomes by a single sperm which endoreplicates its genome, or two sperm simultaneously, to form the androgenetic zygote exclusively with paternal genome (Fig 1.6) (Candelier, 2016, Slim and Wallace, 2013). The loss of the maternal genome but doubling of the paternal genome leads to the absence of maternally expressed genes and overexpression of the paternally expressed genes in post-fertilisation. Therefore, CHM is characterised by defective fetal growth and trophoblastic hyperplasia. Notably, in rare cases (1% CHM), patients can have recurrent CHM from which complete parental genomes are contained, indicating normal fertilisation between sperm and oocyte, known as biparental CHM (BiCHM) (Fig 1.6). Women with this type of CHM often have a family history in which other females in the family also have BiCHM (Candelier, 2016, Slim and Wallace, 2013).

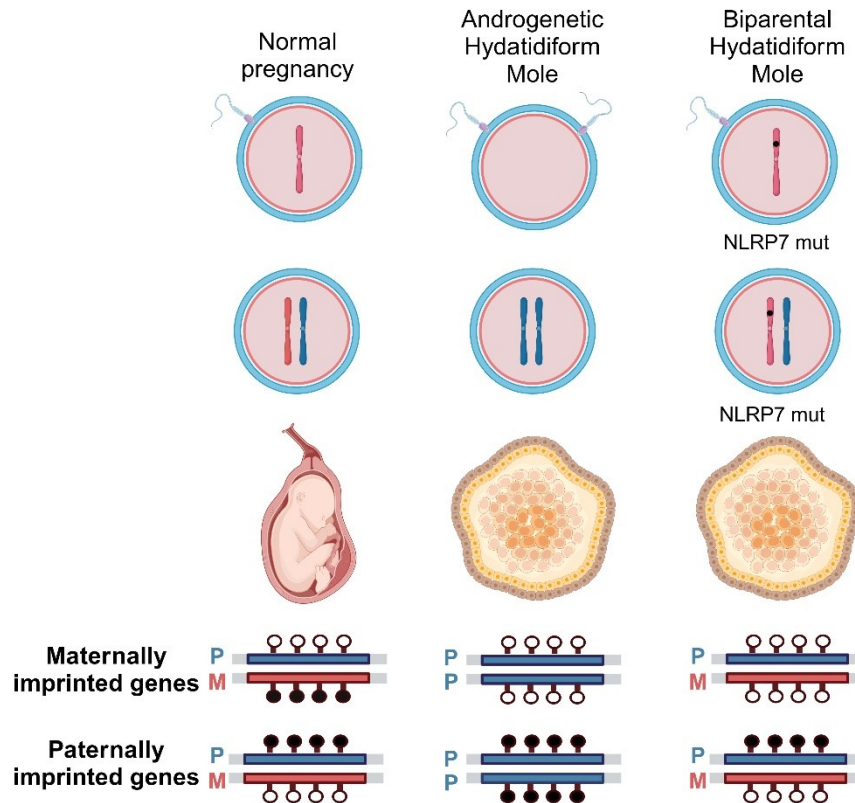


Figure 1.5 Methylation status in normal pregnancy, androgenetic CHM and BiCHM.

Androgenetic CHM and BiCHM originate through distinct mechanisms. The methylation profiles at the DMRs of both maternally and paternally imprinted genes, differ between normal embryos and molar tissues derived from androgenetic CHM and biparental CHM.

‘M’ represents maternal alleles, and ‘P’ represents paternal alleles. Open and closed circles indicate unmethylated CpG sites and methylated CpG sites, respectively.

1.2.5.2. Recessive *NLRP7* mutation in women accounts for BiCHM

Genetic linkage analysis within women having BiCHM has found that the majority of these cases are associated with biallelic *NLRP7* mutations in women (Moglabey et al., 1999). These mutation sites are mainly distributed on the LRR region of *NLRP7* which may imply the importance of LRR region (Fig 1.7) (Wang et al., 2009). Interestingly, these mutations in the *NLRP7* gene do not manifest any discernible phenotypes in somatic cells and women carrying these mutations can grow normally without any effects. However, they rarely have normal pregnancies but often experience recurrent BiCHM (Van den Veyver and Al-Hussaini, 2006, Qian et al., 2007). In contrast, men with biallelic mutation in *NLRP7* do not have abnormal phenotypes and are able to

have healthy offspring. This suggests that NLRP7-associated BiCHM is a maternal effect. In fact, *NLRP7* is the first maternal-effect gene identified in humans (Slim and Wallace, 2013). Additionally, mutations in *KHDC3L* account for approximately 5-10% of BiCHM. Recent studies have shown that deficient *KHDC3L* can lead to genome-wide DNA demethylation in oocytes, particularly affecting maternal gDMRs (Demond et al., 2019). It would be interesting to explore whether mutations in *NLRP7* could lead to a similar phenomenon as those observed with *KHDC3L* in future research.

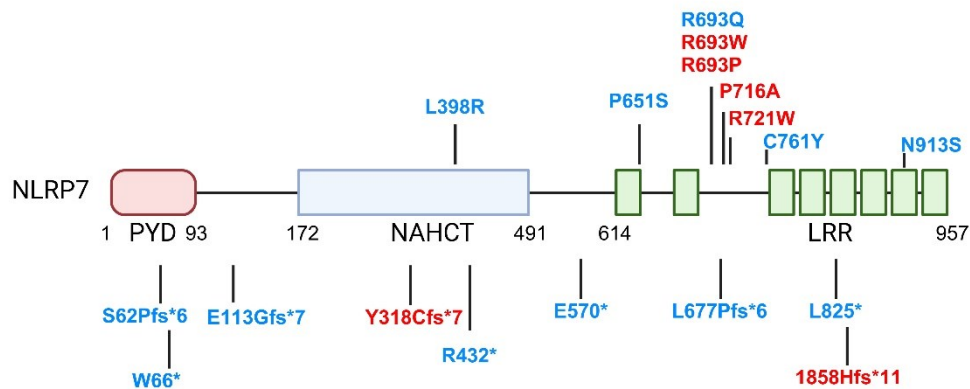


Figure 1.6 Reported NLRP7 mutations in CHM disease

This figure showed all mutation sites reported in CHM disease. Red color showed mutations found in more than one family; Blue color represents mutation sites currently reported in only a single family; star symbol (*) represents mutation leads to a protein truncation.

1.2.5.3. Loss of maternal imprinting in NLRP7-BiCHM

However, the mechanisms by which NLRP7 mutations induce molar pregnancies remain elusive. Based on the research of NLRP7 being important in the innate immune response (Kinoshita et al., 2005), it was initially proposed that mutations in *NLRP7* among pregnant women could trigger an abnormal immune response from the mother towards the embryo, potentially leading to a CHM. Surprisingly, a woman with biallelic *NLRP7* mutations and a history of recurrent BiCHM successfully delivered a healthy baby following an oocyte donation with normal *NLRP7* (Fisher et al., 2011). This suggests that BiCHM may be due to an abnormal oocyte rather than maternal immune rejection.

The study on artificial androgenetic embryos indicates the methylation level of androgenetic blastocysts closely resembles that of sperm (Xu et al., 2021). Given the similarity in pathology between BiCHM and androgenetic CHM where maternal imprinting is lost, it is anticipated that BiCHM may arise from dysregulated maternal imprinting due to *NLRP7* mutations. Oocytes or their fertilized embryos containing deficient *NLRP7* may lose maternal imprinting, resulting in an androgenetic-like phenotype (Van den Veyver and Al-Hussaini, 2006). Indeed, a genome-wide methylation study of molar tissues collected from patients with *NLRP7* mutations provides strong evidence to support such a hypothesis. The data shows that maternally imprinted genes lose their methylation on the maternal allele, leading to a shift from monoallelic expression to biallelic expression, while paternally imprinted genes remain unaffected (Fig 1.6) (Sanchez-Delgado et al., 2015). Hence, the loss of maternal imprinting due to *NLRP7* mutations is more likely to contribute to BiCHM. However, it remains unknown how *NLRP7* regulates maternal imprinting.

1.2.5.4. *NLRP7* and subcortical maternal complex (SCMC)

The subcortical maternal complex (SCMC) is a protein complex which constitutes of multiple maternal effect proteins. These proteins are expressed in early embryos and oocytes in mammals and play an essential role in the progression of zygote to the 2-cell stage (Yu et al., 2014, Bebbere et al., 2021). The SCMC complex is characterised by the subcortical localization in oocytes and early embryos at cleavage-stage but expelled from cell-cell contacting regions in early embryos (Li et al., 2008). This complex was firstly identified with four members in mouse preimplantation embryos, consisting of Mater/Nlrp5 (Maternal Antigen That Embryos Require), Filia/Khdc3 (KH domain-containing protein 3), Floped/Ooep (Factor Located in Oocytes Permitting Embryonic Development/ oocyte-expressed protein) and Tle6 (Transducin-Like Enhancer of Split 6) (Ohsugi et al., 2008, Li et al., 2008). After that, Padi6 (peptidyl arginine deiminase 6), another maternal-effect protein, is reported to interact with

Floped and Mater; thus, Padi6 was considered as a member of SCMC (Li et al., 2010a, Yurttas et al., 2008, Kim et al., 2010a). Furthermore, Nlrp2, Zbed3 and Nlrp4f have been identified as members of SCMC in mouse (Mahadevan et al., 2017, Gao et al., 2018, Qin et al., 2019). In human, only four key members of SCMC have been identified, which are NLRP5, KHDC3L, OOEP, and TLE6 (Zhu et al., 2015). However, the combined molecular weight of these four proteins is much less than that of the whole SCMC protein complex, indicating that there are more unidentified proteins involved in the SCMC. Based on their similar subcortical localisation, NLRP7, NLRP2 and PADI6 are the potential members of the SCMC. Interestingly, mutations in these genes result in similar phenotype in offsprings (Mahadevan et al., 2017, Akoury et al., 2015, Qian et al., 2018). So far, most research on the SCMC are performed in the mouse model due to the limited availability of human materials. Mutations in any of SCMC proteins in mouse exhibit similar phenotypes in the offspring, including asymmetric division of the zygote, embryo arrest at the two-cell stage, defective distribution of organelle and lack of cytoplasmic lattices (CPLs) in zygotes and early embryos (Lu et al., 2017, Mahadevan et al., 2017, Gao et al., 2018, Qin et al., 2019, Yurttas et al., 2008). Some mutations also result in abnormal DNA methylation at imprinted loci (Mahadevan et al., 2017). All these phenotypes indicate the importance of the SCMC in oocyte-to-embryo transition as well as in preimplantation development of the mouse embryo. Based on limited number of clinical studies, defective SCMC members in human are considered to cause early embryonic arrest, BiCHM and MLID (Qian et al., 2018, Parry et al., 2011, Murdoch et al., 2006, Docherty et al., 2015). For example, BiCHM disease is thought to be caused by defective NLRP7 or KHDC3L (Demond et al., 2019). The biallelic mutations of PADI6 have been reported to be associated with embryonic lethality and recurrent pregnancy loss (Qian et al., 2018). As mentioned above, maternal NLRP2 deficiency can lead to BWS disease, resulting from abnormal DNA methylation at the *KvDMR1* and *PEG1* imprinted regions (Meyer et al., 2009). Moreover, women with NLRP5 mutation suffer from recurrent

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miscarriages and offsprings with MLID (Docherty et al., 2015). These clinical cases indicate that SCMC may play a role in the establishment of genomic imprinting in the oocyte or the maintenance of imprinting in the preimplantational embryo.

1.3.HUMAN EMBRYONIC STEM CELL

Laboratory animals, especially mice, are commonly used as a models to study embryonic development and disease modelling, and genomic imprinting is mainly characterised in the mice model. However, *NLRP7*, as a primate and human-specific gene, is absent in mice and its closest gene in mice *NLRP2* does not exhibit the same phenotypes in deficiency as already discussed in the previous section. Therefore, *NLRP7* has a special role in human embryonic development which is distinct from *NLRP2* in mice. Owing to limited human materials available for research, it is very difficult to study the function of *NLRP7* in the regulation of genomic imprinting, particularly maternal imprinting. Human embryonic stem cells (hESCs) might be the most associated cell model to early embryonic cells and thus could be an alternative model for studying human embryonic development and *NLRP7*.

hESCs isolated from pre-implantation ICM of human blastocysts have the ability for unlimited self-renewal to generate themselves and meanwhile retain their potential to generate all cell types within the human body under appropriate cues, named pluripotency (Smith, 2006). In addition, induced pluripotent stem cells (iPSCs) generated by somatic cell reprogramming have similar properties to hESCs (Zhu and Huangfu, 2013). However, iPSCs may retain some memory of their tissue of origin in terms of DNA methylation states (Marchetto et al., 2009, Kim et al., 2010b). Therefore, hESCs have been used as a cell model to study *NLRP7* function in this study.

1.3.1. Pluripotency in embryonic development

Early embryonic development in mammals is a highly conserved and tightly regulated process (Rossant and Tam, 2022). During embryogenesis, a zygote undergoes multiple cleavages and cell differentiation to form a blastocyst, which consists of three lineages: trophoctoderm, hypoblast, and epiblast (Rossant and Tam, 2022). Epiblast cells, which originate from the blastocyst, exhibit strong pluripotency in preimplantation

blastocysts. When a murine epiblast cell is injected into another recipient blastocyst, it is capable of contributing to all lineages, including the ectoderm, mesoderm, endoderm and PGCs of the offspring (Masaki et al., 2015). Therefore, these epiblast cells are in what is called a developmental ground state, or a naïve state (Zhou et al., 2023). Following implantation, the epiblast cells undergo further patterning and differentiation, and their morphology changes to the epithelium in response to signalling molecules from the surrounding tissues, which are then considered to be in a more advanced “onset of differentiation” state, known as the primed state (Zhou et al., 2023). Generation of three germ layers from naïve epiblast requires the initial differentiation to a primed state prior to all lineages (Guo et al., 2017, Liu et al., 2017).

The first ESCs were derived from the ICM of preimplantation murine blastocysts, termed mouse ESCs (mESCs). These cells exhibit a domed-shaped colony and mainly rely on the *Lif/Stat3* signalling pathway for maintaining their ESC properties, while *Fgf* signalling promotes their differentiation (Nichols and Smith, 2009). After being injected into blastocysts, these cells are capable of integrating into the host ICM and differentiating into all cell lineages to produce chimeras (Orkin and Hochedlinger, 2011). Therefore, mESCs are considered to closely resemble the cells in the ICM of the preimplantation blastocyst, at a naïve pluripotent state. Pluripotent stem cells (PSCs) can also be derived from post-implantation mouse E5.5 epiblast termed mouse epiblast stem cells (mEpiSCs) (Brons et al., 2007, Tesar et al., 2007). In contrast to mESCs, mEpiSCs exhibit a flattened colony morphology and are maintained by the *Fgf/Activin* signalling pathway *in vitro* (Brons et al., 2007, Vallier et al., 2005). Intriguingly mEpiSCs seldom contribute to chimeric mice when injected into blastocysts (Dong et al., 2019). This led to the suggestion that mEpiSCs exhibit a “primed” pluripotent state corresponding to the post-implantation epiblast, differing from the “naïve” blastocyst-like pluripotent state of mESCs (Nichols and Smith, 2009).

The comparative study of primed mEpiSCs and naïve mESCs has revealed the molecular and epigenomic similarities and distinctions between naïve and primed PSCs. Both primed mEpiSCs and naïve mESCs exhibit high expression levels of key pluripotency factors, including *Oct4* and *Sox2* and are capable of differentiating into cells from the three germ layers (Weinberger et al., 2016, Hackett and Surani, 2014). However, they display unique transcriptomic and epigenomic profiles. Naïve mESCs express specific factors such as *Rex1*, *Nr0b1*, *Klfs*, and *Esrrβ*, and exhibit genome-wide hypomethylation. Additionally, female mESCs maintain two active X chromosomes. In contrast, expression of lineage commitment factors like *Otx2*, *Zic2*, and *Brachyury* start to emerge in primed mEpiSCs and these cells show increased global genome methylation. Moreover, in female mEpiSCs, one X chromosome undergoes inactivation (Weinberger et al., 2016).

1.3.2. Naïve vs Primed hESCs

The first hESCs were generated from the ICM of human blastocysts by a similar approach to mESC derivation. However, the morphology of resulting hESCs reveals tightly packed flattened colonies and hESCs require *FGF/Activin* signalling for their indefinite propagation (Thomson et al., 1998). This culture requirement is very different from that of mESCs but similar to mEpiSCs. In addition, hESCs and mEpiSCs also show similar colony morphology.

Further studies show that hESC and mEpiSCs display comparable molecular characteristics. Both of them depend on the MEK-ERK pathway to maintain their pluripotency and their OCT4 expression is controlled only by the OCT4 proximal enhancer without activation of distal enhancer (Angeles et al., 2012, Weinberger et al., 2016). This alignment suggests that hESCs, like mEpiSCs, exist in a 'primed' pluripotent state. In supporting this, the transcriptional profile of monkey ESCs, closely resembling hESCs, is found by single-cell RNA sequencing to be closely comparable

to that of the late post-implantation monkey epiblast, confirming that hESCs are in primed pluripotency, a feature of post-implantation epiblast (Nakamura et al., 2016).

However, it is important that hESCs and mEpiSCs are not completely identical possibly due to different species (Nakamura et al., 2016). For instance, hESCs express certain naïve pluripotent markers at relatively high levels, such as NANOG, PRDM14, and REX1, while they are absent or only minimally present in mEpiSCs (Chia et al., 2010). Moreover, a cross-species comparison of single-cell RNA sequencing between hESCs and mEpiSCs revealed that hESCs share similar expression profiles with the late post-implantation monkey epiblast. In contrast, mEpiSCs resemble the early post-implantation mouse epiblast (Nakamura et al., 2016). Therefore, hESCs may represent a later stage of post-implantation epiblast in embryonic development compared to mEpiSCs.

Interestingly, many studies have shown that PSCs in naïve and primed states, regardless of mouse or human, can be exchangeable by culturing them under conditions that support naïve or primed pluripotency (Nichols and Smith, 2009, Dong et al., 2019). In mice, switching medium from active *Lif* combined with inhibited *Fgf* to active *Fgf/Activin* could convert mESCs from naïve to primed pluripotency, and vice versa (Hayashi et al., 2008). In humans, it is more complex as most of the hESCs are cultured in a primed state, while the naïve state is less clearly defined. Over the past decade, various groups have developed protocols to convert primed hESCs to a naïve pluripotency (details in the next section). The converted hESCs by some methods achieve a naïve-like pluripotent state, characterized by transcriptome similarities with preimplantation human epiblast, global DNA hypomethylation, and reactivation of the X chromosome in female hESCs (Kisa et al., 2017). Characteristics between naïve and primed hESCs are summarised in Table 1.2. Although chimeric formation, the gold standard for naïve pluripotency, is not feasible to be done with human embryos, a chimeric monkey experiment in a recent study has shown that monkey naïve ESCs

converted using a similar approach for humans can integrate into early monkey embryos and produce chimeric embryos with a high level of cellular contribution (Cao et al., 2023). Therefore, these converted ESCs can be considered to be in a naïve state, closely resembling the cells in the ICM of preimplantation blastocysts.

Table 1.2 Characteristics between naïve and primed status in hESCs

Properties	Naïve state (hESCs)	Primed state (hESCs)
Corresponding embryonic cells	Preimplantation blastocyst (ICM)	Post-implantation Epiblast
Culture conditions	5iLAF/PXGL/4CL	Conditioned medium with bFGF
Dominant OCT4 enhancer	Distal	Proximal
Pluripotency markers	REX1, KLF2, PRDM14, TCL1B, NLRP7, DNMT3L	OCT4, NANOG, SOX2
Blastocyst chimaeras	Yes	No
XX status	XaXa	XaXi
Genome-wide DNA methylation	Hypomethylation	Hypermethylation
Colony morphology	Domed	Flattened

1.3.3. Naïve conversion of hESCs

Over several years, research into reprogramming mEpiSCs back to a naïve pluripotent state has shown that overexpression of transcriptional factors such as Klf4 or Myc in mEpiSCs in Lif-containing medium can lead to the generation of naïve mESCs (Hanna et al., 2009, Guo et al., 2009). These reprogrammed mEpiSCs display characteristics of naïve pluripotency comparable to those of mESCs, including the reactivation of the X chromosome and a significant contribution to chimeric mice (Dong et al., 2019). The success of creating naïve-like mEpiSCs prompts the question of whether similar strategies could be employed with hESCs to achieve human naïve pluripotency. Over the past decade, various groups have developed protocols that rely on either transgenes dependent or independent methods to induce and sustain the naïve state

in human PSC (Li et al., 2009, Takashima et al., 2014, Gafni et al., 2013, Theunissen et al., 2014).

Based on the strategy to induce naïve pluripotency in mouse PSCs, the first human naïve-like PSCs were generated in 2009. This was achieved by overexpressing a combination of OCT4, SOX2, NANOG and Lin28 in human fibroblast cells, cultured under mESCs conditions (2i/L) supplement with ALK5 inhibitors. These cells formed domed-shaped colonies reminiscent of mESCs (Li et al., 2009, Hanna et al., 2009). Another popular method is t2iLGö which can generate naïve hESCs by transiently overexpressing NANOG and KLF4 in primed hESCs followed by culture in 2iL medium with protein kinase C inhibitor (Takashima et al., 2014). It is noteworthy that the majority of these cells were cultured in conditions supplemented with 2iL (Zhou et al., 2023).

Beyond the use of overexpressing exogenous transcriptional factors, researchers have also developed transgene-independent methods for converting hESCs into their naïve state as well, which is summarized in Table 1.3. However, only 5iLAF, PXGL and 4CL showed the key criteria of the naïve state, the global DNA hypomethylation, after naïve conversion.

Table 1.3 Naïve conversion methods without transgenesis

Method	Basal Medium	Growth Factors/Inhibitors	Reference
NHSM	KO-DMEM	MEKi, GSK3i, p38i, JNKi, aPKCi, ROCKi, LIF, Activin A, FGF2	(Gafni et al., 2013)
3iL	TeSR1	MEKi, GSK3i, BMPi LIF, FGF2, TGFβ	(Chan et al., 2013)
RT	DMEM/F12 +KSR	1. HDACi for 2 passages 2. MEKi, GSK3i, FGF2	(Ware et al., 2014)
5iLAF	N2B27	MEKi, GSK3i, SRCi, bRAFi, ROCKi LIF, Activin A, FGF2	(Theunissen et al., 2014)
PXGL	N2B27	1. HDACi, MEKi, LIF for 3 days 2. WNTi, MEKi, PKCi, LIF for 6 days	(Guo et al., 2017)
4CL	N2B27	HDACi, MEKi, Tankyrase inhibitor EZH2 inhibitor, LIF, Activin A	(Mazid et al., 2022)

*NHSM (Naïve Human embryonic Stem cell Media)

**RT (Reverse toggle media)

To effectively activate the naïve-specific OCT4-distal enhancer, the 5iLAF method was developed to generate naïve hESCs. This method utilizes a combination of inhibitors—MEKi, GSK3i, RAFi, SRCi, and ROCKi—along with LIF, activin A, and bFGF (Theunissen et al., 2014). hESCs cultured in 5iLAF medium exhibit significant upregulation of naïve markers and a more pronounced reduction in global DNA methylation close to it in cells from ICM (Theunissen et al., 2014, Pastor et al., 2016). However, this method results in the erasure of genomic imprinting. In primed hESCs, imprinted genes are expressed monoallelically, but this expression shifts to biallelic after naïve conversion (Theunissen et al., 2014). This loss is not reversed when the naïve hESCs are reconverted to their primed state (Guo et al., 2017, Pastor et al., 2016). In contrast, hESCs derived from the inner cell mass (ICM) maintain genomic imprinting, unlike those derived from 5iLAF treatment, even though both show reduced global DNA methylation (Guo et al., 2014). The underlying mechanisms that induce these differences remain unclear and require further investigation.

Based on the t2iLGö culture condition, the transgene-free version of t2iLGö has been developed to revert primed hESCs back to the naïve state called PXGL (Guo et al., 2017, Bredenkamp et al., 2019). Initially, hESCs undergo treatment with HDACi, MEKi and LIF, followed by a transition to the 2iLGö medium supplement with WNTi for further induction. PXGL method efficiently produces naïve hESCs that exhibit transcriptomic, DNA methylation profile, X chromosome reactivation, and loss of imprinting similar to 5iLAF converted hESCs (Guo et al., 2017).

4CL is a novel culture condition for naïve hESCs, whose cells exhibit characteristics similar to those of the 8-cell stage embryo. This method involves cultivating naïve hESCs with MEKi, HDACi, tankyrase inhibitor, EZH2 inhibitor, and human LIF (Mazid et al., 2022). The resulting transcriptome reveals not only an increased expression of naïve pluripotent markers but also of totipotency genes such as *TPRX1*, *DUXA*, and *ZNF280A* (Theunissen et al., 2014, Mazid et al., 2022, Cao et al., 2023, Zhou et al., 2023). Although 4CL-generated hESCs, similar to totipotent stem cells, have limited self-renewal capacity, they maintain a normal karyotype (Mazid et al., 2022, Surani et al., 2007, Macfarlan et al., 2012). Furthermore, the global DNA methylation levels in 4CL-generated hESCs closely resemble those of cells from the 8-cell stage embryo, albeit slightly higher than those in cells from the ICM (Mazid et al., 2022). However, it remains unclear whether genomic imprinting is altered after 4CL treatment.

Numerous protocols have been developed for creating naïve hESCs, with experiments like chimeric formation and teratoma assays used to evaluate their developmental potential (Cao et al., 2023). Notably, hESCs created under the 5iLAF and PXGL (t2iLGö) conditions closely mimic the naïve state of the ICM in preimplantation blastocysts. Under 4CL conditions, these cells likely exhibit pluripotency akin to that of the 8-cell stage embryo (Theunissen et al., 2014, Mazid et al., 2022, Cao et al., 2023, Zhou et al., 2023).

Since naïve hESCs, when converted using 5iLAF or PXGL methods, exhibit a loss of germline imprinting, this erasure of genomic imprinting during naïve conversion presents a valuable model for exploring the role of NLRP7 in the establishment and maintenance of human genomic imprinting.

1.4.ASSOCIATED PRELIMINARY DATA OF THIS PROJECT

Our laboratory has been attempting to investigate the role of NLRP7 in maternal imprinting using hESCs, using prime-to-naïve conversion by 5iLAF as a global demethylation model. In our lab, it has been determined that the H1 and H7 hESC lines contain SNPs in two maternally imprinted genes (*NAP1L5* and *PEG10*) and one paternally imprinted gene (*MEG3*). These SNPs are identified by screening five maternally imprinted genes (*LIN28B*, *PEG10*, *NAP1L5*, *ZDBF2* and *MEST*) and two paternally imprinted genes (*H19* and *MEG3*). The identification of SNPs enables us to differentiate between two alleles in these cells. Both maternally and paternally imprinted genes are shown to be expressed mono-allelically in these hESCs, assuming a parental-specific pattern, but becoming biallelic expression after naïve conversion, indicating loss of imprinting. Therefore, using the naïve conversion of hESCs as a model offers a promising approach to investigating the function of NLRP7 in regulating maternal imprinting.

1.5.AIM AND HYPOTHESIS

The overall aim of this project is to investigate the role of NLRP7 in the regulation of maternal imprinting during global demethylation using naïve conversion of hESCs as a cell model. Particularly, I want to investigate whether the NLRP7 protect maternal imprinting from the global DNA demethylation during the naïve conversion of hESCs and try to explore the underlying mechanisms. To achieve the goal, I have carried out following studies:

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- 1) Investigating the role of NLRP7 on maternal imprinting during naïve conversion of hESCs
- 2) Investigate whether the deficient NLRP7 can rescue the loss of maternal imprinting during naïve conversion of hESCs
- 3) Explore the approach of NLRP7 to regulate maternal imprinting during naïve conversion of hESCs

Chapter 2

Materials and Methods

2.1 CELL CULTURE

2.1.1. List of materials

Cell lines in this project		
Name	Origin	
H1 hESCs	Male hESC Line (WA-01) Distributed By Wicell	
H7 hESCs	Female hESC Line (WA-07) Distributed By Wicell	
HEK293T	Immortalized Human Embryonic Kidney Cell Line (Cat#ATCC® CRL-3216TM)	
Name	Company	Catalogue number
Cell Culture Medium Components		
100x N2 Supplement	Life Technologies	17502048
100x Non-Essential Amino Acids (NEAA)	Sigma-Aldrich	M7145
100x Penicillin-Streptomycin (Pen/Strep)	Sigma-Aldrich	P0781
200 mM L-Glutamine (LG)	Sigma-Aldrich	G7513
30% Bovine Serum Albumin (BSA) Solution	Sigma-Aldrich	A9576
50x B27 Supplement	Life Technologies	17504044
DMEM/F12	Thermo Fisher	21331020
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich	D0819
Heat-Inactivated Fetal Bovine Serum (FBS)	Sigma-Aldrich	10500064
Knockout DMEM	Life Technologies	10829018
Knockout Serum Replacement (KSR)	Life Technologies	10828028
Neurobasal medium	Life Technologies	21103-049
β-Mercaptoethanol for Tissue Culture (50 mM)	Thermo Fisher	31350-010
Growth Factors and Inhibitors		
Recombinant human/murine/rat Activin A	Qkine	QK001
Chloroquine Disulphate Cystalline	Sigma-Aldrich	C6628
Human Basic Fibroblast Growth Factor (bFGF)	Peptotech	100-18B
Human Leukemia Inhibitory Factor (hLIF)	Cell Guidance Systems	GFH200
IM-12 (GSK3i)	Sigma-Aldrich	SML0084
PD0325901 (MEKi)	Sigma-Aldrich	PZ0162
Puromycin	Sigma-Aldrich	P8833
SB590885 (BRAFi)	Bio-Tech	2650
WH-4-023 (SRCi)	Bio-Tech	5413
Y-27632 (ROCKi)	Cell Guidance Systems	SM02
Dissociation Reagents		
Accutase	Sigma-Aldrich	A6964
Collagenase IV	Life Technologies	17104019
Trypsin-EDTA	Sigma-Aldrich	T3924
Cell Culture Associated Reagents		
2% Gelatin in H ₂ O	Sigma-Aldrich	G1393
Calcium Phosphate Transfection Kit	Sigma-Aldrich	CAPHOS-1KT
Chondroitin Sulphate	Sigma-Aldrich	C4384
Dimethyl Sulphoxide (DMSO)	Sigma-Aldrich	D2650
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich	D8537
Hexadimethrine Bromide (Polybrene)	Sigma-Aldrich	H9268

Matrigel® Matrix Growth Factor Reduced	SLS	354230
Thymidine	Sigma-Aldrich	T1895

2.1.2. Stock Solutions and Medium

DMEM medium recipe

Reagent	Volume	Final Con.
DMEM	500 ml	
FBS	50 ml	10%
100x Pen/Strep	5 ml	1X

KSR medium recipe

Reagent	Volume	Final Con.
Knockout™ DMEM	400 ml	80%
Knockout™ Serum Replacement (KSR)	100 ml	20%
200 mM L-Glutamine	2.5 ml	1 mM
100x Non-Essential Amino Acids	5 ml	1 x
50 mM β-Mercaptoethanol	1 ml	0.1 mM
100x Pen/Strep	5 ml	1 x

N2B27 base medium recipe (Theunissen et al., 2014)

Reagent	Volume	Final Con.
DMEM/F12	240 ml	48%
Neurobasal	240 ml	48%
100x N2 Supplement	5 ml	1 x
50x B27 Supplement	10 ml	1 x
200 mM L-Glutamine	5 ml	2 mM
Non-Essential Amino Acids	5 ml	1%
50mM β-Mercaptoethanol	1 ml	0.1 mM
30%BSA	87 µl	50 µg/mL
100 x Pen/Strep	5 ml	1 x
KSR	2.5 ml	0.5%

5iLAF cocktail

Reagent	Solvent	Stock Con.	Working Con.
PD0325901 (MEKi)	DMSO	1 mM	1 μ M
IM-12 (GSK3i)	DMSO	1 mM	1 μ M
SB590885 (bRAFi)	DMSO	1 mM	0.5 μ M
WH-4-023 (SRCi)	DMSO	1 mM	1 μ M
Y-27632 (ROCKi)	dH ₂ O	10 mM	10 μ M
Activin A	0.2% BSA in dH ₂ O	10 μ g/ml	20 ng/ml
rhLIF	0.2% BSA in dH ₂ O	10 μ g/ml	20 ng/ml
bFGF	0.2% BSA in dH ₂ O	10 μ g/ml	10 ng/ml

Dissociation enzymes

Reagent	Solvent	Final Con.
Collagenase IV	Knockout DMEM	200 unit/ml

Thymidine stock solution

Reagent	Solvent	Final Con.
Thymidine	dH ₂ O	200mM

2.1.3. Methods

2.1.3.1. *hESC culture and propagation*

i. Generation of irradiated mouse embryonic fibroblast (iMEF) and iMEF-Conditioned Medium (MEF-CM)

MEFs were derived from embryos in E13.5 day pregnant CD-1 mouse which is terminated by cervical dislocation under sterile condition. Each embryo was washed with PBS containing 2 x Pen/Strep and the viscera was cut from the embryo with sterile scissors. Each carcass was washed with PBS three times and finely minced. After that, the minced tissues were trypsinized to break up the clumps into single cells with a regular vortex. All suspension was transferred into a T75 flask with DMEM medium top-up to 15ml. Followed by two days culture at 37°C in a 5% CO₂, MEFs were collected by trypsin for cell counting. 1 x 10⁷ cells were frozen in each tube containing

DMEM medium with 10% DMSO, defined as passage 0, and stored in liquid nitrogen for further use for up to one year.

To produce the MEF-CM, one vial of MEF was resuscitated into a T75 flask and expanded with a ratio of 1:3-6 when confluency reached 90%. After three passages, all MEFs were dissociated by trypsin and collected into 50 ml tubes with DMEM medium. To mitotically inactivated MEFs, cells were irradiated at 40 grays and resuspended with fresh medium for cell counting later. The iMEFs as a feeder layer for the naïve conversion of hESCs were frozen down at this stage. Cell suspensions were transferred into a labelled cryotube and the cold DMEM medium with 20% DMSO was added dropwise into the cryotube in a 1:1 ratio. The following freezing procedure was the same as frozen hESCs which was described in the following section.

To make MEF-CM, 2×10^7 iMEFs were seeded on a 0.5% gelatin-coated T225 flask with 40 ml DMEM medium. The next day, all flasks were replaced with 150 ml KSR medium with freshly added 4 ng/mL bFGF after rinsing with DPBS, which was collected on the following day, labelled as Day 1. Flasks were then topped up with 150ml KSR medium again for next-day collection. This circle was repeated for 6 days, and all medium was stored at -80°C for future use. When needed, MEF-CM was thawed at 4°C overnight and 1x Pen/Strep and 2 mM LG were added before going through the Corning® Filter system (0.22 µm). The MEF-CM was stored at 4°C and finished within one month.

ii. Matrigel coating plates

The hESCs could be cultured on Matrigel-coated plates. The Matrigel® stock was slowly thawed in an ice box in a cold room overnight to avoid solidification, which was then diluted 1:1 with cold Knockout™ DMEM and aliquoted the mixture into 1ml per 15ml tubes which were stored at -20°C. To make Matrigel-coated plates, the diluted 1ml Matrigel was further diluted with cold Knockout™ DMEM at a 1:25 ratio and 1ml Matrigel solution was coated in each well of 6-well plate. The plates should be used

after overnight incubation at 4°C and the rest plates were stored up to one week. Matrigel solution should be removed immediately before use.

iii. Culture and passage hESCs

hESCs (H1 and H7) could be cultured at 37°C in a 5% CO₂ with MEF-CM daily replacement on Matrigel coated plates. For one well of a 6-well plate, 2-3ml MEF-CM supplemented with freshly added 10ng/ml bFGF was consumed every day. When cells reached over 90% confluency, they were split in a 1:3 ratio by Collagenase IV.

Cells were treated with 200 units/ml Collagenase IV per well of a 6-well plate for 5-10 mins to de-attach differentiated cells from the plate. Cells were resuspended in 1ml MEF-CM and gently scraped with a 5ml pipette. The suspension cells could be gently pipetted to dissociate cells into small clusters and were equally seeded on a Matrigel-coated plate.

iv. hESCs frozen and thawed

When hESCs were over 90% confluency, they were treated with Collagenase IV like the passaging procedure and scraped mechanically into large clumps in 1ml cold KSR. The cell suspension was further transferred into a labelled cryotube and 10% DMSO was added dropwise. All cryotubes were placed into Mr. Frosty freezing container which was cooled down overnight in a -80°C freezer. For long-term storage, frozen cryotubes were transferred into liquid nitrogen later.

To thaw hESCs, the frozen cryotube with hESCs was immediately thawed in a 37°C water bath with gentle shaking until small ice remained in the tube. Then cell suspensions were added into pre-warmed MEF-CM dropwise and pelleted down in a centrifuge at 1500 RPM for 5 minutes. The cell pellet was re-suspended into MEF-CM with 10 ng/ml bFGF and an additional 10 µM ROCKi was added into the medium to improve cell viability. Cell suspensions were transferred into Matrigel coating plates and incubated at 37°C in a 5% CO₂.

2.1.3.2. Naïve conversion and propagation of naïve hESCs

i. iMEF feeder coating plates

Naïve conversion of hESCs requires an iMEF feeder layer to support this process. Before seeding hESCs, the iMEF plate should be prepared one day before. 1.5×10^5 – 2.5×10^5 iMEFs were seeded per well of a 6-well culture plate which was already coated with 0.25% Gelatin at room temperature for 1 hour. After overnight incubation at 37°C in a 5% CO₂, plates were available for naïve conversion.

ii. Naïve conversion and passaging

To perform naïve conversion of hESCs, routinely cultured hESCs were dissociated into single cells by the Accutase-Rocki split method. Cells were treated with 1ml Accutase per well at 37°C for 5-10mins. Then, the cell suspension was gently pipetted by P1000 to dissociate cells into single cells and a culture medium was added to the cell suspension to neutralise Accutase. Cells were pelleted down after centrifuging at 1500 RPM for 5 minutes and resuspended into fresh MEF-CM media supplemented with 10 µM ROCKi and 10 ng/ml bFGF. The cell suspension flowed through the 40µm cell strainer (Corning 431750) to ensure that only single cells were collected. For each well of iMEF coated 6-well culture plate, 1.5 - 2.5×10^5 cells were plated and cultured in MEF-CM with 10 ng/ml bFGF for another day. Two days after cell seeding, the medium was changed to 5iLAF medium (N2B27 base medium supplemented with 5iLAF cocktail) for daily change. The domed-shaped colonies of naïve hESCs should be visible around Day 10 after changing to 5iLAF medium and naïve hESCs could be passaged in a ratio at 1:2-3 depending on the confluency every week via the Accutase method.

iii. Naïve hESCs frozen and thawed

Naïve hESCs were treated with Accutase at 37°C for 5 mins to dissociate them into single cells and cell suspension was neutralised with culture medium and precipitated by the centrifuge. The cell pellet was further resuspended with 1ml cold KSR which

was then transferred into a labelled cryotube and 10% DMSO was added into tubes dropwise. All cryotubes were placed into Mr. Frosty which was cooled down overnight in a -80°C freezer and moved to liquid nitrogen for long-term storage. The procedure of de-freezing naïve hESCs was the same as that of hESCs.

2.1.3.3. HEK293T cell culture

HEK293T cell was cultured in DMEM medium at 37°C, 5% CO₂. The medium was changed every two days and passaged in a ratio of 1:10 when over 90% confluency by Trypsin-EDTA. Cells were frozen in DMEM medium with 10% DMSO and the procedure was the same as hESCs frozen.

2.1.3.4. Gene editing in cell lines

i. Calcium Phosphate Transfection

Calcium phosphate transfection was a method for introducing DNA into HEK293T cells for both transient and stable transfection. For a 10 cm dish, 2 x 10⁶ cells were seeded one day before transfection. Cells were replaced with fresh medium 2 hours before transfection and the reagent was prepared during this 2 hour. The reagent consisted of tube A including 30 µg DNA and 62 µl CaCl₂ with H₂O up to 500 µl and tube B containing 500 µl 2x HeBS buffer. The tube A should be gently added into tube B dropwise while making bubbles to better mix them. Following 20 minutes of incubation, the reagent mixture was added over the cell culture medium on the plate evenly and the plate was gently agitated after that. After overnight incubation, cells were changed with fresh medium for the culture of the following days. Cells could be collected 48 or 72 hours post-transfection for further experiments.

ii. Lentiviral transduction

Lentiviral transduction is a potent method to manipulate genes in hESCs for stable expression. After infection, the viral RNA which contains the interested gene would be

reverse transcribed and incorporated into the host genome. To produce the lentivirus, it was separated into three plasmids including a lentiviral vector including the gene of interest, a helper construct pCMV Δ 8.91 and an envelope construct VSV-G for a safety reason. As a lentivirus factory, 7×10^6 HEK293T cells were seeded on a T225 flask for calcium phosphate transfection according to the protocol last section mentioned, and the ratio of DNA added for lentivirus production is shown as follows: 45 μ g lentiviral vector/ GFP-lentiviral vector, 30 μ g pCMV Δ 8.91 and 15 μ g VSV-G. Followed by 18h incubation after transfection, the transfected cells were replaced with 24 ml fresh medium. On the following day, the supernatant was collected after 24h incubation and precipitated by centrifuge at 1,500 g for 5 mins. The supernatant was then flowed through a 0.45 μ m sterile filter unit, and 120 μ l Polybrene (20 mg/ml) and 120 μ l Chondroitin Sulphate (20 mg/ml) were added to the filtered supernatant followed by a 20 mins incubation at 37°C. The virus particles were then precipitated by centrifugation at 10,000 g for 20 mins at room temperature and the pellet at the bottom of the tube was resuspended by an appropriate volume of DPBS. For short-time storage, the virus could be kept at 4° C and for long-time storage, the virus could be kept at -80° C.

To calculate the number of viruses, a titration experiment was performed with GFP lentiviral particles. A serial dilution of GFP concentrated virus (1:1, 1:10, 1:100, 1:1000) was diluted in DPBS. Then, 20 μ l of the above-diluted viruses were mixed with 1 ml DMEM medium containing 8 μ g/ml Polybrene, which was cultured in 5×10^4 HEK293T cells/well of a 24-well plate for 24h incubation. The fresh medium was changed the other day. The GFP-infected cells were observed under the fluorescent microscope 48h post-infection to determine the percentage of GFP signals. The number of viral particles was calculated via the formulation listed below:

$$\text{Virus titer calculation: Titer} = [(F \times Cn) / V] \times DF$$

F: The frequency of GFP-positive cells

Cn: The total number of HEK293T cells infected.

V: The volume of the inoculum.

DF: The virus dilution factor.

To transduce hESCs, 2.5×10^5 single hESCs were plated on the 6-well plate with MEF-CM (with 10 ng/ml bFGF) containing 1.25×10^6 virus in medium suspension and 8 µg/ml Polybrene. The fresh MEF-CM with 10 ng/ml bFGF was changed for the following day culture and transduced cells were selected by the medium including designed antibiotics (2 µg/ml) after 48h post-transduction.

2.1.3.5. Sequential double thymidine block

Thymidine, an effective inhibitor of DNA synthesis, can arrest cells in either G1 prior to DNA replication or S phase. To synchronise hESCs at metaphase during cell mitosis, sequential double thymidine block and release treatment is an effective method that does not interfere with the spindle fibre. When hESCs reached confluency for splitting, they were seeded into a new 6-well culture plate at approximately 20% confluency per well. After incubating for 18-24 hours, 2 ml of medium with freshly added 2 mM thymidine was replaced, initiating the first synchronisation, and the cells were incubated for 16 hours. After this incubation, the thymidine-containing medium was completely removed, and the cells were washed twice with pre-warmed 1X PBS. Then, 2 ml of fresh medium without thymidine was added, and the cells were incubated for about 8 hours to allow for cell division. Following this, 2 ml of medium with fresh 2 mM thymidine was added for another 16 hours to perform the second round of synchronisation. Following the incubation, the medium was discarded, and the cells were washed twice with 1 ml of pre-warmed 1X PBS buffer. Cells were then released for cell division by adding a 2 ml medium without thymidine. After a 10-hour incubation,

most of the hESCs were synchronised at metaphase and were ready for immunostaining or other techniques.

2.2 DNA AND RNA TECHNIQUES

2.2.1. List of materials

Name	Company	Catalogue number
Reagents, Chemical and Kits		
2-Log DNA Ladder	NEB	N3200S
50 bp DNA Ladder	NEB	N3236S
6x Loading Dye	NEB	B7025
Agarose	Sigma-Aldrich	A9539
dNTP Mix (10 mM each)	Thermo Fisher	R0192
Ethanol Absolute	VWR	20821.330
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich	E9884
EZ DNA Methylation-Gold TM Kit	Zymo	D5005
Isopropanol	Thermo Fisher	BP2618212
KiCqStart® SYBR® Green qPCR ReadyMix	Sigma-Aldrich	KCQS02-250RXN
Molecular Grade Water	Thermo Fisher	R0581
Monarch® DNA Gel Extraction Kit	NEB	T1020S
Murine RNase Inhibitor	Thermo Fisher	EO0381
Oligo(dT)18 100 mM	Thermo Fisher	10753741
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma-Aldrich	P2069
PureLink™ Genomic DNA Mini Kit	Thermo Fisher	K182001
Q5® Hot Start High-Fidelity 2X Master Mix	NEB	M0494S
Sodium Chloride (NaCl)	Sigma-Aldrich	S3014
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	L3771
SYBR Safe DNA Gel Stain	Thermo Fisher	S33102
TOPO™ TA Cloning™ Kit	Thermo Fisher	450030
TRI-Reagent	Sigma-Aldrich	T9424
Enzyme		
BstUI	NEB	R0518S
DNase I	Thermo Fisher	EN0521
Protoscript II Reverse Transcriptase	NEB	M0368
ZymoTaq™ DNA Polymerase	Zymo	E2001
Plasmids		
Name	Source	Purpose
pLVTHM-CAG-Puro-2A-HA-NLRP7	Lab plasmid	NLRP7 stable overexpression
pLVTHM-CAG-EGFP	Lab plasmid	control plasmid

2.2.2. Buffers and solutions

Buffer Name	Components
50X Tris-Acetate-EDTA Buffer (TAE)	242 g Tris Base 57.1 ml Acetic Acid 18.6 g EDTA Fill dH ₂ O up to 1L
10X Tris-Borate-EDTA Buffer (TBE)	108 g Tris Base 55 g Boric Acid 9.3 g EDTA

	Fill dH ₂ O up to 1L
Lysogeny Broth (LB) Medium	10 g Bacto-tryptone 5 g yeast extract 10 g NaCl Fill dH ₂ O up to 1L
LB Agar	10 g Bacto-tryptone 5 g yeast extract 10 g NaCl 15 g Agar Fill dH ₂ O up to 1L
P1 Resuspension Buffer (Qiagen)	100 mM Tris-HCl pH 8.0 10 mM EDTA 100 µg/ml RNase A
P2 Lysis Buffer (Qiagen)	200 mM NaOH 1% SDS
P3 Neutralization Buffer (Qiagen)	3 M Potassium Acetate pH 5.5

2.2.3. List of Primers

2.2.3.1. Bisulfite primers:

Name	Primer 5' to 3'	Location
<i>NAP1L5</i> -DMR	F (Ori seq): GGGGCTCCTCAGCCATCTGACCAG R (Ori seq): CAAGATCTCTCTGGACCAGCTCT	Chr4: 88697589- 88697830
<i>GRB10</i> -DMR	F: GGTTCTCATGGCAACCCTGA F (Ori seq): GGTTTTATGGTAATTTGAAGAATTTT R: TCAGTTTAAATGGGACCCTACTTTG R (Ori seq): TCAATTTAAATAAAAACCCTACTTTATCC	Chr14: 50781029- 50783615
<i>KCNQ1OT1</i> -DMR	F: TGTGAGTGGAATTAGTAAATTGTATTA F (Ori seq): TGTGAGTGGAATCAGCAAATTGTATCA R: AAAAAACAAAACCTCTTAAAAAACCC R (Ori seq): AAGGGGACAAGGCCTCTTAAAGGGGCC	Chr11: 2721505- 2721876
<i>L3MBTL</i> -DMR	F: TTAATTAGGATTTTTTAGAGGATGATAG F (Ori seq): CCAATCAGGACCCCCCAGAGGATGATAG R: CTCCTCTAATAAACTCTAATATTCCTA R (Ori seq): CTCCTCCTCTGATGGGCTCTGGTATTCCCTG	Chr20: 43515039 - 43515397
<i>NAP1L5</i> -DMR	F: GGGGTTTTTTAGTTATTTGATTAG F (Ori seq): GGGGCTCCTCAGCCATCTGACCAG R: CAAATCTCTCTAAACCAACTCT R (Ori seq): CAAGATCTCTCTGGACCAGCTCT	Chr4: 88697589- 88697830
<i>PEG3</i> -DMR	F: GGAAAGAAAATTTTTATAGGTAGGATAGT F (Ori seq): GGAAAGAAAACCCCTACAGGCAGG R: AAACCCTAAACCTCCTAAACTAAATCTAA R (Ori seq): GAGCCCTGGGCCTCCTG	Chr14: 56839940- 56840149

PLAGL1-DMR	F: TTGAAAAATATTGTTTTTAAGGATGAA F (Ori seq): CTGAAAAACATTGTTCTTAAGGATGAATAC R: TAAAAATCAAAAAATAAAAACACCCAAC R (Ori seq): TAAAAATCAAAAAATAGAAACACCCAGC	Chr6: 144007011- 144007441
SNURF/SN RPN-DMR	F: TAGGTTGTTTTTTGAGAGAAGTTAT F (Ori seq): CAGGCTGTCTCTTGAGAGAAGCCAC R: AAAAAAACTAAAACCCCTACACTAC R (Ori seq): GAGGGAGCTGGGACCCCTGCACTGC	Chr15: 24954865- 24955100

2.2.3.2. Genotyping and allelic-expression analysis primers

Name	SNP	Primer 5' to 3'	Length	MAF*
<i>KCNQ1OT1</i>	rs151212	F: GCCTTCTTCCTCTCTTGATG R: GCAAACCTACAGTGAGAAGG	453 bp	C=0.33 088
<i>MAGEL2</i>	rs9785	F: CGTTCAAAGAACAACCGAT R: CCATAAGAAAGATCCACAGAG	503 bp	G=0.42 4488
<i>MEG3</i>	rs11160608	F: GCAAGCCAGGATGAGAGTGT R: TGACGGGCCTCAAAATCTATGT	268 bp	A=0.43 8001
<i>NAP1L5</i>	rs710834	F: GGTGAGCTCTTGATCTTGG R: GCGGCTTCTCCTCTAACATG	389 bp	C=0.47 1801
<i>NDN</i>	rs2192206	F: CTACACAATAGTACCCAAATGAAT R: TACCAGCGCGTCCCATA	407 bp	A=0.15 956
<i>PEG10</i>	rs13073	F: ACAGAGATGTAAGAGGCAGGC R: CTAGTCACCACTTCAAAACACAC	228 bp	A=0.26 8031
<i>SNRPN-Geno</i>	rs705	F: CATCAGTCCTAAGTGTGTC R: GATCACTGCACATGCTGGCAAAC	423 bp	T=0.44 6903
<i>SNRPN-Trans</i>	rs705	F: CAGGCATTCTTAGCTGAGAC R: CATCTTGCAAGGATACATCTC	294 bp	-

*MAF (minor allele frequency) is the second most common allele of a SNP that occurs in a given population.

2.2.3.3. qRT-PCR primers

Primer name	5' to 3'
<i>qDNMT1</i>	F: GGTTCTTCCTCCTGGAGAATGTC R: GGGCCACGCCGTACTG
<i>qDNMT3A</i>	F: CAATGACCTCTCCATCGTCAAC R: CATGCAGGAGGCGGTAGAA
<i>qDNMT3B</i>	F: CCATGAAGGTTGGCGACAA R: TGGCATCAATCATCACTGGATT
<i>qDNMT3L</i>	F: GGGACAACTGAAGCATGTGGT R: AAGATCGAAGGGTCCCCACT
<i>qH19</i>	F: CAGGAGTGATGACGGGTGG R: GCCAGCTACACCTCCGTTG
<i>qKLF2</i>	F: CACACAGGTGAGAAGCCCTA R: CAGTCACAGTTTGGGAGGGG

qMEG3	F: AAGACCATGGTTCATCCGCT R: TGACGGGCCTCAAAATCTATG
qNANOG	F: TGATTTGTGGGCCTGAAGAAAA R: GAGGCATCTCAGCAGAAGACA
qNAP1L5	F: CTCCTAGACCTCTGCGGCT R: AGGCAGGCTCTCGATAAAGTC
qNLRP7	F: GACGGACAGGTGCAAGAAAT R: AGAATGGAATGAACCGTTGG
qOCT4	F: TCGAGAACCGAGTGAGAGGC R: CACACTCGGACCACATCCTTC
qPEG10	F: GAGGCAGGCTTTAATTGCCA R: TTGAAAGCCTAGTCACCACTTCA
qPRDM14	F: TCTGTGATGTGGGAGATCTTTGA R: ACTCTTCAGAGGGCCCAGAT
qREX1	F: TCACAGTCCAGCAGGTGTTT R: GCCATCACATAAGGCCACACA
qRPL22	F: TCGCTCACCTCCCTTTCTAA R: TCACGGTGATCTTGCTCTTG
qTCL1B	F: GGGCAGCAGATATGAACCCA R: CCCAGAACTGGAATCCGCT
qTET1	F: ACCCCCTGTACCTGCTGAGG R: GCGATGGCCACCCACCAAT
qTET2	F: TCACACCAGGTGCACTTCTC R: GGATGGTTGTGTTTGTGCTG
qTET3	F: TCTCCCCAGTCTTACCTCCG R: CCAGGCTTCAGGGAACCTCAG
q β -actin	F: TGTCTGGCGGCACCACCATG R: AGGATGGAGCCGCCGATCCA

2.2.4. Methods

2.2.4.1. RNA extraction and cDNA generation

i. RNA extraction from adherent hESCs

When the confluency of cells reached 90% in a 6-well culture plate, cells were rinsed with DPBS and lysed in 1 ml of TRI reagent with regular pipetting. Then, cell lysate in TRI reagent was transferred into a DNase-free and RNase-free Eppendorf tube, which was then mixed with 200 μ l of chloroform: isoamyl alcohol by vigorously shaking. After centrifugation at 13,000 g for 15 mins at 4 ° C, the mixture was separated into three layers whose top layer containing nucleic acids was transferred into a new tube and mixed with an equal volume of isopropanol to precipitate nucleic acids. Followed by 10

mins incubation at room temperature, the tube was centrifuged at 13,000 g for 10 min at 4 °C and the pellet of nucleic acids was visible at the bottom of the tube. The pellet was then washed with 1ml 75% ethanol and centrifuged at 13,000 g for 10 min at 4 °C. Finally, the nucleic acids pellet was resuspended with 20 µl of RNase-free water and treated with DNase I to remove genomic DNA according to the chart below:

Component	Amount
RNA	20 µl
10X Reaction buffer with MgCl ₂	2.5 µl
DNase I, RNase-free	2.5 µl

The mixture was incubated at 37 °C for 30 mins to perform function. Then, 2.5 µl 50 mM EDTA was added into a tube to inactivate DNase I at 65 °C for 10 mins. The concentration of RNA could be measured by NanoDrop® Spectrophotometer and the RNA samples were kept at -80 °C for long-term storage.

ii. cDNA generation by reverse transcription polymerase chain reaction

The RNA was mixed with all components listed below in a sterile RNase-free microfuge tube to denature RNA and primer for 5 minutes at 65°C.

Component	Volume
RNA	1.5 µg
Oligo(dT)18 100 mM	2 µl
10 mM dNTP	1 µl
Nuclease-free Water	Up to 10 µl

After that, the rest components listed below were further added into the tube. The total 20 µl reaction was incubated at 42°C for one hour, followed by inactivating enzyme at 65°C for 20 mins. Finally, the cDNA reaction was diluted 1:10 and long-term stored at -20°C. The cDNA was prepared to use after dilution with ddH₂O in a ratio of 1:10.

Component	Volume
5X ProtoScript II Buffer	4 µl
0.1 M DTT	2 µl
ProtoScript II RT (200 U/µl)	1 µl
RNase Inhibitor (40 U/µl)	0.2 µl
Nuclease-free Water	2.8 µl

iii. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

qRT-PCR is a routine method to analyse the RNA expression of different genes. The reaction was prepared in MicroAmp™ Fast Optical 96-Well Reaction Plate and the component of each well was shown below. The StepOnePlus™ Real-Time PCR System was used to perform this experiment under the program shown below.

Component	Volume
KiCqStart SYBR Green qPCR ReadyMix, with ROX(2X)	5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
cDNA template	0.5 µl
Nuclease-free Water	Up to 10 µl

qPCR cycles set up:		
Initial Denaturation	95 °C	2 mins
Denaturation	95 °C	15 sec
Annealing	58 °C	30 sec
Extension	72 °C	30 sec
Collect data at the end of the extension step		
40 Cycles		
Melting curve from 70 °C to 95 °C, read every 0.5 °C, hold 1 sec		

Two housekeeping genes, β -actin and RPL22, were used in this project and they both showed similar pattern. Ct values in genes of interest were normalized to the Ct value of the housekeeping gene as ΔCt^* . The relative expression level was calculated using $\Delta (\Delta Ct)$ method**, relative to the primed hESCs:

$$\text{Expression of the target gene relative to } \beta\text{-actin} = 2^{-\Delta Ct}$$

$$\text{Relative gene expression level after naïve conversion} = 2^{-\Delta (\Delta Ct)}$$

$$* \Delta Ct = Ct_{(\text{interested gene})} - Ct_{(\text{housekeeping gene: } \beta\text{-actin})}$$

$$** \Delta (\Delta Ct) = \Delta Ct_{(\text{naïve})} - \Delta Ct_{(\text{primed})}$$

All the RT-qPCR data required for statistical analysis (eg: qRT-PCR data) was analysed by Excel software for initial analysis to obtain the relative expression level to housekeeping genes (β -actin or RPL22). The two-tail unpaired t-test was used to calculate the significance of two data sets with three biological replicates for each set and $p < 0.05$ was used as a threshold. Each graph was drawn and presented by GraphPad prism further.

2.2.4.2. *Genotyping and imprinting analysis*

The potential SNPs in imprinted genes were chosen based on the highest minor allele frequency (MAF) through the NCBI Variation viewer (<https://www.ncbi.nlm.nih.gov/variation/view/overview/>). PCR primers were designed to amplify the interested regions containing the SNP site from genomic DNA generated from the H1 and H7 hESCs. Genomic DNA should be amplified by Q5® Hot Start High-Fidelity 2X Master Mix with different sets of primers whose flanking regions contain the SNP site as designed. Reagents were mixed according to the following table and performed by Bio-Rad Dyad DNA Engine thermocycler based on the program shown below and the annealing temperatures depend on different primer designs. The PCR product could be purified and checked by agarose gel electrophoresis and extracted from the gel using an NEB Monarch gel extraction kit. The purified product was ready for Sanger sequencing to identify the genotype of potential SNPs in H1 and H7 hESCs. Sequencing results were interrogated using SnapGene Viewer. Those SNPs shown heterozygous results were available to use for further allelic expression analysis using RT-PCR and direct Sanger sequencing which is the same as the genotyping procedure but uses cDNA as samples.

Component	Amount
Q5 High-Fidelity 2X Master Mix	12.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template gDNA/cDNA	2 µl
Nuclease-free Water	Up to 10 µl

Q5® Hot Start High-Fidelity DNA Polymerase PCR cycles		
Initial Denaturation	98 °C	30 sec
Denaturation	98 °C	10 sec
Annealing	Ta	30 sec
Extension	72 °C	30 sec
25-35 Cycles		
Final Extension	72 °C	2 mins
Hold at	4 °C	∞

2.2.4.3. Bisulfite Sequencing Polymerase Chain Reaction

i. Genomic DNA (gDNA) extraction

Cells were treated with Accutase to dissociate from the culture plate and resuspended with 200 µl PBS after centrifugation. Then the resuspended cell sample was processed with PureLink™ Genomic DNA Mini Kit for gDNA extraction according to the instructions from the manufacturer. The concentration and purity were checked by NanoDrop® Spectrophotometer.

ii. Bisulfite conversion

Bisulfite conversion is a popular method to detect and quantify methylation on the DNA. The principal idea of this method is to convert the unmethylated C to U, while the methylated C is maintained. To quantify the methylated level of interested regions, the EZ DNA Methylation-Gold™ Kit was utilized following the manufacturer's protocol to covert gDNA.

iii. Polymerase Chain Reaction (PCR)

To quantify the methylated level of interested DMR regions, the bisulfite-converted gDNA should be amplified by *ZymoTaq*™ DNA Polymerase with different sets of primers which target the DMRs of imprinted genes. Reagents were mixed according to the following table and kept on ice until put into the machine. Bio-Rad Dyad DNA Engine thermocycler was set up based on the program shown below and the annealing temperatures depend on different primer designs. The PCR products could be checked by agarose gel electrophoresis with 1.8% agarose gel and purified by Monarch® DNA Gel Extraction Kit following the instructions from the manufacturer.

Reagent	Volume	Final Con.
2X Reaction Buffer	10 µl	1X
dNTP Mix	0.5 µl	0.25 mM each dNTP
Forward Primer (10 µM)	2 µl	1 µM
Reverse Primer (10 µM)	2 µl	1 µM
Template DNA	1 µl	
<i>ZymoTaq</i> TM DNA Polymerase (5 U/µl)	0.2 µl	1 U/20 µl
ddH ₂ O	4.3 µl	
Total volume	20 µl	

<i>ZymoTaq</i> TM DNA Polymerase PCR cycles		
Initial Denaturation	95 °C	10 mins
Denaturation	95 °C	30 sec
Annealing	T _a	40 sec
Extension	72 °C	60 sec
35-40 Cycles		
Final Extension	72 °C	7 mins
Hold at	4 °C	∞

iv. Cloning PCR products into TOPO[®] vector

Since the *ZymoTaq*TM DNA Polymerase can add an additional A at the 3' end of PCR products, PCR products are ready for TOPO[®] cloning without a need for an A-tailing reaction. The purified PCR products were ligated to TOPO[®] vectors as described in the following tables:

Reagent	Volume
Purified PCR product	1 µl
Salt Solution	1 µl
Water	3 µl
TOPO [®] vectors	1 µl
Total volume	6 µl

The reaction was mixed gently and incubated for 5 minutes at room temperature. After that, the reaction was placed consistently on ice before transformation, otherwise, stored at -20 °C for long-term storage.

v. Bacterial transformation

2 µl of the ligation reaction was added into 100 µl DH5α *E. coli* after it was slowly thawed on ice. The tube containing the DNA-bacterial mixture was gently flicked and incubated

on ice for 30 minutes. The mixture was then heat-shocked in a 42 °C water bath for 40 seconds and immediately placed on ice for another 2 minutes. Following this, 200 µl of LB broth was added, and the mixture was incubated at 37 °C with shaking at 250 rpm for 1 hour. The bacterial mix was then plated onto an LB agar plate containing 100 ng/ml ampicillin

vi. Plasmid amplification and extraction

The surviving white colonies on the LB agar plates containing ampicillin indicated successful PCR product insertion into the vector. To analyse the methylation levels of DMRs, at least ten successfully transformed bacterial colonies were selected and amplified. Each white colony was picked and cultured in 5 ml of LB broth with 100 ng/ml ampicillin at 37 °C overnight with shaking at 250 rpm. A 1.5 ml aliquot of the bacterial culture was then pelleted by centrifugation at 6,000 g for 15 minutes. The pellet was resuspended in 250 µl of P1 buffer and vortexed vigorously. An equal volume of P2 lysis buffer was added, followed by a 5 mins incubation. Subsequently, 250 µl of P3 neutralization buffer was added. Bacterial debris was removed by centrifugation at 13,000 g for 5 minutes, and the supernatant was transferred to a new tube and mixed vigorously with an equal volume of isopropanol. The plasmid DNA was pelleted by centrifugation at 13,000 g for 5 minutes, air-dried, and resuspended in 40 µl of ddH₂O. The plasmid DNA concentration was measured using a NanoDrop® spectrometer. The extracted plasmids were then sent for Sanger sequencing, and the sequencing results were compared with the original sequence of DMRs to determine the methylation status of the imprinted genes.

vii. Restriction digestion of Bisulfite-PCR product

All Bisulfite-PCR products were designed including at least one CGCG restriction cutting site for BstUI restriction enzyme. The restriction enzyme was provided by NEB company and plasmids digestions were conducted based on the chart below and instructions from the manufacturer (<https://nebcloner.neb.com/#!/redigest>). The

digested plasmid was separated through agarose gel electrophoresis. The gel images were visualised and captured by Syngene Geneflash Bio-Imaging Gel Doc System. These images were analysed by ImageJ software and methylation level was calculated based on the intensity of each band.

Component	Amount
10x CutSmart Buffer	1 μ l
BstUI	1 μ l
Bisulfite-PCR product	100 ng
Nuclease-free Water	Up to 10 μ l

2.3 PROTEIN TECHNIQUES

2.3.1. List of Materials

Name	Company	Catalogue number
Reagents, Chemical and Kits		
30% Acrylamide/Bis Solution-37.5:1	BioRad	1610159
4',6-Diamidino-2-Phenylindole (DAPI)	Sigma-Aldrich	D9542
Ammonium Persulfate (APS)	Sigma-Aldrich	A3678
Bicinchoninic Acid (BCA) Assay Protein Quantification Kit	Thermo Fisher	23227
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A5611
Bromophenol Blue	Sigma-Aldrich	B1026
Clarity ECL Western Blotting Substrate	BioRad	1706061
CL-Xposure Film	SLS	MOL7016
Glycerol	Sigma-Aldrich	G5516
Goat Serum	Sigma-Aldrich	G9023
Immobilon® Forte Western HRP Substrate	Millipore	WBLUF0500
Immobilon® Polyvinylidene Fluoride (PVDF) Membrane	Millipore	431175
Methanol	VWR	85800
Mowiol 4-88	Calbiochem	475904
Nonidet P-40 (NP-40)	Anachem	E109-100ML-R
Phenylmethanesulfonyl Fluoride (PMSF)	NEB	8553S
Pierce™ 16% Formaldehyde	Thermo Fisher	28908
Prestained Protein Ladder	NEB	p7712L
Protease Inhibitor Cocktail	Sigma-Aldrich	P8340
Skimmed Milk Powder	Sigma-Aldrich	70166
Sodium Azide	Sigma-Aldrich	S2002
Sodium Dodecyl Sulphate (SDS)	Sigma-Aldrich	L3771
Tetramethylethylenediamine (TEMED) Sigma-	Sigma-Aldrich	T7024
Triton X-100	Sigma-Aldrich	T8787
Tween-20	Sigma-Aldrich	P9416
β-Mercaptoethanol 14.3M	Sigma-Aldrich	M3148

2.3.1.1. List of Antibodies

Name	Company	Catalogue number	Dilution
Anti-mouse IgG-HRP	Startech	115-035-174	1:5000
Anti-rabbit IgG (H+L)-HRP	Thermo Fisher	31460	1:5000
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate	Invitrogen	A11029	1:400
Goat anti-Mouse-Secondary Antibody, Alexa Fluor 568	Invitrogen	A-11004	1:400
Goat anti-Rabbit IgG (H+L) Cross Secondary Antibody, Alexa Fluor™ 568	Invitrogen	A-11011	1:400
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor™ 488	Invitrogen	A-11008	1:400
Mouse anti- α -tubulin	Cell signalling	3873S	IF: 1:2000
Mouse anti-NLRP7	Santa Cruz	sc-377190	IB: 1:500 IF: 1:100
Mouse anti- β -actin	Sigma-Aldrich	A5316	IB: 1:5000
Rabbit anti-HA	Sigma-Aldrich	H6908	IB: 1:1000

2.3.2. Buffers and Solutions

2.3.2.1. Western Blotting

Buffer Name	Components	Final conc.
10X SDS Running Buffer	30.3 g Tris base 144.2 g Glycine 50 ml 20% SDS add ddH ₂ O up to 1 L	250 mM 1.9 M 1% w/v
4% Polyacrylamide Stacking Gel	2.5 ml 0.5 M Tris-HCl pH 6.8 1.3 ml 30% Acrylamide/Bis 100 μ l 10% SDS 50 μ l 12% APS 12 μ l TEMED add ddH ₂ O up to 10 ml	125 mM 4% v/v 0.1% w/v 0.06 % w/v 0.12% v/v
5 x Sample Buffer	1.25 ml 0.5M Tris-HCl pH 6.8 2.5 ml glycerol 2 ml 10% SDS 400 μ l 0.5% bromophenol blue 500 μ l β -mercaptoethanol (14.3M) Autoclaved water to 10 ml	50 mM 25% v/v 2% w/v 0.02% w/v 715 mM
7.5% Polyacrylamide Running Gel	2.5 ml 0.5M Tris-HCl pH 8.8 2.5 ml 30% Acrylamide/Bis 100 μ l 10% SDS 50 μ l 12% APS	125 mM 7.5% v/v 0.1% w/v 0.05% w/v

	12 µl TEMED add ddH ₂ O up to 10 ml	0.12% v/v
Radio Immunoprecipitation Assay (RIPA) Buffer	25 ml 1M Tris-HCl pH 8.0 15 ml 5M NaCl 5 ml NP-40 2.5 g Sodium deoxycholate 0.5 g SDS Add ddH ₂ O up to 500 ml	50 mM 150 mM 1% v/v 0.5% w/v 0.1% w/v
Transfer Buffer	5.82 g Tris base 2.93 g Glycine 3.75 ml 10% SDS 200 ml methanol add ddH ₂ O up to 1 L	48 mM 39 mM 0.04% w/v 20% v/v
Tris Buffered Saline with Tween-20 (TBS-T)	20 ml 1M Tris-HCl pH 7.6 26 ml NaCl 1 ml Tween-20 add ddH ₂ O water to 1 L	20 mM 130 mM 0.1% v/v

2.3.2.2. Immunocytochemistry

Buffer Name	Components	Final conc.
Fixation Buffer	10 ml 16% PFA solution 30 ml DPBS	4% w/v
Blocking Buffer	1 ml goat serum 0.25 g BSA 30 µl Triton X-100 add PBS up to 10ml	10% v/v 2.5% w/v 0.3% v/v
Antibody Dilution Buffer	0.1 g BSA 30 µl Triton™ X-100 add PBS up to 10ml	1% BSA 0.3% Triton™ X-100
Mowiol mounting solution	12 ml 200 mM Tris-HCl pH 8.5 6 g glycerol 2.4 g Mowiol 4-88 6 ml ddH ₂ O Heat at 60°C to dissolve Mowiol	135 mM

2.3.3. Methods

2.3.3.1. Western Blotting

After washed in DPBS, hESCs cultured on a 6-well plate were directly added with ice-cold 100 µl RIPA buffer including 1% protease inhibitor cocktail and 1% PMSF. Followed by cell lysis on ice for 30 mins, the mixture was collected into a new microfuge

tube and centrifuged at 14,000rpm for 30 min at 4°C. The supernatant containing solubilized proteins was transferred and aliquot into tubes for long-term storage at -80°C. To measure the concentration of the protein sample, a BCA assay protein quantification kit was used under the instructions of the manufacturer.

10-20 ug protein was resolved in 5x sample buffer supplemented with H₂O to dilute to 1x sample buffer. After incubation at 95 °C for 5 min, the protein mixture was loaded on 7.5% SDS-polyacrylamide gel under 180 v for 60 min. The protein was further transferred to a PVDF membrane through electroblotting (wet transfer, 110 v, 60 min). Then, the membrane was blocked in 5% skimmed milk in TBS-T for 1 hour at room temperature and later probed with primary antibodies in 1% milk in TBS-T overnight at 4°C. After washing three times with 1% TBS-T, the membrane was incubated with secondary antibodies in 1% milk at room temperature for 1h. Followed by washing three times with 1% TBS-T, the membrane was performed chemiluminescent substrate reaction via ECL substrate and ready for exposure onto CL-XPosure film in Optimax X-ray film processor.

2.3.3.2. Immunostaining of hESCs

Matrigel-coated Thermanox coverslips were prepared in a 6-well plate in advance. The next day, hESCs were seeded onto the prepared plates. After culturing for a couple of days, cells were washed with PBS and fixed in 4% formaldehyde at room temperature for 10 min. Followed by washing with PBS, cells were incubated in blocking buffer for 1 hour and incubated in diluted primary antibody overnight at 4°C. On the following day, cells were washed three times with PBS and incubated with fluorophore-conjugated secondary antibody in a dark condition for 1 hour. Then, cells were incubated with 1 ng/ml DAPI and Phalloidin staining for another 15 minutes. The coverslip-seeded cells were mounted with Mowiol before the observation under the Leica SP5 confocal microscope.

2.4 EQUIPMENT, SOFTWARE, AND ONLINE TOOLS

Equipment
Bio-Rad Dyad DNA Engine thermocycler
Bio-Rad Mini Trans-Blot® Cell
Leica SP5 confocal microscope
Mini-Sub Cell GT System
Nanodrop® Spectrophotometer
Nikon Inverted Fluorescence Microscope
Optimax X-Ray Film Processor
StepOnePlus™ Real-Time PCR System
Syngene Geneflash Bio Imaging Gel Doc System

Software
Image J
Serial Cloner
SnapGene
LAS AF Lite
Adobe Photoshop 2023
StepOne Software v2.3

Online tools	
T _m Calculator	http://tmcalculator.neb.com
Basic Local Alignment Search Tool (BLAST)	http://blast.ncbi.nlm.nih.gov/Blast.cgi
NCBI Variation viewer	https://www.ncbi.nlm.nih.gov/variation/view/ov
NEB Double Digest Finder	https://nebcloner.neb.com/#!/redigest
NEBcutter V2.0	http://nc2.neb.com/NEBcutter2/
Primer-BLAST	https://www.ncbi.nlm.nih.gov/tools/primerblast/
MethPrimer	http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi

Chapter 3

**Identification of SNP sites from maternally
imprinted genes in H1 and H7 hESCs**

3.1 INTRODUCTION

Genomic imprinting is an epigenetic phenomenon which results in a parent-of-origin-specific expression (Ferguson-Smith, 2011). Most identified imprinted genes are physically clustered and regulated by ICRs, which regulate allele-specific expression of imprinted genes (Kelsey and Feil, 2013). At an imprinted locus, the ICR is differentially modified by epigenetic marks, predominantly DNA methylation, between paternal and maternal alleles (Rugg-Gunn et al., 2007). The transcriptional machinery of the cells could recognize the differentially methylated ICRs (DMRs) to determine which allele should be expressed and which should be repressed (Rugg-Gunn et al., 2007). In imprinting, a maternally imprinted gene is only expressed from the paternal allele but silenced on the maternal allele. Conversely, a paternally imprinted gene is only expressed from the maternally inherited allele (Hanin and Ferguson-Smith, 2020). Therefore, monoallelic expression of imprinted genes is the gold standard for determining whether genes are imprinted.

Over the past few years, comparing the transcriptome and methylome of human androgenetic embryonic stem cells (hA ESCs) with biparental hESCs has uncovered more candidate paternally imprinted genes in humans. Similarly, more maternally imprinted genes have been discovered through comparisons between parthenogenetic embryonic stem cells (hP ESCs) and biparental hESCs. (Stelzer et al., 2015, Sagi et al., 2019). Recently, quantitative whole-transcriptome sequencing combined with identified single nucleotide polymorphisms (SNPs) has provided another reliable strategy for discovering novel imprinted genes in humans (DeVeale et al., 2012). SNPs are common genetic variations between paternal and maternal alleles at specific sites within a genome (Syvanen, 2001). By analysing readings of differential nucleotides at SNP sites in sequencing data, imprinted genes can be identified if they exhibit monoallelic expression (DeVeale et al., 2012).

Since hESCs are valuable human embryonic materials for the study of human embryonic development, including genomic imprinting, they have been widely used in research of genomic imprinting. hESCs exhibit a substantial degree of genomic imprinting stability in culture and most of the stable imprinted genes are expressed in hESCs and display monoallelic expression even after differentiation. However, some imprinted genes show variable expression patterns across different hESC lines, with underlying mechanisms yet to be understood (Kim et al., 2007, Rugg-Gunn et al., 2007, Bar et al., 2017, Sun et al., 2006). Therefore, validating the imprinting status of targeted imprinted genes in selected hESC lines is crucial, as occasional loss of imprinting can occur among different hESC lines (Rugg-Gunn et al., 2005, Sun et al., 2006).

To apply hESCs for the study of imprinting, one important issue is to distinguish maternal and paternal alleles on imprinted genes in the selected hESCs. Previous work in our lab has screened six maternally imprinted genes for SNPs and expression patterns in H1 and H7 hESCs and identified two maternally imprinted genes (*PEG10* and *NAP1L5*) that contain SNPs in their coding region and are imprinted in both hESC lines (Zhi, 2021). However, I feel that the number might be limited. Hence, I aim to explore the possibility of expanding the study to more maternally imprinted genes. In this chapter, I take a more systemic approach to address the following questions:

- i. How many imprinted genes have been identified in human?
- ii. Do identified maternally imprinted genes contain allele-specific SNP sites in H1 and H7 hESCs?
- iii. Do these maternally imprinted genes exhibit monoallelic expression in H1 and H7 hESCs?

3.2 RESULT

3.2.1. Identifying potential maternally imprinted genes in H1 & H7 hESCs

The transition from imprinted to non-imprinted status in genomic imprinting could be investigated by examining allele-specific expression of imprinted genes which is shown as monoallelic expression to biallelic expression (Bar et al., 2017). Thus, to be able to use hESCs for the study of maternal imprinting, it is important to identify SNP sites within maternally imprinted genes. Identifying allele-specific SNPs would facilitate the determination of allele-specific gene expression in these imprinted genes.

To maximise the number of maternally imprinted genes to be studied in hESCs, I firstly analysed public online data, including database from www.geneimprint.com; www.otago.ac.nz and published data of imprinted genes in human, to determine the number of maternally and paternally imprinted genes in human (Tucci et al., 2019). A total of 227 imprinted genes were identified in human comprising 152 maternally imprinted genes and 48 paternally imprinted genes (Table 3.1). However, approximately half of them were tissue-specific and isoform-specific imprinted genes, which were not imprinted in hESCs, e.g. a placental-specific imprinted gene *LIN28B* (Zhi, 2021), herein they were excluded from my study. Then, 101 imprinted genes remained, including 68 maternally imprinted genes and 33 paternally imprinted genes (Table 3.1). Then, I utilized our unpublished RNA sequencing data from H1 hESCs to determine whether these genes are expressed in our hESCs. The analysis revealed that 24 maternally and 22 paternally imprinted genes are expressed in our H1 hESCs (Table 3.1).

However, not all imprinted genes would have active imprinting status in hESCs, and even in different hESC lines, the imprinting status of imprinted genes could vary (Kim et al., 2007, Bar et al., 2017, Mai et al., 2011). Thus, I summarised imprinted genes that were confirmed to be imprinted in hESC lines from two published papers to find

the imprinted genes for my study. The first group analysed allele-specific expression of imprinted genes among different hESC lines and identified 28 Maternally imprinted genes and 6 paternally imprinted genes (Bar et al., 2017), while the other group compared the transcriptome of hPESCs and hESCs to identify 14 maternally imprinted genes in hESCs (Mai et al., 2018). The number of maternally and paternally imprinted genes identified by these two papers are also summarized in Table 3.1.

Table 3.1 Summary of imprinted genes identified in different databases.

	Maternally	Paternally	Total	Reference
Imprinted genes in human	152	48	227	(Tucci, 2019)
Imprinted genes in human (Excluded placental specific)	68	33	101	www.geneimprint.com www.otago.ac.nz
H1 hESCs	24	22	46	(Li, unpublished)
Imprinted genes in hESCs	28	6	34	(Bar, 2017)
	14	-	-	(Mai, 2018)

I then checked whether these genes were expressed in our H1 hESCs. I cross-compared the imprinted genes that are expressed in our H1 hESCs with these two published datasets about hESC-expressing imprinted genes. The result revealed that six maternally imprinted genes (*KCNQ1OT1*, *MAGEL2*, *NDN*, *PEG10*, *PEG3*, *SNRPN/SNURF*) were expressed in all three datasets. In addition, seven maternally imprinted genes (*DLK1*, *FAM50B*, *IGF2*, *LRRTM1*, *NNAT*, *SGCE*, *ZDBF2*) were found between our H1 hESC and Bar's studies (Figure 3.1A) (Bar et al., 2017). For paternally imprinted genes, four genes (*MEG3*, *ZNF597*, *H19* and *NAA60*) were expressed between our H1 hESCs and Bar's studies (Figure 3.1B).

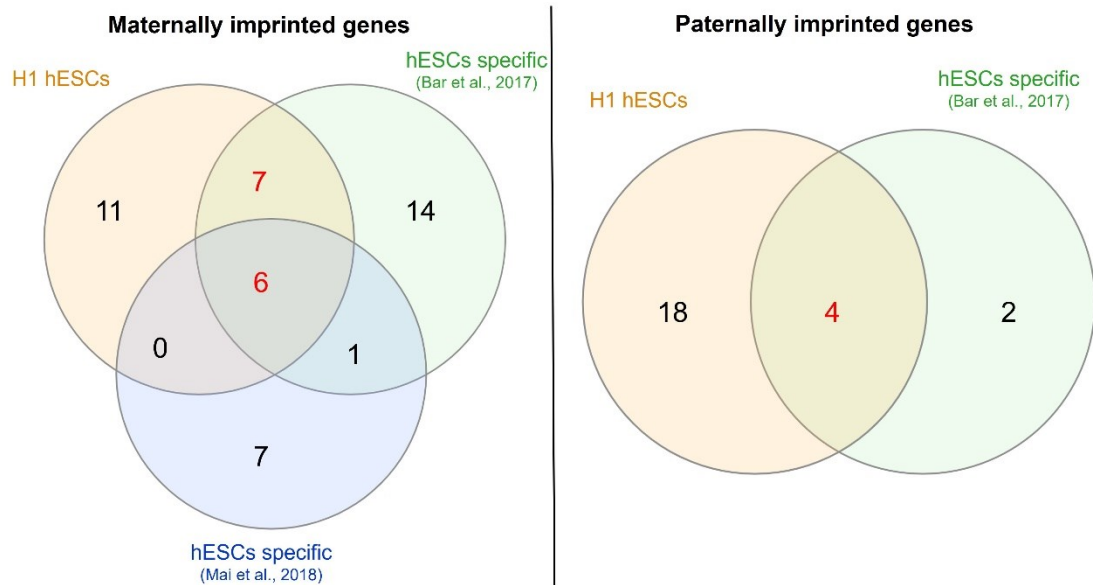


Figure 3.1 Number of imprinted genes expressed in hESCs in various studies

Venn diagram showing similarities and differences on hESC-expressing imprinted genes between the three indicated studies. **(A)** Maternally imprinted genes. **(B)** Paternally imprinted genes. Red colour presents maternally and paternally imprinted genes shared in 2 or more studies.

3.2.2. Screening allele-specific SNPs in hESC-expressing maternally imprinted genes in H1 and H7 hESC lines

Since this project mainly focused on maternal imprinting, I next focused on the 13 maternally imprinted genes that exhibit expression not only in our hESCs but also in at least one of these two hESC studies (Figure 3.1A) for SNP analysis to validate their imprinting status in our hESC lines.

Minor allele frequency (MAF) represents the frequency at which the second most common allele occurs within a specific population (Hernandez et al., 2019). An SNP site with an MAF of at least 0.05 indicates a common occurrence within the population, otherwise, it is classified as a rare variant (Sidore et al., 2015). In addition, SNPs may be located in the intron region of certain genes, which cannot be used to validate allelic expression patterns. Therefore, I first inspected the MAF score and the SNP location in the 13 maternally imprinted genes and then eliminated the one with $MAF < 0.05$ or not located within the exon for further analysis. I also included *NAP1L5* in this analysis

(Table 3.2) since it showed to be imprinted in the H1 and H7 hESCs in our previous analysis and Bar's study (Zhi, 2021, Bar et al., 2017).

After applying the selection criteria, seven genes were the potential candidates of maternally imprinted genes in H1 and H7 hESCs, in which *PEG10*, *NAP1L5* and *ZDBF2* have been previously checked in our group to contain SNPs in H1 and H7 hESCs (Zhi, 2021). Herein, I had four genes (*KCNQ1OT1*, *MAGEL2*, *NDN* and *SNRPN/SNURF*) to be investigated for their SNPs in H1 and H7 hESCs.

Table 3.2 Summary of SNP sites within selected maternally imprinted genes.

Gene name	Location	Gene types	SNP	MAF
<i>NAP1L5</i>	4q21-q22	protein coding	rs710834	MAF=0.47
<i>SNRPN/SNURF</i>	15q11.2	protein coding	rs705	MAF=0.447
<i>ZDBF2</i>	2q33.3	protein coding	rs7582864	MAF=0.421
<i>MAGEL2</i>	15q11.2	protein coding	rs9785	MAF=0.42
<i>KCNQ1OT1</i>	11p15.5	LncRNA	rs151212	MAF=0.33
<i>PEG10</i>	7q21.3	protein coding	rs13073	MAF=0.27
<i>NDN</i>	15q11.2	protein coding	rs2192206	MAF=0.159
<i>DLK1</i>	14q32.2	protein coding	Rare SNP	MAF<0.05
<i>FAM50B</i>	6p25.2	protein coding	Rare SNP	MAF<0.05
<i>IGF2</i>	11p15.5	protein coding	Rare SNP	MAF<0.05
<i>LRRTM1</i>	2p12	protein coding	Rare SNP	MAF<0.05
<i>NNAT</i>	20q11.23	protein coding	Rare SNP	MAF<0.05
<i>PEG3</i>	19q13.43	protein coding	Rare SNP	MAF<0.05
<i>SGCE</i>	7q21.3	protein coding	Rare SNP	MAF<0.05

Red colour labelled: SNP sites already identified.

To identify SNPs within imprinted genes in hESC, gDNA was extracted from both H1 and H7 hESCs, followed by PCR amplification using primers flanking regions containing the SNPs within the maternally imprinted genes. The amplified PCR products were sequenced to check the nucleotide composition at these SNP sites. If the sequencing results show two different nucleotides being equally detected at the SNP site, they indicate the existence of the SNP in the gene. That is a difference in nucleotides between the paternal and maternal alleles at that position. Conversely, if

only one nucleotide is detected at the site, it indicates that both paternal and maternal alleles of the gene contain the same nucleotide at that site.

The sequencing result revealed that the *SNRPN/SNURF*, *KCNQ1OT1*, and *MAGEL2* genes contain two peaks at their SNP sites in either H1 or H7 hESC lines (Fig 3.2A). Although the electropherogram labelled the sites containing two peaks as “N”, the identity of the nucleotides can be recognised from the colour, corresponding to the dbSNP database. For example, two peaks representing nucleotides T (red colour) and G (black) were detected at the SNP site of *SNRPN/SNURF* in the H1 hESC line, consistent with the allelic nucleotide information provided by the dbSNP database from NCBI. These results demonstrated the presence of allele-specific SNPs within *SNRPN/SNURF* and *KCNQ1OT1* in H1 hESCs and *MAGEL2* in H7 hESCs. By contrast, the maternally imprinted gene *NDN* only revealed a clear A and C peak at the SNP site in H1 and H7 hESC lines, respectively, indicating that SNP within *NDN* is absent in both hESC lines (Fig 3.2B). Interestingly, *SNRPN/SNURF*, *MAGEL2*, and *NDN* are all localised in the same cluster, which is regulated by the same SNURF-gDMR. Therefore, the SNPs of *SNRPN/SNURF* and *MAGEL2* could represent the imprinted status of SNURF-gDMR in H1 and H7 hESCs, respectively.

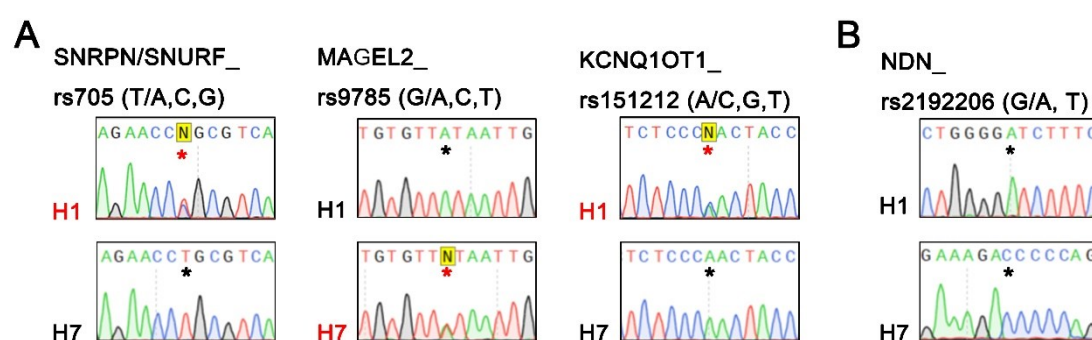


Figure 3.2 SNP identification in the indicated maternally imprinted genes in H1 and H7 hESCs

(A) Electropherogram of genomic DNA-PCR sequencing showing existence of SNP (red star) within the indicated maternally imprinted genes only in either H1 or H7 hESC lines. **(B)** Electropherogram showing no SNP in the SNP site (black star) of the *NDN* gene in H1 and H7 hESCs.

3.2.3. Identifying the maternal imprinting status of the SNP-containing imprinted genes in hESCs

Although the *PEG10*, *NAP1L5* and *ZDBF2* genes all contain SNPs in their protein-coding region in both H1 and H7 hESCs, their mRNA sequencing results revealed that only *PEG10* and *NAP1L5* exhibited monoallelic expression, i.e. active imprinted status, while *ZDBF* showed biallelic expression in H7 hESCs and partial biallelic expression in H1 hESCs (Zhi, 2021). These results agree with the previous reports that not all the human imprinted genes are imprinted in hESCs (Kim et al., 2007, Bar et al., 2017). Therefore, it is necessary to assess the imprinting status of the newly identified SNP-containing maternally imprinted genes in our cultured H1 and H7 hESCs.

To investigate the imprinting status of these newly identified SNP-containing maternally imprinted genes, RT-PCR was performed to amplify the mRNA regions containing SNP sites and the resulting RT-PCR products were sent for Sanger sequencing. If Sanger sequencing detects only one nucleotide at the SNP site, it demonstrates monoallelic expression of this gene, indicating its maternal imprinting status. Conversely, if both SNP nucleotides are detected at the SNP site, it represents that the gene is expressed from both the paternal and maternal alleles, indicating an absence of imprinted status. Sequencing results revealed that *SNRPN/SNURF* and *MAGEL2* showed a single nucleotide at their SNP sites in their SNP-containing H1 and H7 hESCs, respectively, demonstrating a monoallelic expression pattern of these genes (Fig 3.3A) and indicating that these genes were imprinted in the hESCs. Differently, *KCNQ1OT1* exhibited transcript expression from both alleles by A and C two peaks at the SNP site albeit the A peak being more predominant than the C peak, suggesting a partial imprinting in H1 hESCs (Fig 3.3B) (Morcos et al., 2011).

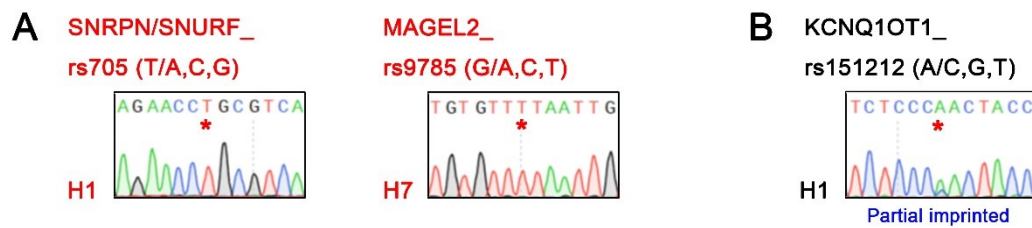


Figure 3.3 Allelic expression of indicated maternally imprinted genes in H1 and H7 hESCs by RT-PCR sequencing containing SNPs.

Electropherogram of RT-PCR fragments of maternally imprinted genes in hESC lines. Positions of SNPs are indicated by star label. **(A)** maternally imprinted genes are imprinted in hESC line. **(B)** maternally imprinted genes are partial imprinted in hESC line.

Taken together (Table 3.3), In addition to the previously identified *PEG10* and *NAP1L5* in both H1 and H7 hESCs, *SNRPN/SNURF* and *MAGEL2* genes also contain allele-specific SNPs and exhibit correct imprinting states in H1 and H7 hESCs, respectively. Consequently, four maternally imprinted genes (*PEG10*, *NAP1L5*, *SNRPN/SNURF*, *MAGEL2*) and the paternally imprinted gene *MEG3* can be used to assess maternal and paternal imprinting in future experiments.

Table 3.3 Summary of imprinting status of genes in H1 and H7 hESCs.

	Genes	Location	SNP	MAF	H1 hESCs	H7 hESCs
Maternally imprinted genes	<i>PEG10</i>	7q21.3	rs13073	0.268	✓	✓
	<i>NAP1L5</i>	4q21-q22	rs710834	0.35	✓	✓
	<i>MAGEL2</i>	15q11.2	rs9785	0.42		✓
	<i>SNRPN/SNURF</i>	15q11.2	rs705	0.447	✓	
	<i>KCNQ1OT1</i>	11p15.5	rs151212	0.33	partial	
	<i>ZDBF2</i>	2q33.3	rs7582864	0.485	partial	
Paternally imprinted genes	<i>MEG3</i>	14q32.2	rs11160608	0.438	partial	✓

“✓”: monoallelic expression

“Partial”: one allele expressed more than another.

3.3 DISCUSSION

In this chapter, I compared our RNA sequencing data from H1 hESCs with all identified imprinted genes in human from Tucci et al. (2019) to determine the expressed imprinted genes in H1 hESCs. The analysis revealed that 24 maternally and 22 paternally imprinted genes were expressed in H1 hESCs (Table 3.1). Cross-comparing these genes with hESC-expressing imprinted genes from two published datasets (Bar et al., 2017, Mai et al., 2018), indicated that 13 maternally imprinted genes and 4 paternally imprinted genes were expressed in our H1 hESCs and at least one of these two hESC studies showed these genes retained imprinting status in hESCs (Figure 3.1). However, 21 maternally imprinted genes, excluding *NAP1L5*, and 2 paternally imprinted genes were not expressed in our H1 hESCs (Figure 3.1A), although they were expressed and imprinted in the studies by Bar et al. (2017) and/or Mai et al. (2018). For example, maternally imprinted genes such as *IPW*, *DIRAS3* and *ZIM2*, and paternally imprinted genes like *KLF14* and *KCNQ1DN*, were absent in our H1 hESCs. Since hESCs are derived from embryos of different individuals with distinct genetic identities, their gene expression profiles are variable between different hESC lines. The *IPW* gene exhibits monoallelic expression in various hESC lines in multiple studies (Li et al., 2010b, Kim et al., 2007), but is undetectable in our H1 hESCs and also in H9 hESCs from RNA-seq data (Tang et al., 2015). Another possibility is that culture conditions can influence gene expression in hESCs. Comparison of RNA-seq data from H1 hESCs in MEF-CM condition (Wu et al., 2010) and commercial mTeSR medium (Lister et al., 2009) showed almost absent expression of *DIRAS3* and *ZIM2* in MEF-CM condition (7.3×10^{-7} of b-actin expression for both genes), but scanty expression in mTeSR medium (1.5×10^{-4} of b-actin expression for *DIRAS3*; 1.9×10^{-3} of b-actin expression for *ZIM2*). Therefore, these factors may affect the gene expression in hESCs, possibly including imprinted genes, which results in the absence of some hESCs-expressing maternally imprinted genes in the H1 hESC line.

In addition to previously identified *NAP1L5* and *PEG10* in both H1 and H7 hESCs, SNPs of *SNRPN/SNURF* and *KCNQ1OT1* were identified exclusively in H1 hESCs, while *MAGEL2* SNP was found in H7 hESCs (Figure 3.2). Given that H1 and H7 hESCs originate from the embryos of different individuals with distinct genetic identities, some SNPs are commonly undetectable in some hESC lines (Abeyta et al., 2004). Both *SNRPN/SNURF* and *MAGEL2* exhibited monoallelic expression in H1 and H7 hESCs, respectively, indicating that these genes were imprinted in their respective hESC lines (Figure 3.3A). In contrast, *KCNQ1OT1* showed unequally biallelic expression in H1 hESCs, suggesting partial loss of imprinting in our hESCs (Figure 3.3B). The loss of allele-specific expression is commonly observed in certain imprinted genes across different hESC lines. This phenomenon, reported in several publications, suggests epigenetic instability in these genes (Johannesson et al., 2014, Nazor et al., 2012, Bar et al., 2017). One potential explanation is that hESCs are derived by expanding individual undifferentiated colonies without clonal selection (Thomson et al., 1998). The loss of imprinting stability in a few single cells could confer a growth advantage under environmental stress, allowing these cells to dominate the population. For instance, abnormal biallelic expression of *KCNQ1OT1* reduces the expression of *CDKN1C*, a cyclin-dependent kinase inhibitor. This reduction releases cells into cell proliferation at a high frequency (Papulino et al., 2020). Therefore, increased *KCNQ1OT1* biallelic expression due to imprinting instability may provide a selective advantage during routine culture.

In summary, I have cross-compared three datasets on imprinted genes in human and hESCs with our RNA-seq data from H1 hESCs to identify hESC-expressing imprinted genes in H1 hESCs. In addition to the previously identified *PEG10* and *NAP1L5*, the genes *SNRPN/SNURF* and *MAGEL2* also contain allele-specific SNPs and exhibit correct imprinting status in H1 and H7 hESCs, respectively. Consequently, four maternally imprinted genes (*PEG10*, *NAP1L5*, *SNRPN/SNURF*, *MAGEL2*) and the

paternally imprinted gene *MEG3* can be used to assess maternal and paternal imprinting in later experiments.

Chapter 4

**Characterisation of NLRP7 expression in hESCs
during their naïve conversion**

4.1 INTRODUCTION

NLRP7, as a member of the rNLRPs, plays an important role in human reproduction and embryonic development, and its deficiency in women can result in BiCHM (Van den Veyver and Al-Hussaini, 2006). Investigation of the molar tissues collected from patients with defective *NLRP7* revealed that recessive mutations of *NLRP7* in the maternal genome correlate with the loss of maternal imprinting in the embryos, which is likely to account for BiCHM given its similar phenotype as that of androgenesis (Sanchez-Delgado et al., 2015). Since maternal imprinting is established in oogenesis and maintained during global demethylation in pre-implantation embryonic development, NLRP7 probably plays a vital role in the establishment and/or maintenance of maternal imprinting in oocyte maturation and embryonic development, respectively. However, it is largely unknown how NLRP7 regulates the establishment and maintenance of maternal imprinting in germ cells and early embryos, respectively. Since *NLRP7* is absent in the genome of common laboratory animals and human embryonic materials have very restricted availability (Tian et al., 2009), I thought that hESCs might be a useful *in vitro* model to explore the role of NLRP7 in the regulation of maternal imprinting. Thus, our laboratory has attempted to use hESCs to study NLRP7 on maternal imprinting, as hESCs are *in vitro* human materials that resemble closely to human embryonic tissues. In particular, I am interested in using the transition between primed and naïve states in hESCs as a model.

Routinely cultured hESCs, known as primed hESCs, resemble post-implantation epiblast cells which are hypermethylated and the ICRs on imprinted genes also exhibited differential methylation between maternal and paternal alleles to be DMRs, herein many of the imprinted genes exhibit a parental allele-specific expression pattern (Tucci et al., 2019). Interestingly, these primed hESCs can be converted into a naïve state by altering the culture medium, which closely resembles a pre-implantation epiblast with hypomethylation. Therefore, during this naïve conversion,

hypermethylated primed hESCs undergo a global DNA demethylation process to become hypomethylated naïve hESCs, even resulting in the loss of imprinted states in imprinted genes (Theunissen et al., 2014, Pastor et al., 2016). I think that this naïve conversion of hESCs provides us with a global demethylation model, which might be useful for us to study *NLRP7*.

In this project, I aimed to perform the naïve conversion of hESCs to recapitulate the global demethylation process and investigate the expression of *NLRP7* during this process, especially their relationship with the loss of imprinting.

Specifically, I aim to address the following objectives:

- To establish naïve hESCs in both H1 and H7 hESC lines and characterise these converted hESCs in terms of morphology, gene expression and imprinting status to confirm their naïve state
- To examine the expression and localisation of *NLRP7* in hESCs during naïve conversion

4.2 RESULTS

4.2.1. Conversion of hESCs to the naïve state using 5iLAF culture condition

Recently, various protocols have been developed to convert routinely cultured hESCs to the naïve state, exhibiting characteristics similar to cells in the ICM, in terms of morphology, transcription profile and epigenetic features (Nichols and Smith, 2009). However, genome-wide characterisation revealed that not all the methods can successfully achieve true conversion but the 5iLAF method has been validated by several laboratories (Liu et al., 2017). The 5iLAF method uses N2B27 as a base medium which is supplemented with MEKi, GSK3i, SRCi, bRAFi, ROCKi, LIF, Activin, and bFGF for converting primed hESCs to naïve hESCs (Theunissen et al., 2014). Surprisingly, Naïve hESCs converted by the 5iLAF method have revealed not only global DNA hypomethylation but also a reduction of DNA methylation at DMRs of imprinted genes, leading to a loss of genomic imprinting (Theunissen et al., 2014, Pastor et al., 2016).

Naïve conversion of hESCs using the 5iLAF method requires MEF-feeders, which were prepared one day before the naïve conversion, for cell survival. The next day, hESCs were dissociated into single cells by Accutase and subsequently plated onto the iMEF feeders in the presence of ROCKi and cultured in hESC culture medium (MEF-CM supplemented with bFGF). After two days, the medium was switched to the 5iLAF culture medium for daily medium refreshment (Fig 4.1A).

Under this 5iLAF culture condition, widespread cell death was clearly observed in H7 hESCs among the first three days. By day 4, hESC colonies began to condense, and by around day 8, a small number of dome-shaped naïve hESC-like colonies started to emerge (Fig 4.1B). The naïve conversion was complete around 15 days when most hESCs appeared the dome-shaped colonies and these naïve like hESCs can be

passaged for a long time culture. To validate whether the naïve conversion of hESC was successful, the gene expression of pluripotent and naïve markers in hESCs was analysed before and after the naïve conversion. Expression of the pluripotent markers (*NANOG* and *OCT4*) and naïve markers (*REX1*, *KLF2* and *PRDM14*) showed a significant upregulation after naïve conversion (Fig 4.1C). These results indicate the successful conversion of H7 hESCs to a naïve state using this method.

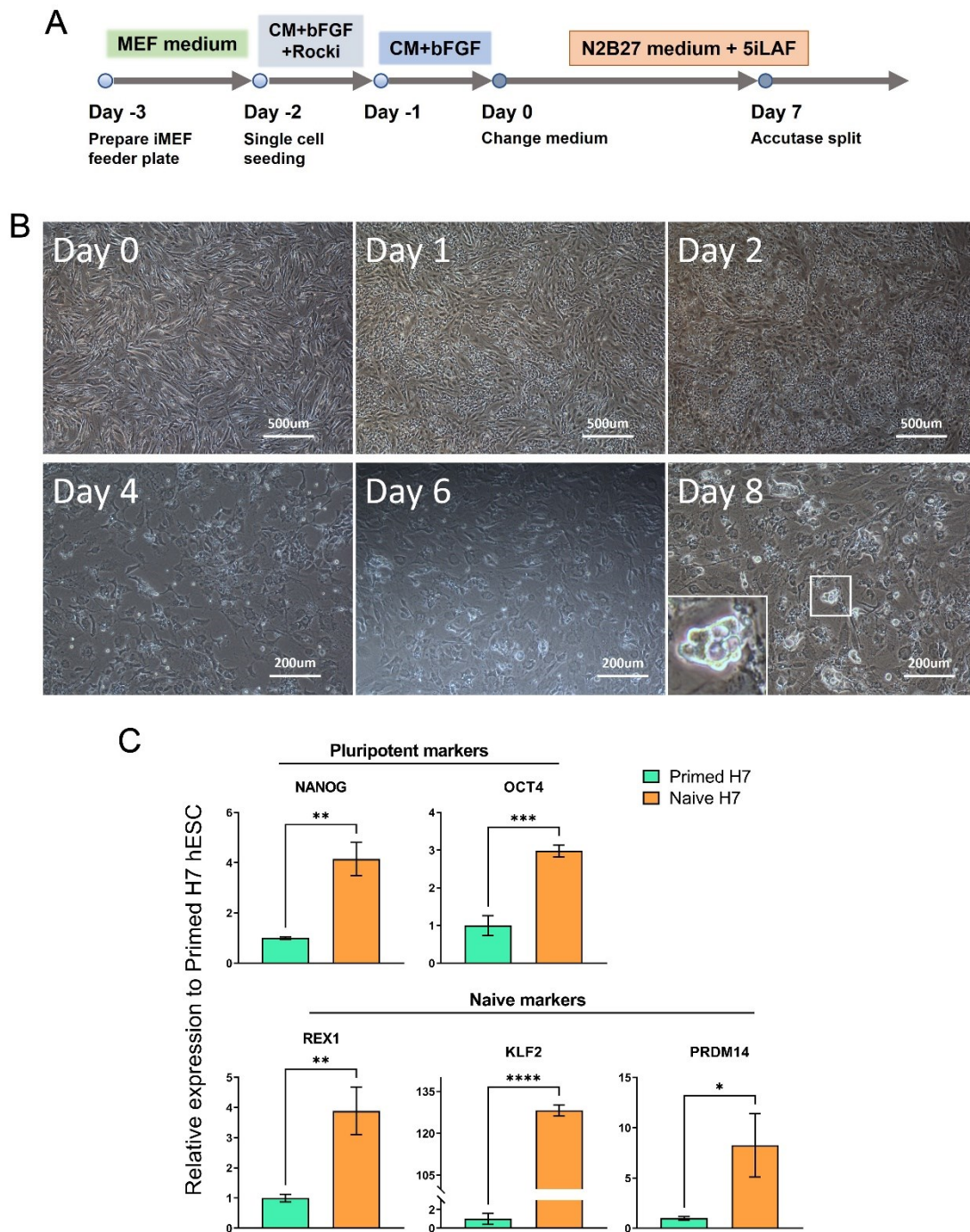


Figure 4.1 Conversion of hESCs to naïve state using 5iLAF method.

(A) Schematic depicting experimental procedure to convert primed hESCs to naïve hESCs using 5iLAF method. **(B)** Morphological changes in H7 hESCs during their naïve conversion; Days in conversion are indicated. **(C)** qRT-PCR analysis of pluripotent markers (*OCT4*, *NANOG*) and naïve markers (*REX1*, *KLF2*, *PRDM14*) in converted naïve H7 hESCs (passage 2) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tail unpaired t-test, $n = 3$ biological replicates).

4.2.2. Loss of imprinting in hESCs after naïve conversion

To investigate whether there are any changes in imprinting status after naïve conversion of hESCs, I examined the allelic expression of the imprinted genes that contain SNPs in H7 hESCs, as loss of imprinting is one of the key characteristics reported in naïve hESCs generated by the 5iLAF method (Pastor et al., 2016). RT-PCR crossing the SNP sites was performed in H7 hESCs before and after the naïve conversion and allelic-specific expression was visualised by Sanger sequencing of the RT-PCR products.

In H7 hESCs, both maternally imprinted genes, *PEG10*, *NAP1L5* and *MAGEL2*, and paternally imprinted gene *MEG3* contain SNPs in the genome and exhibited monoallelic expression in primed hESCs but became biallelic expression after naïve conversion, indicating these genes were imprinted in the primed H7 hESCs but lost their imprinting after the naïve conversion as previously reported (Fig 4.2A&B). This result demonstrates that both maternal and paternal imprinting are erased after naïve conversion in H7 hESCs, agreeing with previously published reports and confirming that my naïve conversion is successful. This finding also aligns with our previous observations that the methylation level of CpGs in DMRs of imprinted genes decreased after naïve conversion in hESCs (Zhi, 2021). Therefore, naïve conversion via the 5iLAF method indeed erases genomic imprinting, likely through the demethylation of DMRs in imprinted genes.

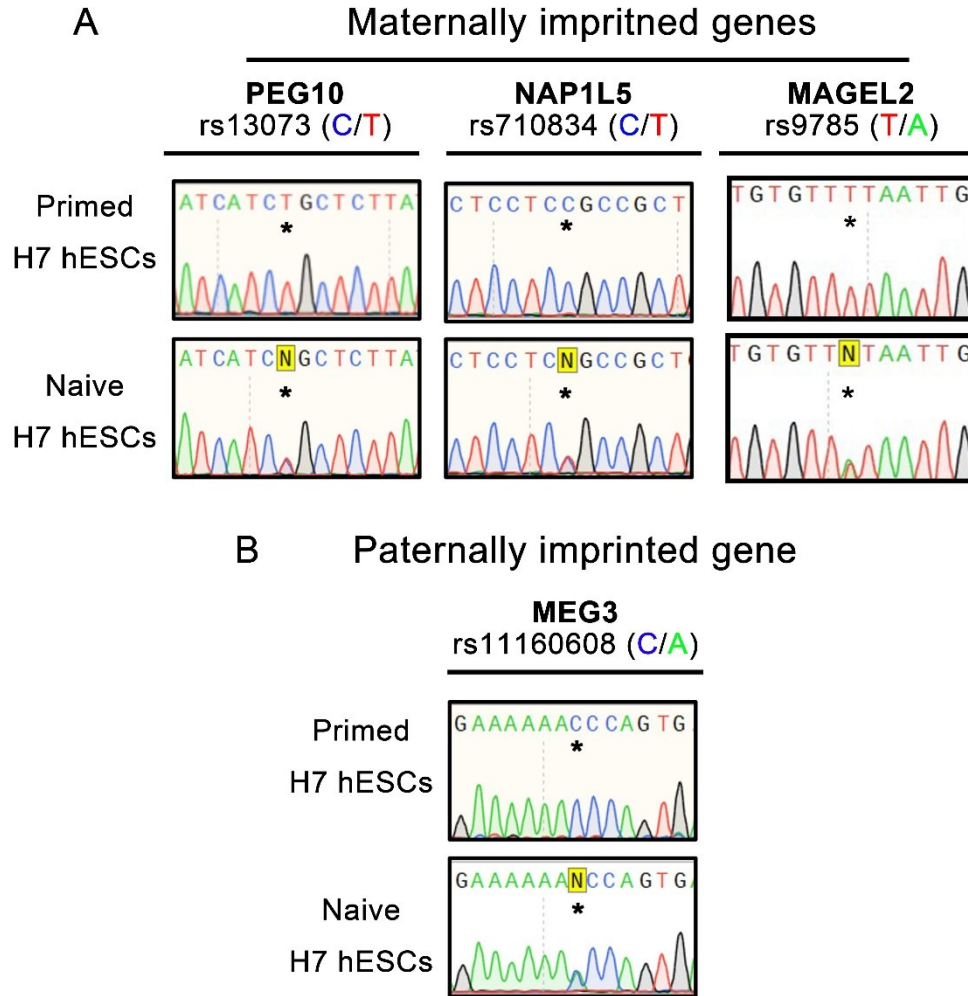


Figure 4.2 Alteration of imprinted genes from monoallelic to biallelic expression in H7 hESCs after naïve conversion.

Comparison of allelic expression of maternally imprinted genes (*PEG10*, *NAP1L5* and *MAGEL2*) (**A**) and paternally imprinted genes (*MEG3*) (**B**) in H7 hESCs between primed and naïve states (black star is the SNP site of the imprinted genes).

4.2.3. Differential dynamic in expression levels between maternally and paternally imprinted genes after naïve conversion

Although it has become evident that hESCs lose their genomic imprinting after naïve conversion in which imprinted genes change their expression pattern from monoallelic to biallelic expression, it remains unclear whether the expression levels of these imprinted genes are altered during this process. To investigate this, I compared the mRNA expression of paternally (*MEG3* and *H19*) and maternally (*PEG10*, *NAP1L5*, *SNRPN/SNURF*, *KCNQ1OT1*, and *MAGEL2*) imprinted genes in H7 hESCs before and after naïve conversion using qRT-PCR. Notably, the expression of paternally imprinted genes, *MEG3* and *H19*, exhibited significant increases, from 12 to over thousands of folds, following the naïve conversion in H7 hESCs (Fig 4.3A). However, the maternally imprinted genes (*PEG10*, *NAP1L5*, *SNRPN/SNURF*, *MAGEL2* and *KCNQ1OT1*) showed no significant changes in their expression after naïve conversion (Fig 4.3B). These results suggest that loss of imprinting leads to the upregulation of paternally imprinted genes but has no significant effects on maternally imprinted genes.

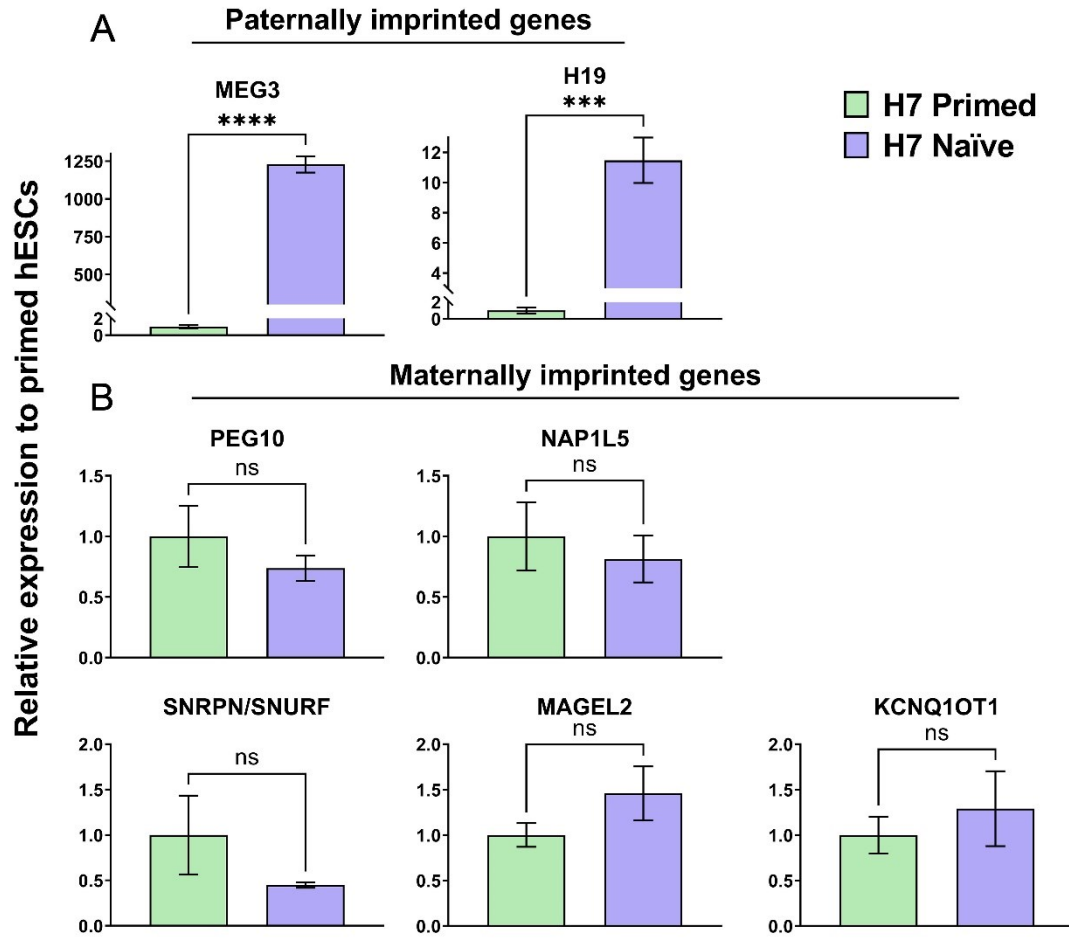


Figure 4.3 Distinct dynamic in expression levels between maternally and paternally imprinted genes in H7 hESCs after naïve conversion.

(A) Significant upregulation of paternally imprinted genes after naïve conversion. **(B)** Maternally imprinted genes exhibited variable changes in their expression levels. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tail unpaired t-test, $n=3$ biological replicates).

4.2.4. Dynamic expression of DNMTs and TETs during naïve conversion of hESCs

Although naïve conversion of hESCs is a global DNA demethylation process, it is not fully understood the underlying mechanism by which this demethylation takes place. To investigate this, I analysed dynamic expressions of DNA methyltransferases DNMTs and 5mC dioxygenase TETs during the naïve conversion of hESCs.

To gather this information, H7 hESCs were collected every two days during the 5iLAF conversion procedure for gene expression analysis. DNMT1, responsible for methylation maintenance in newly synthesized DNA after DNA replication, maintained its expression in the first two days of the conversion and then was decreased to 70% on day 6 before being upregulated to the initial level by day 10 (Fig 4.4A, upper panel). This result showed that DNMT1 had a transient downregulation during the naïve conversion process. Since DNMT1 is responsible for DNA methylation maintenance, its reduction may contribute to global DNA demethylation by being incapable of methylating newly replicated DNA in cell divisions during naïve conversion.

The expression levels of *de novo* methyltransferases DNMT3A/3B/3L were generally elevated during naïve conversion, but their dynamic trends were variable. DNMT3A showed a progressive increase with two waves of upregulation: the first increase to 1.8-fold occurred on the initial two days and the second started on day 6 (1.7-fold increase), peaking at 2.5-fold by day 8 (Fig 4.4A, lower panel left). DNMT3B exhibited a transient spike to 3.1-fold on day 2, followed by a drop to the initial level on day 6, and then a gradual increase to 1.7-fold by day 10 (Fig 4.4A, lower panel middle). Interestingly, DNMT3L exhibited a continuous upregulation with a surge from day 6 and reached to approximately 1400-fold increase by day 10 (Fig 4.4A, lower panel right). These results suggest that *de novo* methyltransferases DNMT3 group were all upregulated during naïve conversion, albeit to a variable extent, indicating that *de novo*

methylation may also occur during naïve conversion of hESCs, but further experiments are required to support this statement.

The 5mC dioxygenase TET proteins were responsible for active demethylation (Wu and Zhang, 2017). Following naïve conversion, the expression levels of all three TET proteins increased significantly. However, the increase of TET1 expression fluctuated with two waves and finally increased over 4-folds (Fig 4.4B left). In contrast, TET2 exhibited a robust and continuously increase throughout the naïve conversion process, being upregulated 18-fold by day 6 and approximately 55-fold by day 10 (Fig 4.4B middle). Interestingly, TET3 showed a similar expression pattern as that of DNMT3B, which exhibited a transient spike to 30-fold at day 2 followed by a gradual reduction by day 6 to 9-fold. After that, its expression fluctuated with an upregulation to 15-fold by day 8, then reduced to around 9-fold thereafter (Fig 4.4B right). As all TET genes showed varying increases during naïve conversion, the upregulation of TETs might be a contributor to the global demethylation process in naïve conversion of hESCs.

Overall, my results were consistent with our preliminary data showing that all DNMTs and TETs were increased in hESCs after naïve conversion (Zhi, 2021). These results suggest that all DNMTs and TETs, except for DNMT1, were upregulated during naïve conversion of hESCs to variable extent, indicating that the reduction of DNMT1 and upregulation of TETs were associated with the global DNA demethylation during naïve conversion of hESCs. In addition, the elevated DNMT3 group demonstrated that *de novo* methylation also occurred when the DNA methylation was globally erased.

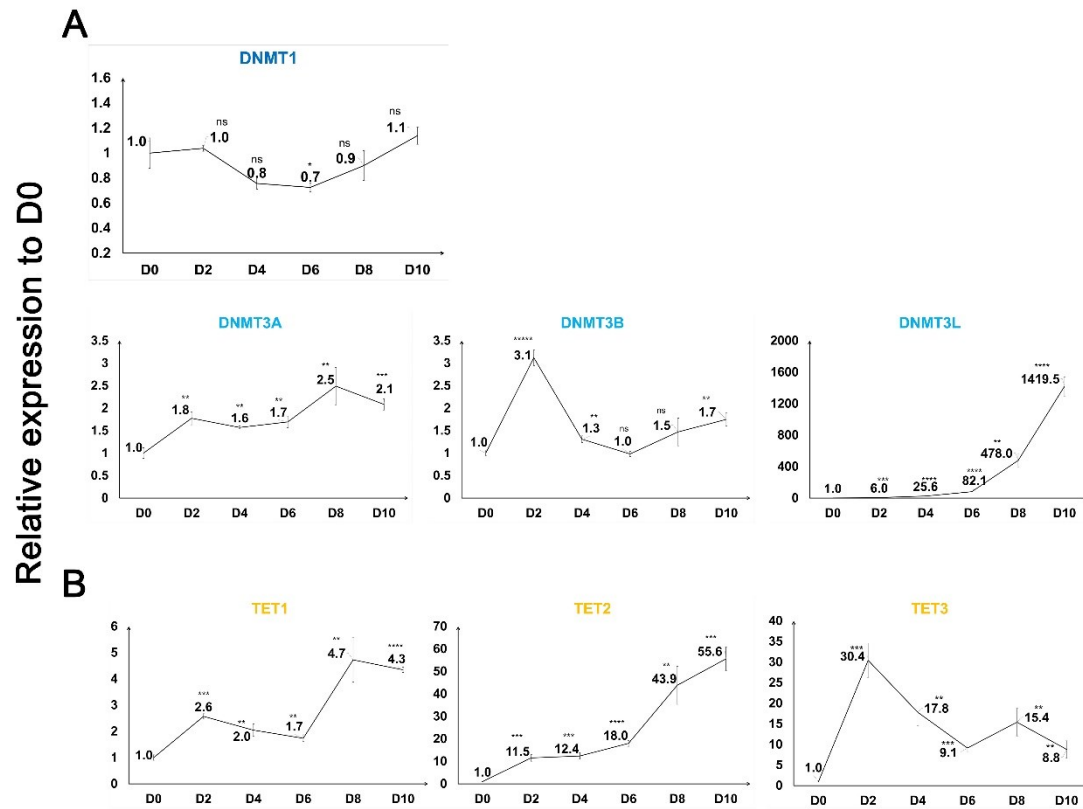


Figure 4.4 Dynamic expression of DNMTs and TETs during naïve conversion of H7 hESCs.

Dynamic expression of **(A)** indicated DNMT genes and **(B)** TET genes during naïve conversion by RT-qPCR. Data are presented as mean $\pm$ SD from three independent conversion experiments ($n = 3$) with the mean being shown. The statistical analysis was performed using One-way ANOVA and every value on each day was compared with value on D0 in each gene group *, ** and *** represent $p < 0.05$, 0.01 and 0.001

4.2.5. Dramatic upregulation of NLRP7 in hESCs after naïve conversion

In order to explore whether NLRP7 has any function in the regulation of maternal imprinting during this global demethylation process, I first examined NLRP7 expression in H7 primed and naïve hESCs. qRT-PCR revealed that *NLRP7* transcript was expressed at an extremely low level in primed hESCs but was dramatically increased approximately 3000 folds after naïve conversion complete (Fig 4.5A). This upregulation in expression after naïve conversion was very reproducible and was also supported at the protein level by western blot in which NLRP7 was barely detectable in primed hESCs but was abundant in naïve hESCs (Fig 4.5B).

Further analysis of the dynamic NLRP7 expression during naïve conversion revealed that dramatic upregulation of NLRP7 was a relatively late event. Although it started early in the conversion and gradually increased on the initial days, the exponential upregulation of NLRP7 only began on day 6 (53-fold increase), reaching a 283-fold increase by day 8 and approximately 1500-fold increase by day 10 (Fig 4.5C). Since NLRP7 expression was extremely low in primed hESCs, it remained at a low level during the initial days of naïve conversion despite several-fold increases. Therefore, a functional level of NLRP7 may only be reached at the later stages of naïve conversion. These results suggest that the NLRP7 level at the initial stage of naïve conversion might be too low to be functional during the global demethylation occurring in the naïve conversion.

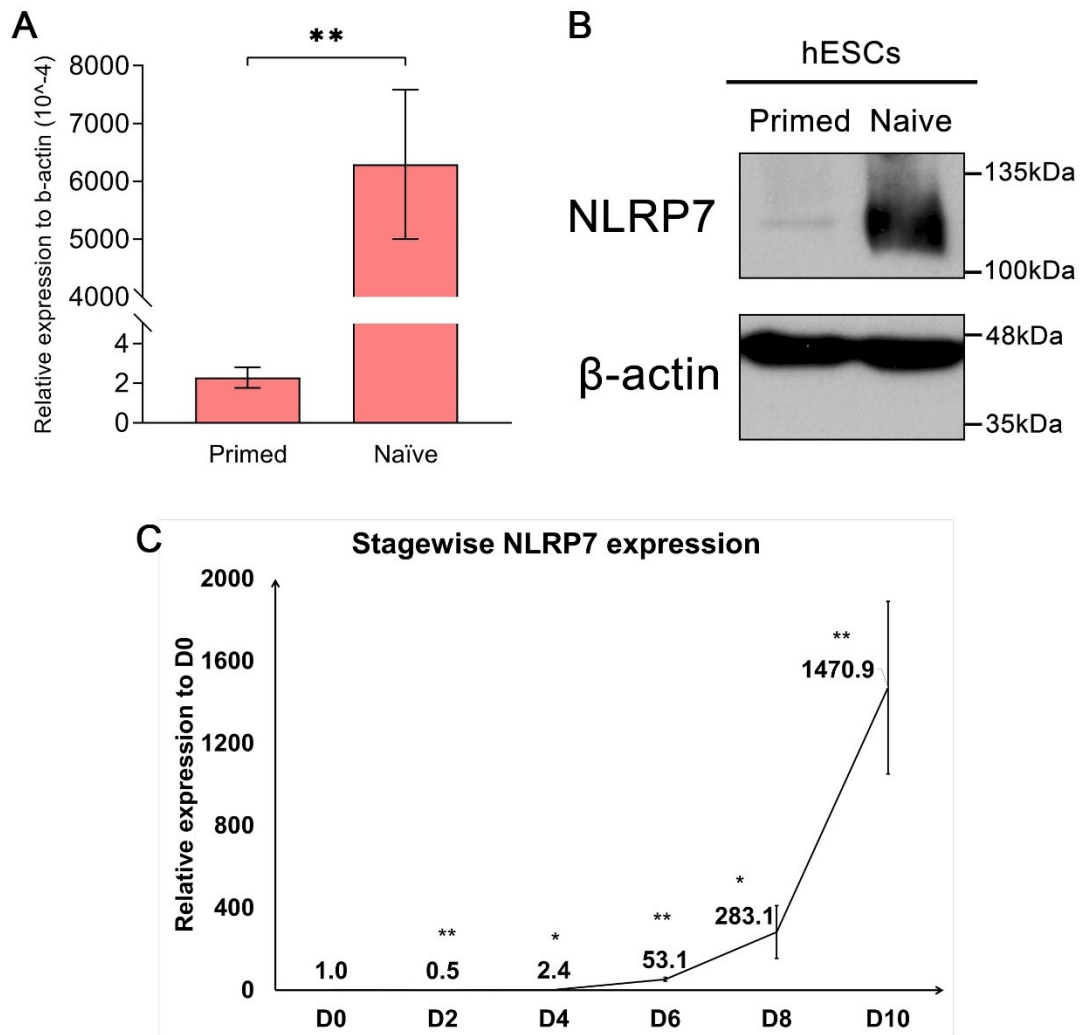


Figure 4.5 Expression analysis of NLRP7 in primed and naïve hESCs.

(A) Endogenous NLRP7 expression in primed and naïve hESCs by qRT-PCR. (B) Western blot analysis comparing the NLRP7 expression level in primed and naïve hESCs. (C) RT-qPCR analysis of NLRP7 mRNA expression during naïve conversion. In all the RT-qPCR, data are presented as mean $\pm$ SD from three independent conversion experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tail unpaired t-test, $n = 3$ biological replicates).

4.2.6. High expression of NLRP7 is not limited to cytoplasmic and subcortical regions in naïve hESCs

NLRP7 protein has been found to be predominantly located in the subcortical region of human oocytes and preimplantation embryonic cells but it is difficult to completely exclude its expression in the nucleus (Akoury et al., 2015). If NLRP7 is indeed restricted to the subcortical region of the cells, how can it regulate maternal imprinting? Therefore, we propose that NLRP7 may not be exclusively expressed in the subcortical region of a cell. The previous report supports our concept and shows that ectopic overexpression of NLRP7 in HEK293T cells exhibited its expression in the nuclear compartment in addition to the cytoplasmic region (Mahadevan et al., 2014). However, this nuclear localization has only been observed in an artificial setting by NLRP7 overexpression, which does not represent physiological conditions. Therefore, it is necessary to investigate the subcellular localisation of NLRP7 in naïve hESCs given its high expression levels.

The immunostaining showed that NLRP7 protein was undetected in primed hESCs (Fig 4.6A) but exhibited strong signals in naïve hESCs (Fig 4.6B) This is consistent with its mRNA expression by qRT-PCR and protein expression by western blotting (Fig 4.5 A&B). While the NLRP7 protein was predominantly localised in the cytoplasm (Fig 4.6Bi), and a small amount of NLRP7 was also detected in the nucleus (Fig 4.6Bii). The nuclear localisation of the NLRP7 protein in naïve hESCs is supported by immunoblotting on cell cytoplasmic and nuclear fractions separately, which showed positive signals in the nuclear fraction although the majority of NLRP7 residing in the cytoplasm (Zhi, 2021). Together, both our data and the published report (Mahadevan et al., 2014) demonstrate that NLRP7 protein can be located in the nucleus when expressed at high levels. Therefore, it is possible that the nuclear localised NLRP7 may be in close contact with genomic DNA and affect its expression.

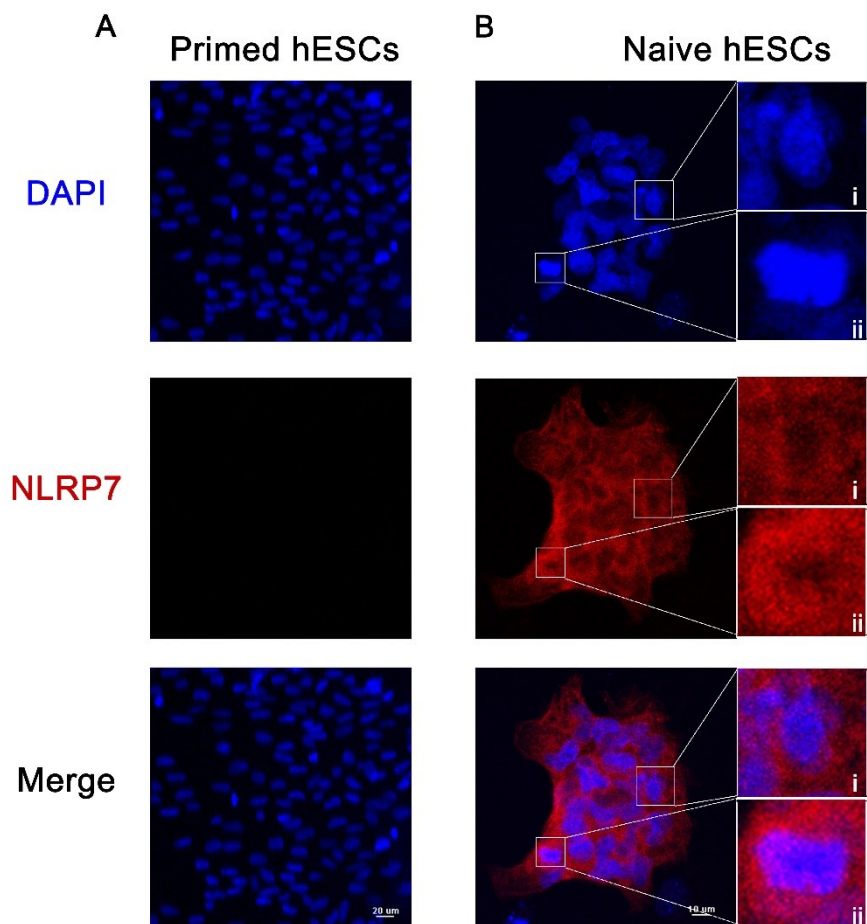


Figure 4.6 The NLRP7 protein localisation in primed & naïve H7 hESCs.

The immunostaining of NLRP7 in primed **(A)** & naïve **(B)** hESCs including the close inspection images which were the larger image of selected block region **(i & ii)**. Blue colour is DAPI representing nucleus; Red colour is NLRP7 protein.

4.3 DISCUSSION

In this chapter, I have successfully converted H7 hESCs to the naïve state using the 5iLAF method. Following naïve conversion, the hESCs acquired several typical characteristics of naïve ESCs, including changes in morphology, transcription profiles, and epigenetic signatures. Initially, hESCs displayed a large, flattened colony morphology. After conversion, the cells formed small, dome-shaped colonies, a morphology similar to mESCs (De Los Angeles et al., 2012) (Fig 4.1B). Gene expression analysis showed elevated levels of pluripotent markers (*NANOG* and *OCT4*) and naïve markers (*REX1*, *KLF2* and *PRDM14*), confirming the acquisition of the naïve state in H7 hESCs after conversion (Fig 4.1C). The upregulation of *NANOG* and *KLF2* is crucial for a successful naïve conversion, as even a short-time overexpression of these transgenes in hESCs can induce the transition from primed to naïve state under 2iL/PKCi conditions (Takashima et al., 2014). Moreover, H7 hESCs transitioned from monoallelic expression to biallelic expression of imprinted genes after naïve conversion, indicating a loss of imprinting (Fig 4.2A&B). This loss of imprinting status is consistent with the previous research showing that naïve hESCs exhibit a hypomethylated state similar to cells from the ICM. However, unlike ICM cells, which maintain genomic imprinting, naïve hESCs lose this imprinting. (Pastor et al., 2016). Thus, H7 hESCs retained the characteristics of naïve ESCs after naïve conversion. Due to some technical issues, the naïve conversion of H1 hESCs showed a transitional status, characterized by a high proportion of spontaneously differentiated cells and reduced expression levels of pluripotent and naïve markers. Therefore, the naïve conversion of H1 hESCs should be conducted in the future to eliminate the bias of cell line identity.

Interestingly, the expression levels of paternally imprinted genes were significantly elevated, more than doubled, in naïve hESCs resulting from loss of imprinting (Fig 4.3A), whereas maternally imprinted genes showed no significant changes in their

expression levels (Fig 4.3B). These results are consistent with the RNA-seq data from published studies, which indicates a dramatic upregulation of paternally imprinted genes and relatively constant expression levels of maternally imprinted genes during naïve conversion of hESCs (Pastor et al., 2016, Bi et al., 2022). The upregulated expressions of paternally imprinted genes likely resemble transcriptional bursting, a phenomenon that describes gene transcription showing intermittent and stochastic bursts rather than at a constant rate (Lipniacki et al., 2006). Recent research found transcriptional bursting was positively correlated with chromatin accessibility, which is highly increased in hESCs after naïve conversion (Ayyamperumal et al., 2024, Theunissen et al., 2014). Maternally imprinted genes during naïve conversion may exhibit behaviours similar to dosage compensation, a process that balances the expression of sex-linked and autosomal genes in the heterogametic sex (Brockdorff and Turner, 2015). Since dosage compensation in the X chromosome is regulated by lncRNA *XIST* to randomly inactivate one of the two X chromosomes, maternally imprinted genes probably have a similar trans-regulatory mechanism to equalise their expression after naïve conversion (Sahakyan et al., 2018). The differential expression changes between paternally and maternally imprinted genes after primed-to-naïve transition in hESCs may indicate distinct transcriptional regulatory mechanisms governing these genes respectively. However, since this experiment was conducted only in one single cell line, potential biases related to cell line identity should be addressed by repeating this study in another hESC line to validate these findings in the future.

During naïve conversion, all DNMTs and TETs, except for DNMT1, were significantly increased to variable extents during naïve conversion process. In comparison with published RNA-seq data from naïve hESCs using the 5iLAF method (Pastor et al., 2016, Bi et al., 2022), my results showed divergent expression patterns in DNMT1 & 3B, though DNMT3A and TETs expression were consistent with published data (Fig

4.4). Both studies showed that DNMT1 was reduced by half in both mRNA and protein levels in naïve hESCs, while my results only showed a transient 30% reduction (Fig 4.4A upper panel). Moreover, DNMT3B was shown to be reduced to 10% of that in primed hESCs after naïve conversion in other studies, in contrast to an overall increase in my study (Fig 4.4A, lower panel middle). These differences might be due to different cells being used. Their studies used purified naïve hESCs sorted by naïve specific surface markers (Pastor et al., 2016) or transgenes driven by naïve specific OCT4 promoter (Bi et al., 2022), while I used the unpurified cell population which may contain other cell types in addition to naïve hESCs. This may affect the gene expression.

DNMT1 which is responsible for DNA methylation maintenance, showed a transient downregulation and return to the initial level throughout the process of naïve conversion (Fig 4.4A upper panel). Given that the downregulation of DNMT1 is the driving force for the passive mechanism of DNA demethylation, mediated by DNA replication-dependent 5mC dilution (Kagiyada et al., 2013), it is plausible that reduced expression of DNMT1 could induce the passive demethylation during naïve conversion. Although there is no relevant data showing the percentage reduction of DNMT1 expression could trigger passive demethylation, some data exhibited the half reduction of DNMT1 was sufficient to induce passive demethylation in PGC development and naïve conversion of hESCs (Tang et al., 2015, Guo et al., 2015, Pastor et al., 2016). Therefore, even a 30% reduction in DNMT1 expression has the possibility to trigger passive demethylation during naïve conversion of hESCs. All TETs expressions were significantly increased during naïve conversion. Since all TETs can oxidase 5mC to 5hmC and following oxidative products (Tahiliani et al., 2009, Ito et al., 2010, Ito et al., 2011), active demethylation could also contribute to the global demethylation process in naïve conversion. Surprisingly, TET3, responsible for the active demethylation during preimplantation embryonic development, exhibits a similar expression pattern to the naïve conversion process (Fig 4.4B right), showing a dramatic upregulation post-

fertilisation and reduced after the 2-cell stage, even absent after 8-cell stage (Yan et al., 2013, Petropoulos et al., 2016). Given that expression patterns of TET1 and TET2 are continuously increased, with transient high expression of TET3 during naïve conversion, the upregulation of all three TETs contributes to the machinery of active demethylation during naïve conversion possibly. Interestingly, continually increased TET2 was shown to maintain a hypomethylated status on *NANOG* promoter in hESCs, activating the high expression of *NANOG* to maintain the pluripotency (Langlois et al., 2014). Since upregulation of *NANOG* could trigger the naïve conversion process (Takashima et al., 2014), a high level of TET2 is probably not only responsible for active demethylation but also induces the naïve conversion in hESCs as well. Additionally, all three *de novo* methyltransferases DNMT3A/3B/3L were upregulated during naïve conversion with variable tendency. Although DNMT3L was dramatically upregulated at the late stages of naïve conversion (Fig 4.4A, lower panel right), the lack of an active catalytic domain hindered its *de novo* methylation function (Li and Zhang, 2014). DNMT3A showed a slight increase (Fig 4.4A, lower panel left), while DNMT3B exhibited a dramatic increase at initial days but repressed to the initial level during naïve conversion (Fig 4.4A lower lane, middle). This result indicates that *de novo* methylation may play a role during the naïve conversion process but to a lesser extent.

NLRP7 exhibits a gradual increase at the initial stages of naïve conversion in hESCs, reaching approximately a 1500-fold increase on day 10 during naïve conversion of hESCs (Fig 4.5C). However, despite this substantial increase at the later stages, the initial expression level in primed hESCs was extremely low, with only 0.02% of β -actin levels (Fig 4.5A). This is much lower than the lowest NLRP7 expression detected during human embryonic development, which is 1% of β -actin at the morula stage (Gkoutela et al., 2015). Even with several-fold increases during the initial stages of naïve conversion, the expression level of NLRP7 may still be too low to perform its function effectively. Therefore, functional levels of NLRP7 might only be reached in the

later stages of naïve conversion when global DNA demethylation has largely occurred or even completed. It is likely that the dramatic upregulation of NLRP7 might be a result of global demethylation-induced genome-wide hypomethylation. Unfortunately, I did not perform proper dot blot experiments with an antibody against 5mC to monitor the dynamic changes in DNA methylation levels during naïve conversion and its relationship with NLRP7 expression due to time restraints. These experiments could provide additional information to illustrate the relationship between DNA methylation and NLRP7 expression.

NLRP7 is well characterised by its cytoplasm and subcortical localisation in oocytes and embryonic cells (Akoury et al., 2015). However, my results indicate that abundant expression of NLRP7 is not only present in the cytoplasm (Fig 4.6Bi), but also detected in the nucleus and juxtanuclear regions (Fig 4.6Bii). The nuclear localization of NLRP7 has also been reported in other studies. For instance, NLRP7 has been shown to interact with the transcription factor Yin and Yang 1 (YY1) predominantly in the cytoplasm, with a smaller proportion in the nucleus in HEK293T cells (Mahadevan et al., 2014). Additionally, a recent study found that in endometrial stromal cells, NLRP7 proteins accumulate in the juxtanuclear region before *in vitro* decidualization but translocate into the nucleus following decidualization (Huang et al., 2017). From a protein structure perspective, the NACHT domain of NLRP7 contains a nuclear localization signal, which may facilitate its translocation into the nucleus (Reddy et al., 2016, Li et al., 2019). These findings suggest that NLRP7 can be detected in the nuclear region of cells when its expression is sufficiently high. Therefore, NLRP7 may have the potential to regulate maternal imprinting during global demethylation, though further experiments are needed to support this hypothesis.

In this chapter, I have established naïve hESCs using the 5iLAF method in both H1 and H7 hESC lines. Following naïve conversion, the epigenetic and gene expression profiles have a dramatic change. NLRP7 expression was low at the early stage of naïve

conversion but eventually increased significantly in naïve hESCs. Additionally, NLRP7 was predominantly localized in the cytoplasm, with a portion also detected in the nucleus.

Chapter 5

**Overexpressing NLRP7 in hESCs extenuates the
loss of maternal imprinting in hESCs after naïve
conversion**

5.1 INTRODUCTION

Genomic DNA methylation is a dynamic process during early embryonic development, playing a pivotal role in establishing and retaining genomic imprinting. Two critical rounds of global DNA demethylation and re-methylation are essential during this process for proper embryonic development and genomic imprinting. One of these demethylation waves occurs in PGCs during their migration to precursor gonads, in which nearly all the methylated DNA CpGs in their genome are erased by the end of this demethylation, including methylation on the ICR of imprinted genes (Tang et al., 2015). During germ cell differentiation and maturation in the gonads, sperms and oocytes acquire distinct methylation patterns in the testis and ovary, respectively (Stewart et al., 2016). The other wave of global demethylation occurs soon after fertilization, with paternal and maternal DNA methylation being erased at different rates due to distinct mechanisms (Zhu et al., 2018, Guo et al., 2014, White et al., 2016). Remarkably, unlike the demethylation in PGCs, the methylation on the ICRs of imprinted genes displays resistance to this round of demethylation, maintaining their parental-specific methylation and imprinting status (Smallwood and Kelsey, 2012).

Single-cell RNA-seq analyses from two independent research have revealed that *NLRP7* transcript is scarcely detected in matured oocytes and zygotes until the late morula stage and peaked at blastocyst stage (Yan et al., 2013, Petropoulos et al., 2016). However, the *NLRP7* protein is abundant in matured oocytes which is synthesised during oocyte maturation (Akoury et al., 2015). This high level of *NLRP7* expression pattern persists in the embryonic cells throughout the preimplantation embryos (Akoury et al., 2015). Furthermore, despite *NLRP7* being highly expressed in the ICM and preimplantation epiblast in the blastocyst, its expression could not be detected in post-implantation epiblast and the emerging PGCs during gastrulation in the human embryo by single-cell RNA-seq (Yan et al., 2013, Petropoulos et al., 2016, Tyser et al., 2020). These *in vivo* data are consistent with my results in Chapter 4

showing that NLRP7 is expressed at very low levels in primed hESCs but highly upregulated in naïve hESCs, given that primed and naïve hESCs resemble closely the pre- and post-implantation epiblasts, respectively.

Taken together, I noticed the following phenomena: 1) Human PGCs and zygotes having distinct NLRP7 expression levels, low and high, respectively, exhibit differential responses to global demethylation on imprinted genes; 2) Primed hESCs with low NLRP7 expression lose their imprinting status after naïve conversion which involves considerable global demethylation; 3) Women with recessive NLRP7 mutation have no normal NLRP7 in their oocytes show loss of maternal imprinting in embryonic tissues due to loss of methylation on the maternal ICRs (Sanchez-Delgado et al., 2015). Therefore, I asked whether the high-level expression of NLRP7 has a function to protect maternally imprinted genes from global demethylation on their ICRs.

To address this question, I aimed to ectopically overexpress NLRP7 in primed hESCs and to investigate its effect on maternally imprinted genes after naïve conversion. Specifically, this chapter focuses on the following objectives:

- To establish H1 and H7 hESC lines that overexpress NLRP7.
- To investigate whether this ectopic overexpression has a protective role for imprinted genes, particularly maternally imprinted genes, from the global demethylation process during naïve conversion of hESCs.
- To explore the underlying mechanisms of NLRP7 in protecting maternally imprinted genes during naïve conversion of hESCs.

5.2 RESULTS

5.2.1. Establishment of hESC lines that overexpress NLRP7

To generate hESC lines with constitutive NLRP7 expression, I used a previously constructed lentiviral expression vector in our lab, in which puromycin and HA-tagged NLRP7 cDNA are linked by 2A sequence, and their expression is under the control of a strong constitutive promoter (Fig 5.1A). The ectopic expression of NLRP7 was achieved by lentiviral transduction after transfection of lentivector carrying the NLRP7 together with the other two lentiviral helper vectors, a 2nd lentiviral transduction system, in HEK293T cells to generate replication-inactive lentiviral particles. These particles were subsequently transduced into the H1 and H7 hESCs followed by puromycin selection. to establish stable NLRP7 overexpression (NLRP7-OE) lines in both H1 and H7 hESCs (details see Chapter 2 -Materials and Methods).

Survival hESCs were tested for NLRP7 expression and compared with untransduced control hESCs. NLRP7 mRNA levels showed approximately a 500- and 100-fold increase in H1 and H7 NLRP7-OE hESCs, respectively, compared to their untransduced controls (Fig 5.1B). Similarly, transgenic NLRP7 proteins also showed strong signals in NLRP7-OE hESCs by immunoblotting with both NLRP7 and HA antibodies, whereas no signal was detected in untransduced control hESCs (Fig 5.1C). Noticeably, more than one band were observed in the immunoblotting, which might be resulted from incomplete cleavage of the 2A peptide, generating a fusion protein of PAC (puromycin acetyltransferase) (21.54 kDa) and HA-NLRP7 and/or post-translational modification of the NLRP7 protein. These results confirm that I have successfully established H1 and H7 hESC lines with constitutive NLRP7 expression.

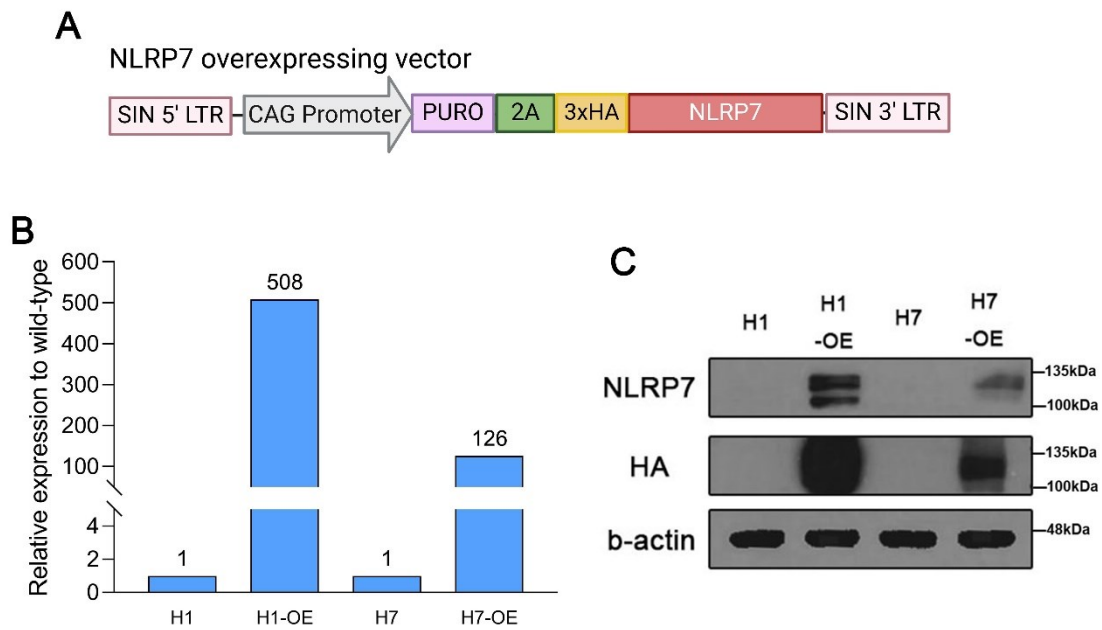


Figure 5.1 Overexpressing HA-tagged NLRP7 cDNA in H1 and H7 hESCs via lentiviral transduction.

(A) Schematic showing the construction of transgenes in the lentiviral expression vector. **(B)** RT-qPCR showing expression of NLRP7 transcripts in the indicated hESCs. **(C)** NLRP7 protein expression in NLRP7-OE and control hESCs (H1 and H7) by immunoblotting.

5.2.2. NLRP7 overexpression in hESCs shows no clear effect on pluripotent gene expression

The hESC colonies of H7 NLRP7-OE hESCs exhibited similar morphology to that of control hESCs, which were tightly packed at the centre of the colonies with a loose boundary, and the cells inside the colonies revealed a large nucleus-to-cytoplasm ratio (Fig 5.2A). To investigate whether ectopic NLRP7 affects the expressions of pluripotent and naïve markers to influence the following naïve conversion experiment, the qRT-PCR was conducted in control and NLRP7-OE H7 hESCs. The expression of pluripotent (*NANOG* and *OCT4*) and naïve markers (*REX1* and *KLF2*) was comparable to that of control hESCs (Fig 5.2B), despite a significant 0.5-fold increase of *REX1* in H7 NLRP7-OE hESCs which was acceptable due to its fluctuating expression in primed hESCs (Pastor et al., 2016, Bi et al., 2022). In addition, the expression levels of DNMTs and TETs in H7 NLRP7-OE hESCs showed no significant differences (Fig 5.2C), indicating that the DNA methylation should not be affected by ectopic NLRP7 expression. In fact, the following experiment measuring the methylation level revealed that the DNA methylation level was not disturbed in NLRP7-OE hESCs (Fig 5.8 & 5.9). Therefore, these results ascertain that NLRP7-OE hESC lines were successfully established and the ectopic NLRP7 expression does not significantly impact cell morphology, pluripotency, and DNA methylation levels in hESCs.

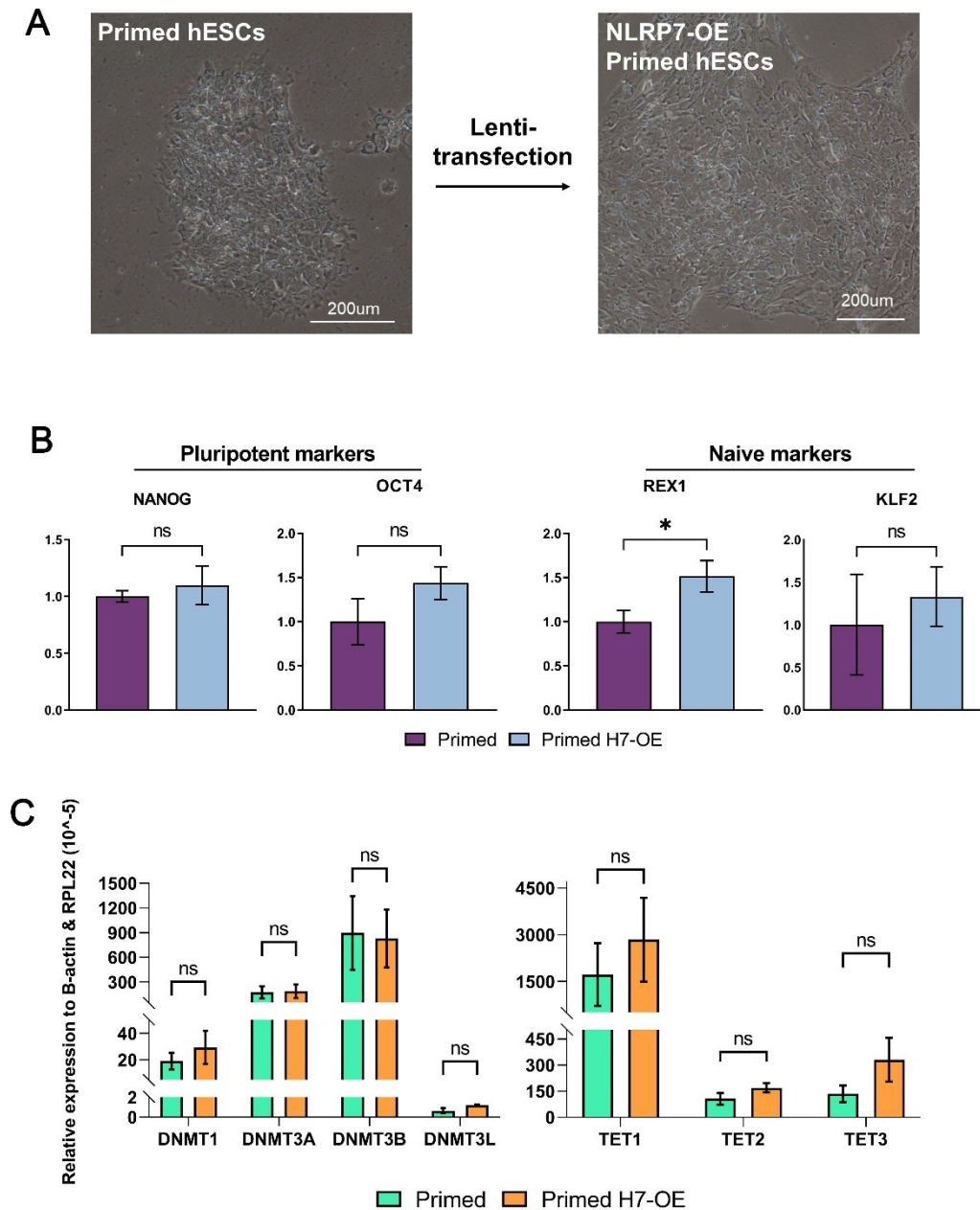


Figure 5.2 Ectopic NLRP7 does not affect characteristics of hESCs.

(A) Morphology of H7 hESCs before and after NLRP7 overexpression by lentivirus transduction. **(B)** The expression level of pluripotent and naïve markers in both H7 and H7-OE hESCs by qRT-PCR. **(C)** Analysis of DNMTs and TETs expression in both H7 and H7-OE hESCs by qRT-PCR. Data are presented as mean $\pm$ SD of 3 biological samples (* p < 0.05, ** p < 0.01, *** p < 0.001 by two-tail unpaired t-test, n = 3).

5.2.3. NLRP7-OE hESCs exhibited significant upregulation of naïve markers after naïve conversion

Following the establishment of NLRP7-OE hESC lines, I asked whether NLRP7-overexpression affects the naïve conversion of hESCs. For this, H7-OE hESCs were converted to the naïve state by the 5iLAF approach as previously described. After 10 days of culture in 5iLAF medium, H7 NLRP7-OE hESCs exhibited domed-shape colonies, which were similar to those observed during the naïve conversion of control H7 hESCs in both the timeline and morphology (Fig 5.3A). As control, both pluripotent markers and naïve markers were significantly elevated after naïve conversion in H7 hESCs. In NLRP7-OE H7 hESCs, RT-qPCR analysis showed that both pluripotent markers (*NANOG* and *OCT4*) exhibited more than 2-fold increase in their expression after naïve conversion compared to primed hESCs and that the increases were even more considerable for naïve markers (*REX1*, *KLF2* and *PRDM14*) which revealed at least 10-fold upregulation after naïve conversion (Fig 5.3B). Although in the primed state, NLRP7-OE H7 hESCs showed comparable or lower expression level in both pluripotent markers and naïve markers, the naïve NLRP7-OE H7 hESC showed higher expression level of naïve markers, compared to the WT H7 hESCs in naïve state. These results indicate that the NLRP7 overexpression has no clear effect on their naïve conversion and NLRP7-OE hESCs can be successfully converted to the naïve state. Therefore, these results suggest that the naïve conversion in H7-OE hESC lines was successful and that ectopic NLRP7 expression does not appear to influence the gene expression profiles of naïve hESCs in the H7-OE hESC line.

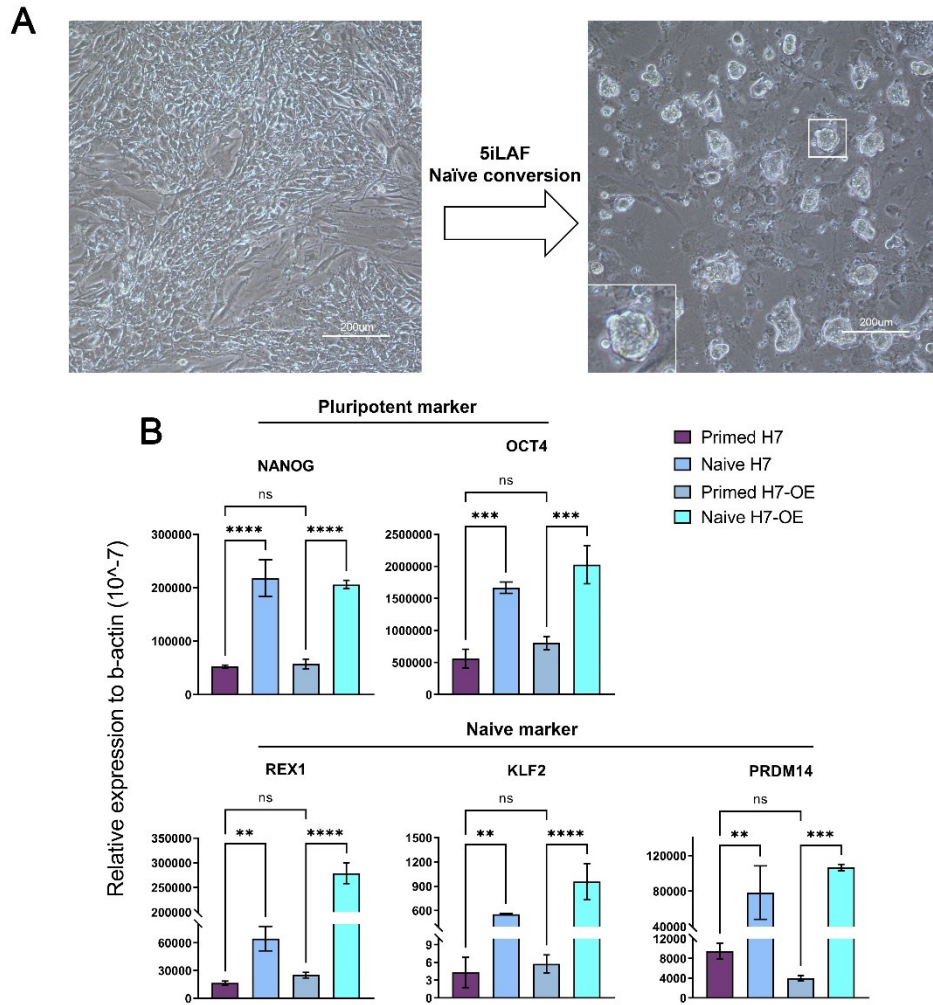


Figure 5.3 Conversion of H7-OE hESCs to naïve state using 5iLAF method.

(A) Morphological changes of H7-OE hESCs during naïve conversion; (B) qRT-PCR analysis of pluripotent markers (*OCT4*, *NANOG*) and naïve markers (*REX1*, *KLF2*, *PRDM14*) in converted naïve H7 and H7-OE hESCs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tail unpaired t-test, $n = 3$ biological replicates).

5.2.4. Ectopic NLRP7 expression alleviates the loss of maternal imprinting in naïve hESCs

Next, I examined the allelic expression patterns of both paternally and maternally imprinted genes in H7 NLRP7-OE hESCs before and after the naïve conversion to investigate whether loss of maternal imprinting was affected in hESCs by NLRP7-OE after naïve conversion. Firstly, I examined the imprinted genes that have been identified in Chapter 3 to contain SNPs in our hESC lines for their allelic-specific expression.

In primed H7 NLRP7-OE hESCs, all three maternally (*PEG10*, *NAP1L5* and *MAGEL2*) and one paternally (*MEG3*) imprinted genes displayed a monoallelic expression pattern (Fig 5.4A&C, upper lane), as previously identified in WT H7 hESCs (Fig 4.2A&B, upper lane), indicating that NLRP7-OE does not affect imprinting status in the primed hESCs. Interestingly, after naïve conversion, maternally imprinted genes exhibited unequally biallelic expression with a much lower expression of the newly activated allele, which was in contrast to the equal biallelic expression detected in WT H7 naïve hESCs (Fig 4.2A, lower lane). For example, the maternally imprinted gene *PEG10* contains a C/T SNP in the H7 line. Its expression in primed hESCs showed a single T peak at the SNP site with no detectable C peak, indicating that only the allele containing T nucleotide at the SNP site was expressed. Given the maternally imprinted nature of *PEG10*, the allele containing T is considered a paternal allele, while the allele containing C is a maternal allele and the maternal imprinting was on in primed hESCs. After naïve conversion, the expression of *PEG10* in NLRP7-OE hESCs exhibited a dominant paternal T peak and a new small maternal C peak at the SNP site, (Fig 5.4A, lower lane), opposing to the equal paternal and maternal peaks of "T" and "C", respectively, in WT H7 naïve hESCs (Fig 4.2A, lower lane). To further quantify the differences, I measured and calculated the ratio between the peak heights of two nucleotides at SNP sites within three maternally imprinted genes. All maternally

imprinted genes showed an increased ratio of maternal to paternal allele expression, changing from 1:1 in naïve H7 hESCs to 1:4 in naïve H7-OE hESCs (Fig 5.4B).

To test the NLRP7 effect on paternal imprinting, the paternally imprinted gene *MEG3* was also examined by RT-PCR in primed and naïve H7 NLRP7-OE hESCs and showed completely different expression patterns from monoallelic expression in primed hESCs to equally biallelic expression after naïve conversion (Fig 5.4C). The differential expression pattern of *MEG3* was consistent with that in WT H7 hESCs (Fig 4.2B, lower lane). These results demonstrated that NLRP7 has no clear effect on paternal imprinting status after the global demethylation process associated with naïve conversion.

Taken together, these results indicate that high NLRP7 expression seems specifically to protect maternally imprinted DMR against naïve conversion-associated demethylation but has little effect on paternal imprinting status.

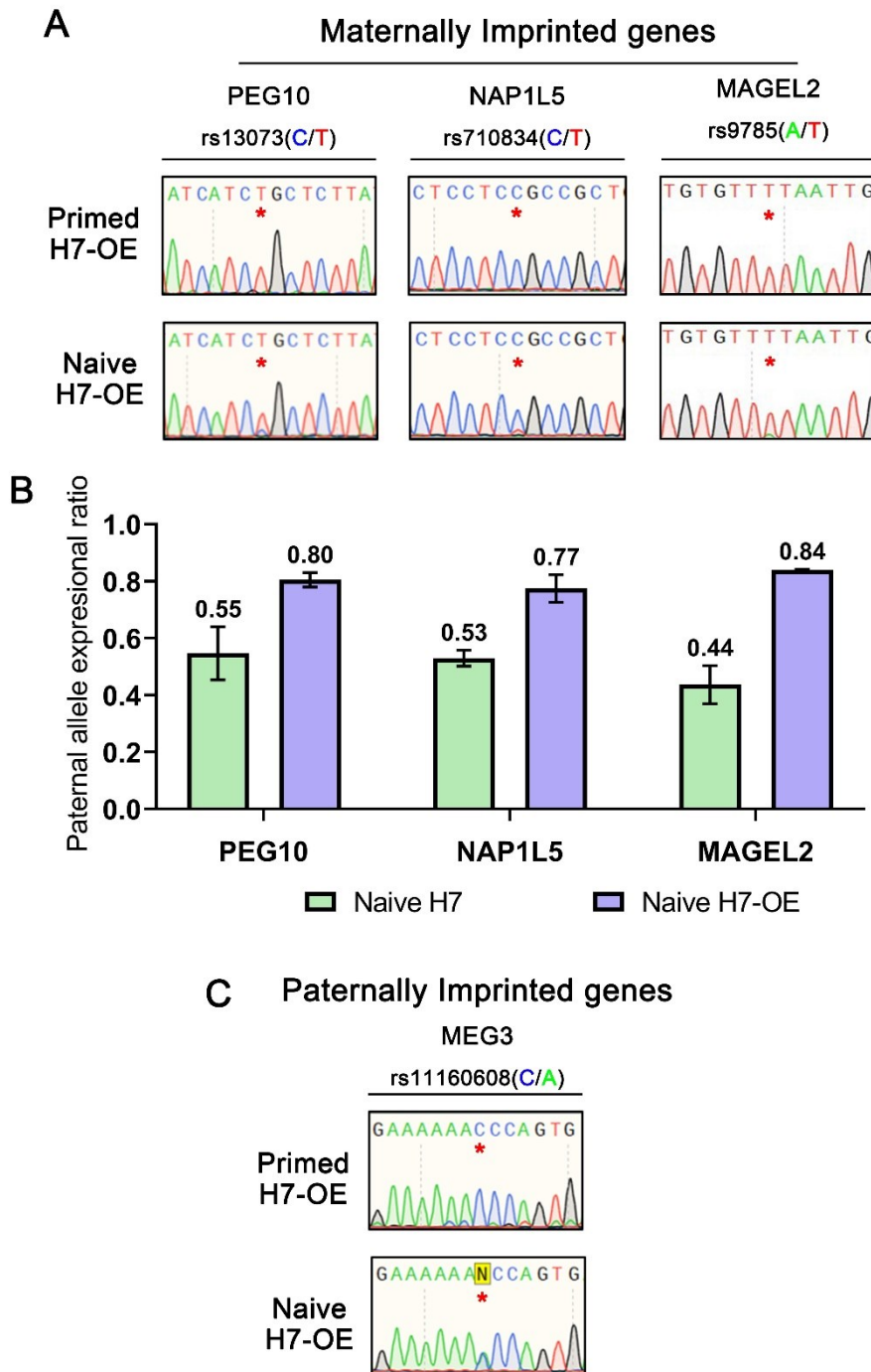


Figure 5.4 Imprinting status of NLRP7-OE H7 hESC before and after naïve conversion.

(A) Allelic expression of maternally imprinted genes (*PEG10*, *NAP1L5* and *MAGEL2*) in NLRP7-OE H7 hESC before and after naïve conversion. (B) Ratio of expression from paternal alleles in maternally imprinted genes in naïve H7 and H7-OE hESCs, calculated by the peak heights of two nucleotides at SNP sites within each gene from Figures 4.2A and 5.4A, from three independent conversion experiment. (C) Allelic expression of the paternally imprinted gene *MEG3* in NLRP7-OE H7 hESCs before and after naïve conversion.

5.2.5. Characterisation of different methods to measure DNA methylation on maternally imprinted DMRs in primed hESCs

In the previous section, I have shown that high expression of NLRP7 mitigates the loss of maternal imprinting during the global demethylation associated with naïve hESC conversion, using an SNP-linked allelic-specific expression approach. Given that genomic imprinting is predominately controlled by differential DNA methylation on DMR (Moore et al., 2013), it is reasonable to assume that enriched NLRP7 may protect maternal DMR from the global DNA demethylation process. However, there is lacked direct evidence to support this statement. Therefore, I wanted to find a reliable method to inspect the methylation status of DMRs, which could not only provide direct evidence of their methylation but also allow the examination of more imprinted genes that do not contain SNPs in hESCs.

Since DNA methylation typically occurs at CpG dinucleotides, bisulphite-PCR is a commonly used approach to measure methylation levels at a single CpG resolution. Thus, I first tried this method to assess the methylation on DMRs of maternally imprinted genes in hESCs. Basically, an unmethylated cytosine (C) is deaminated to uracil (U) after bisulphite conversion, while 5mC remains as C. During PCR amplification, U can be read as thymine (T), whereas 5mC remains as C. The PCR products are then ligated into a cloning vector. A minimum of ten clones are normally to be sequenced to obtain meaningful data. The sequence data will be compared against the reference genome allows researchers to distinguish between methylated CpG (CG) and unmethylated CpG (TG) dinucleotides for the analysis of DNA methylation patterns (Darst et al., 2010) (Fig 5.5A).

To test the accuracy and reproducibility of the bisulphite-PCR method, two maternally imprinted genes (*NAP1L5* and *SNRPN/SNURF*) were selected to quantify their methylation levels on DMRs in H7 WT and NLRP7-OE hESCs as their imprinting status have been confirmed by SNP-associated allelic-specific expression. Given that

NAP1L5 and *SNRPN/SNURF* are known to be imprinted in primed hESCs, their DMRs should exhibit approximately 50% methylation. However, the results showed a 79% methylation level on DMR of *NAP1L5* in primed H7 WT hESCs and 61% in H7-OE hESCs (Fig 5.5B, upper lane), which were much higher than the expected 50% methylation level. Although the methylation level of *SNRPN/SNURF* DMR was shown as 48% in H7 WT hESCs as expected, it was 76% in *NLRP7*-OE counterpart (Fig 5.5B, lower lane), which have the same expression pattern of *SNRPN/SNURF*. These results revealed that the methylation levels measured by the bisulphite-PCR method varied between different cell lines, despite the expectation that they should be around 50%. One issue reported is that PCR products with low levels of cytosine are more difficult to ligate into vectors and propagate in *Escherichia coli* than cytosine-rich fragments (Grunau et al., 2001). Additionally, colony selection is a stochastic process and thus may require a higher number of colonies to be screened, which is costing. These challenges prompted me to explore alternative techniques for quantifying the methylation levels of maternal DMRs.

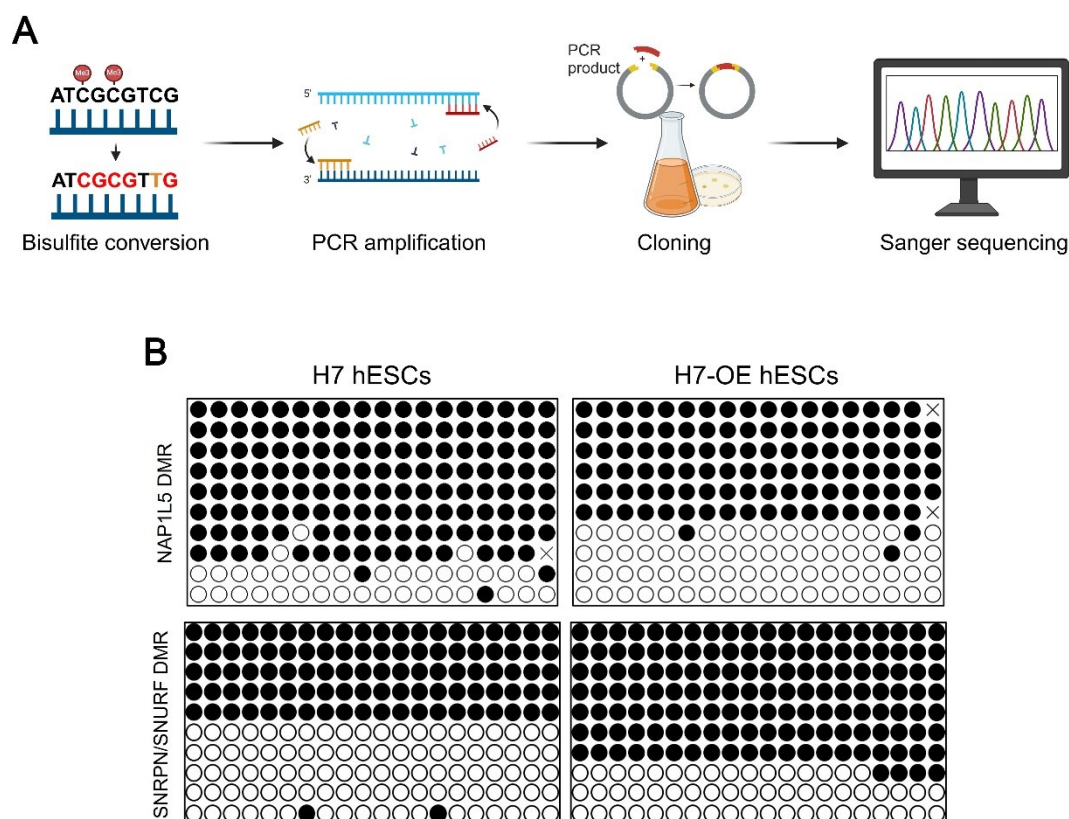


Figure 5.5 DNA methylation at DMRs of indicated maternally imprinted genes in primed H7 WT and NLRP7-OE hESCs by bisulphite-sequencing.

(A) Schematic depicting experimental procedure of bisulphite-sequencing approach. **(B)** Methylation status on the DMRs of *NAP1L5* and *SNRPN/SNURF*, containing 18 and 20 CpG dinucleotides, respectively, in primed H7 WT and NLRP7-OE hESCs. The CpG sites are represented by open (unmethylated) or closed (methylated) circles. Each row represents one clone. Crosses indicate misalignments with other clones. All experiments were done by Madeha Islam.

To avoid plasmid cloning bias and colony-picking stochasticity in the bisulphite-sequencing method, I explored a new approach to analyse methylation status directly with bisulphite-PCR fragment without further cloning and colony-picking. Given that bisulphite treatment converts unmethylated CG to TG but leaves methylated CG unchanged, I sought to find a restriction enzyme that could distinguish between these sequences, therefore, distinguishing methylated from unmethylated DMRs. The BstUI restriction enzyme that recognises the CGCG four base-pair DNA sequence might be suitable for this purpose. I checked the DMRs of the 13 maternally imprinted genes identified in Chapter 3 and found 12/13 genes containing CGCG in their DMRs. If these

sequences are methylated, bisulphite treatment would not change them, then they will be digested by the BstUI, whereas if they are unmethylated, CGCG would be converted to TGTG by bisulphite treatment, rendering them resistant to BstUI digestion. The differential digestion of methylated and unmethylated sequences will result in DNA fragments of different sizes, which could be visualized on an agarose gel by electrophoresis (Fig 5.6A).

The methylation level is calculated by the percentage of intensities of cut fragments over the total intensities of all the fragments. Since the intensity of each band on an agarose gel is determined by the dye/base pair ratios (Zipper et al., 2004), the raw intensity data need to be normalised to the fragment length before calculating the methylation level. Given that all cut fragments originate from the same PCR product, these fragments should exhibit similar values after normalisation. Therefore, at least one cut fragment is sufficient to calculate the methylation level of DMRs. If multiple fragments are available, their average can be used to improve the accuracy. The equation is shown as follows (Fig 5.6B)

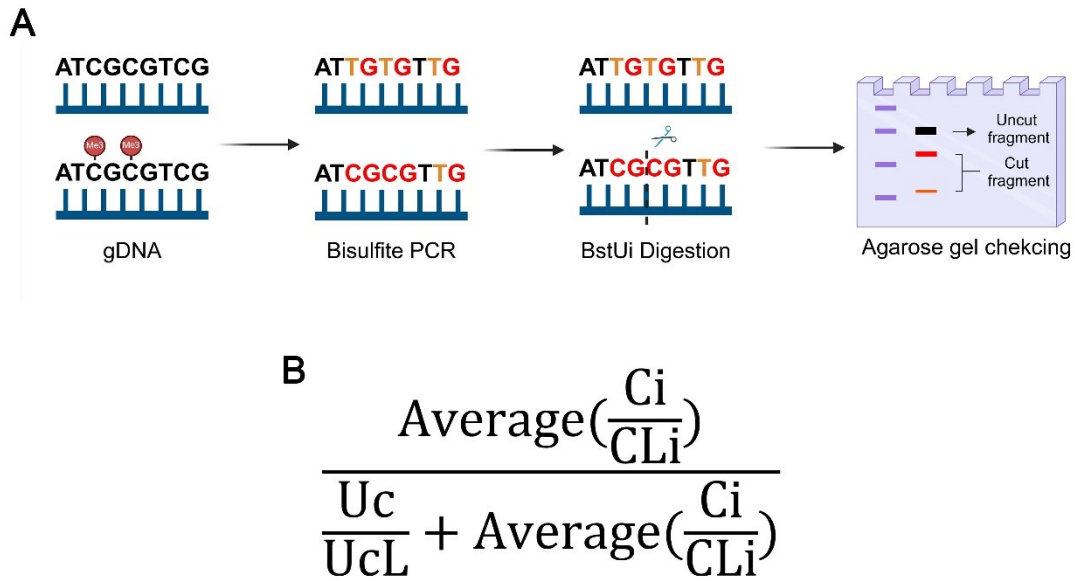


Figure 5.6 Establishment of a bisulfite-digestion method for quantifying methylation level.

(A) Schematic illustrating the experimental procedure for bisulfite digestion to quantify methylation levels in specific regions. (B) The equation to calculate the methylation level of amplified maternal DMRs. Specifically, C_i represents intensity of cut fragments, $i \geq 1$; CL_i represents length of cut fragments, $i \geq 1$; U_c represents intensity of uncut fragment; U_{cL} represents length of cut fragment.

To test the feasibility of this method, I applied this method to analyse *NAP1L5* DMR in primed H7 WT and NLRP7-OE hESCs before and after naïve conversion. *NAP1L5* DMR PCR product generated with dedicated primers (section 2.2.3.1) contains 242 bp and two CGCG sites, which should produce three fragments of 27 bp, 21 bp and 194 bp if these sites are methylated, whereas it would show a 242 bp fragment (Fig 5.7A).

To ensure the efficiency and specificity of the BstUI enzyme, the *NAP1L5* DMR was amplified by PCR without bisulphite conversion so that the PCR fragment could be completely cut into three fragments by BstUI digestion. In addition, to eliminate the possibility of any background interference, I also checked the sample without PCR amplification. The results showed that without PCR amplification, there was no detectable fragment resolved (Fig 5.7B, right). With PCR amplification of the DMR, it generated a fragment of ~250 bp as expected. This fragment completely disappeared and was replaced by a smaller size of ~200 bp fragment after BstUI digestion,

representing the expected fragment of 194 bp (Fig 5.7B, left). The other two digested fragments, 21 bp and 27 bp were too small to be visualised in the gel. This result indicates that the BstUI enzyme is able to specifically recognise the CGCG site in the PCR products and cut them efficiently.

Next, the DNA methylation levels on *NAP1L5* DMR in H7 WT and NLRP7-OE hESCs were examined using the bisulphite-digestion method. The *NAP1L5* DMR was amplified after the gDNAs were converted by bisulphite treatment, and then the PCR products were digested by BstUI and resolved on a gel. The digests from both cell lines showed two bands on the gel: the upper band of 242bp represents the unmethylated DNA sequences and the lower band of 194bp represents the methylated DNA sequence (Fig 5.7C, upper panel). Using the calculation formula described above, the methylation levels were 53% and 52% in WT and NLRP7-OE hESCs, respectively (Fig 5.7C, lower panel). These levels were close to the 50% methylation level expected in primed hESCs, confirming that NLRP7-OE did not affect the methylation levels of imprinted DMRs.

The same experiment was performed in the DNA samples from naïve H7 WT and NLRP7-OE hESCs. As expected, in naïve WT hESCs, the digestion produced two bands consisting of an uncut band of 242 bp and another invisible band at around 200 bp level whose intensity could be measured by software (Fig 5.7D, upper panel left), demonstrating that naïve conversion considerably erases the methylation on maternal *NAP1L5* DMR, which is consistent with its biallelic expression in these cells (Fig 4.2A, lower lane). By contrast, two bands were detected in naïve NLRP7-OE hESCs: one stronger uncut band of 242 bp and one weaker digested fragment of 194 bp, indicating a possibility of lower than 50% methylation level in these cells (Fig 5.7D, upper panel right). The quantification of methylation levels supported the observation, showing 13% and 39% methylation levels in naïve H7 and H7-OE hESCs, respectively (Fig 5.7D, lower panel).

Taken together, these results correlate with the imprinted status of *NAP1L5* in H7 WT and NLRP7-OE hESCs by SNPs expression analysis, where *NAP1L5* was maternally imprinted in primed WT hESCs but lost its imprinted status after naïve conversion; whereas in NLRP7-OE hESCs, this maternally imprinted status was partially retained. This further demonstrates that ectopic NLRP7 might protect maternally imprinted genes from global demethylation by preserving the methylation of their DMRs and that the BstUI digestion method could be used to evaluate methylation levels in DMRs, enabling me to explore more maternally imprinted genes that do not contain SNPs.

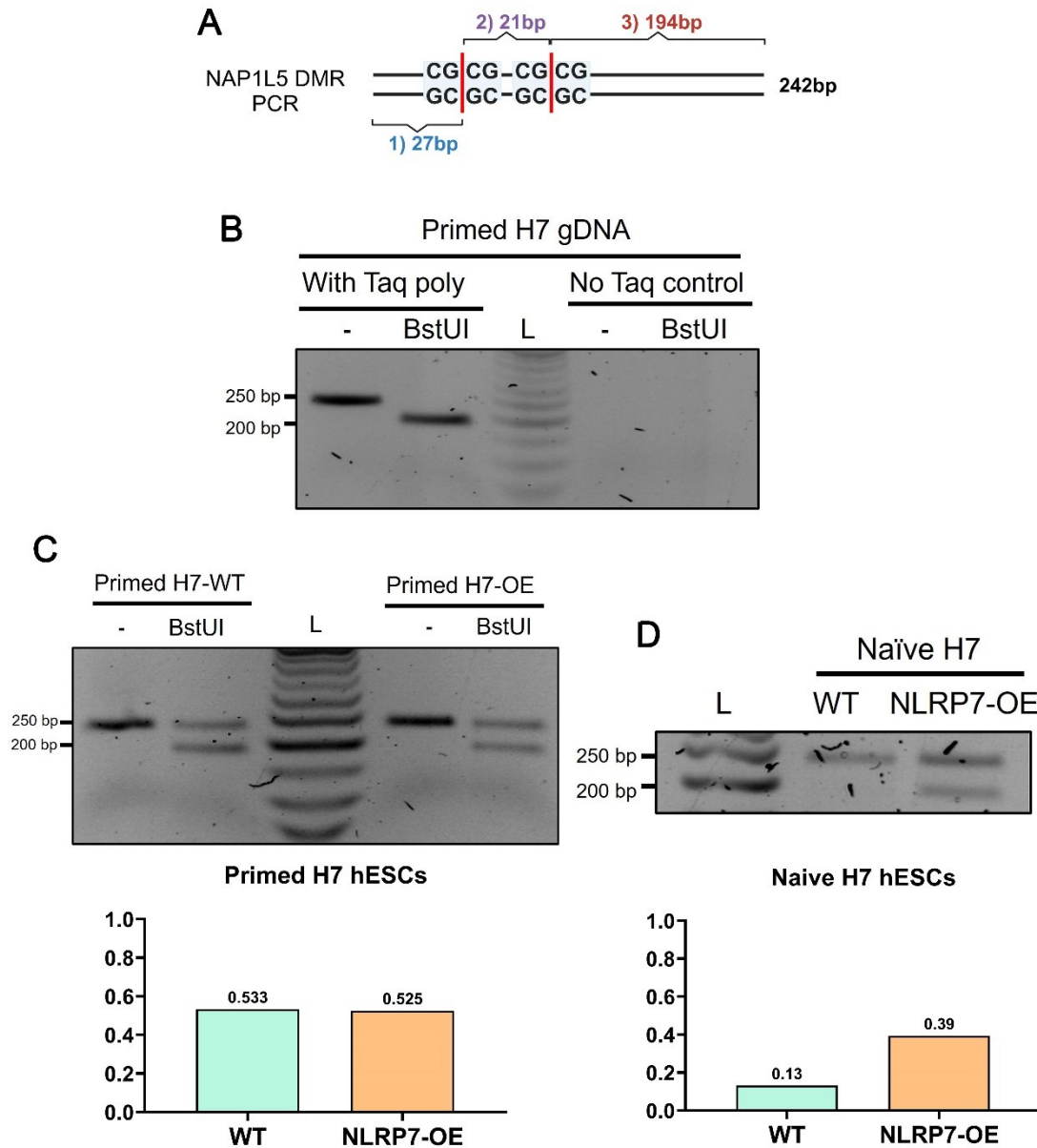


Figure 5.7 Quantifying methylation level on *NAP1L5* DMR by bisulphite-digestion method.

(A) Schematic showing the size of PCR-amplified *NAP1L5* DMR and the resulting digested fragments, with cutting sites labelled. Each colour represents a different DNA fragment. **(B)** Representative gel image showing BstUI-digested PCR products of the *NAP1L5* DMR, amplified using gDNA from wide-type H7 hESCs. **(C&D)** Representative gel images (upper panels) and quantitative analyses of methylation levels (lower panels) for BstUI-digested PCR fragments of the *NAP1L5* DMR, amplified using bisulfite-converted gDNA from H7 and H7-OE hESCs before **(C)** and after naïve conversion **(D)**. Cut: BstUI-digested; Uncut: undigested. All experiments were done by Madeha Islam.

5.2.6. Overexpression of NLRP7 shows to retain maternal imprinting DMR methylation against naïve conversion-associated global demethylation

To further investigate whether ectopic NLRP7 has a function to retain maternal imprinting in hESCs during naïve conversion, I desired to examine more maternally imprinted genes by comparing the methylation on DMRs of maternally imprinted genes during naïve conversion in WT and NLRP7-OE hESCs using the bisulphite-digestion method described in the previous section.

Based on Table 3.2, I summarised the DMRs of each selected maternally imprinted gene (Table 5.1). Since the imprinting status of *PEG10*, *NAP1L5* and *MAGEL2* had been already checked by allelic expression patterns, the rest six maternal DMRs are candidates for analysis. However, *ZDBF2* DMR was found to be localised on the paternal chromosome, rather than the maternal chromosome; additionally, *FAM50B*, *DLK1* and *NNAT* DMRs have multiple CGCG cutting sites which are difficult for bisulphite-specific primer design and following digestive analysis (Table 5.1). Therefore, I chose another three maternally imprinted genes (*PLAGL1*, *L3MBTL1* and *GRB10*) which were highly expressed in H1 hESCs (Table 3.1A). Therefore, in this study, I selected maternal germline DMRs from five genes (*KCNQ1OT1*, *PLAGL1*, *L3MBTL*, *PEG3* and *GRB10*) from the literature (Zink et al., 2018) and designed bisulfite-specific primers based on their DMR sequence.

Table 5.1 Summary of DMRs from identified 13 maternally imprinted genes

Gene name	Location	gDMR
<i>NAP1L5</i>	4q21-q22	NAP1L5
<i>PEG10</i>	7q21.3	PEG10
<i>SGCE</i>	7q21.3	
<i>MAGEL2</i>	15q11.2	SNURF
<i>SNRPN/SNURF</i>	15q11.2	
<i>NDN</i>	15q11.2	
<i>LRRTM1</i>	2p12	Not identified
<i>ZDBF2</i>	2q33.3	GPR1-AS DMR
<i>FAM50B</i>	6p25.2	FAM50B
<i>KCNQ1OT1</i>	11p15.5	<i>KCNQ1OT1</i>
<i>IGF2</i>	11p15.5	
<i>DLK1</i>	14q32.2	IG-DMR
<i>PEG3</i>	19q13.43	<i>PEG3</i>
<i>NNAT</i>	20q11.23	NNAT
<i>PLAGL1</i>	6q24.2	<i>PLAGL1</i>
<i>GRB10</i>	7p12.1	<i>GRB10</i>
<i>L3MBTL1</i>	20q13.12	<i>L3MBTL1</i>

Red colour labelled: genes already checked by allelic expression pattern

Green colour labelled: the additional selected maternally imprinted genes

Blue colour labelled: the selected maternal DMRs for quantification of methylation

As described previously, bisulfite-converted gDNA from four cell lines was amplified by PCR. Ideally, the expected digested band should shown as uncut fragment 431bp and cut fragments 272bp and 159bp (Fig 5.8Ai). Following BstUI digestion, all hESC lines in the *PLAGL1* DMR showed three bands on the gel as expected: an upper band equalled to the untreated PCR product represented unmethylated sequence, and two lower bands represented methylated sequence (Fig 5.8Aii). Methylation levels were quantified using the method described previously. Since both digested fragments could be detected in the *PLAGL1* DMR group, the average value of normalised intensities from both cut bands was used for analysing methylation levels to improve accuracy. The *PLAGL1* DMR was partially methylated in both H7 and H7-OE hESCs, with methylation levels of 38% and 40%, respectively. After naïve conversion, H7 hESCs lost methylation but still retained 25% methylation, while H7-OE hESCs maintained a

partially methylated state at 46%, similar to the primed H7 and H7-OE hESCs (Fig 5.8Aiii). This result revealed that ectopic NLRP7 could protect the methylation on DMRs of maternally imprinted genes from global demethylation associated with naïve conversion.

Similar results were observed in *KCNQ1OT1* DMR and *L3MBTL* DMR. Three bands of expected sizes were detected in all hESC lines: in *KCNQ1OT1* DMR, undigested PCR products at 372bp (unmethylated sequence) and digested fragments at 264bp and 108bp (methylated sequence) (Fig 5.8Bi & ii). Similar to the *PLAGL1* DMR group, primed H7 and H7-OE hESCs showed comparable methylation levels at 40% and 44%, respectively. The methylation level in H7 hESCs decreased to 34% after naïve conversion, whereas H7-OE hESCs maintained a partially methylated state at 49%, not obviously affected by global demethylation during naïve conversion (Fig 5.8Biii). In addition, for the *L3MBTL1* DMR, the undigested PCR product was 356bp (unmethylated sequence) and a digested fragment was 291bp (methylated sequence), with the 65bp fragment being too small to visualize (Fig 5.8Ci & ii). Methylation levels at the *L3MBTL* DMR were similar in both primed H7 and H7-OE hESCs, at 39% and 43%, respectively. After naïve conversion, H7 hESCs showed a decrease in DNA methylation at the *L3MBTL* DMR, with 31% methylation, while H7-OE hESCs maintained methylation levels at 49% (Fig 5.8Ciii).

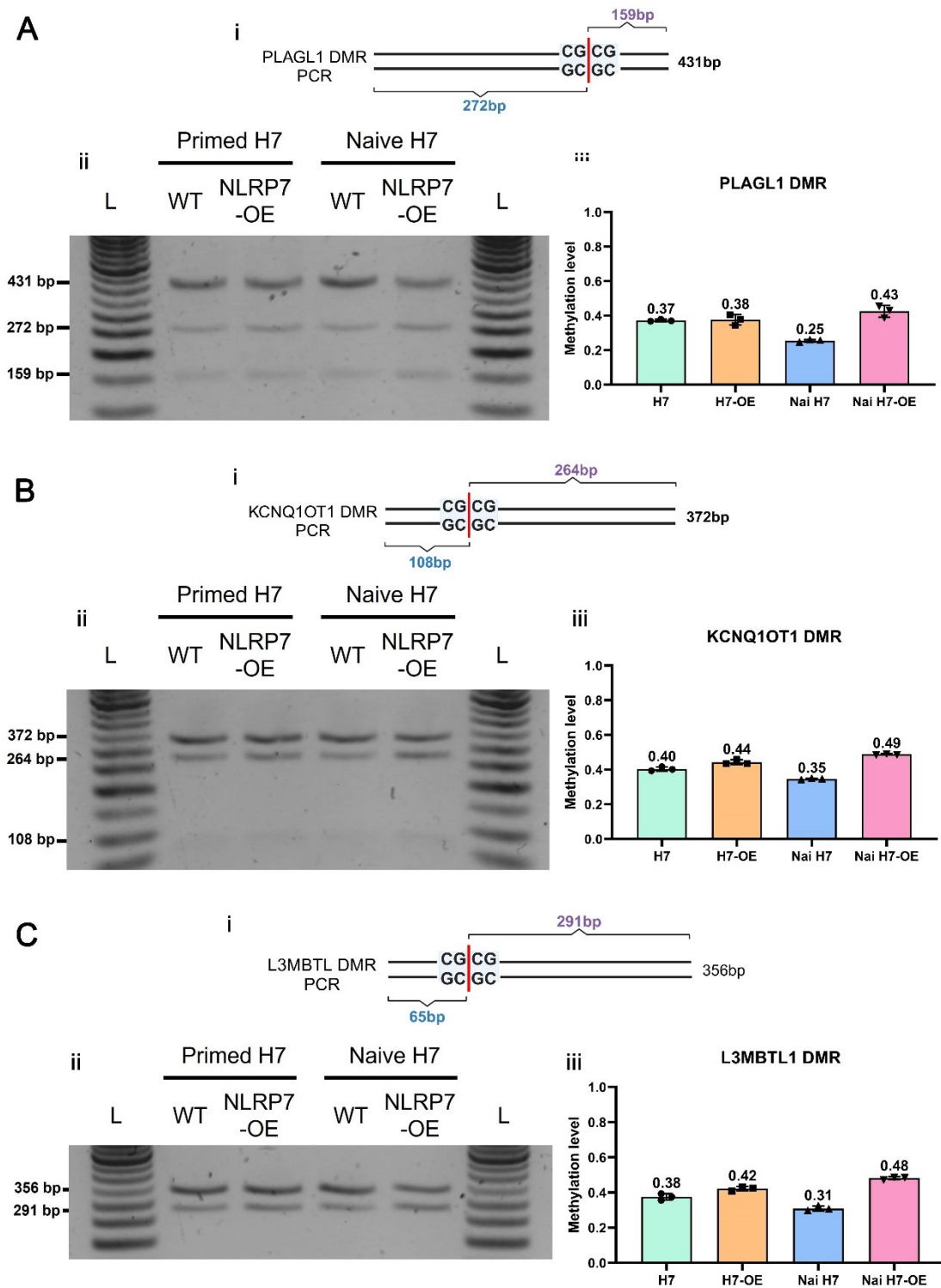


Figure 5.8 Methylation profiles of DMRs at maternally imprinted genes in wide-type and NLRP7-OE H7 hESCs before and after naïve conversion.

Methylation levels of DMR sequence were quantified by bisulfite-digestion method for maternally imprinted genes: *PLAGL1* (A), *KCNQ1OT1* (B), and *L3MBTL* (C) in wide-type and NLRP7-OE H7 hESCs before and after naïve conversion. i: Schematic illustrating the size of PCR-amplified DMRs of the imprinted genes and the resulting digested fragments, with cutting sites labelled. Each colour represents a different DNA fragment. ii: Representative gel images displaying BstUI-digested PCR fragments of DMRs, amplified using bisulfite-converted gDNA from wide-type and NLRP7-OE H7 hESCs before and after naïve conversion. iii: quantitative analyses of methylation levels based on the intensity of each band in the representative gel images. Each experiment was measured three times to ensure the accuracy of quantification.

However, the *PEG3* and *GRB10* DMRs exhibited notably higher and lower methylation levels, respectively, across all tested hESC lines compared to the other three maternal DMRs. In the *PEG3* DMR, three bands were detected in all hESC lines including one uncut band of 172bp representing unmethylated DMR and the other two fragments of 100bp and 72bp representing methylated DMR (Fig 5.9Ai & ii). Surprisingly, the *PEG3* DMR in all cell lines showed extremely high DNA methylation levels. Primed WT and NLRP7-OE hESCs showed similar methylation levels at 83% and 84%, respectively. Although reduced in the naïve hESCs, methylation retained a high level of 69%, significantly higher than the expected 50% in primed hESCs. Naive H7-OE hESCs maintained a methylation level of 79%, similar to primed H7 and H7-OE hESCs (Fig 5.9Aiii). This high methylation level on *PEG3* DMR may result from constantly methylated CGCG cutting sites on both maternal and paternal alleles, as indicated by our preliminary data (Zhi, 2021).

In the *GRB10* DMR, two bands with expected sizes were observed in all hESC lines, an uncut band of 369 bp (unmethylated DMR) and a smaller fragment of 200 bp (methylated DMR), with the 82 and 87 bp fragments being too small to visualize (Fig 5.9Bi & ii). Methylation levels in the *GRB10* DMR were notably lower in all four cell lines compared to the other maternal DMRs. Unexpectedly, primed WT hESCs exhibited a 9% higher methylation level than primed H7-OE hESCs, at 34% and 25%, respectively, indicating divergent methylation levels between different primed hESC

lines (Fig 5.9Biii). This phenomenon is consistent with other studies showing different digestive patterns between mESC clones at some DMRs (Liu et al., 2022). Since the BstUI enzyme is sensitive to the CGCG cutting site, even a single constantly methylated CpG dinucleotide can inhibit its digestive function. Despite this, methylation on the *GRB10* DMR was also reduced in naïve hESCs, showing a 0.2% methylation level, while naïve H7-OE hESCs maintained a methylation level of 22%, comparable to that of primed H7-OE hESCs (Fig 5.9Biii).

Taken together, all quantified methylation levels on each DMR were summarised in Table 5.2. These results for the *PLAGL1*, *KCNQ1OT1*, *L3MBTL*, *PEG3* and *GRB10* DMRs indicate that ectopic NLRP7 can retain methylation levels on maternal DMRs during global DNA demethylation in naïve conversion. This suggests that NLRP7 proteins protect maternally imprinted genes from global demethylation by preserving DNA methylation on maternally imprinted DMRs, thereby partially maintaining their imprinting status.

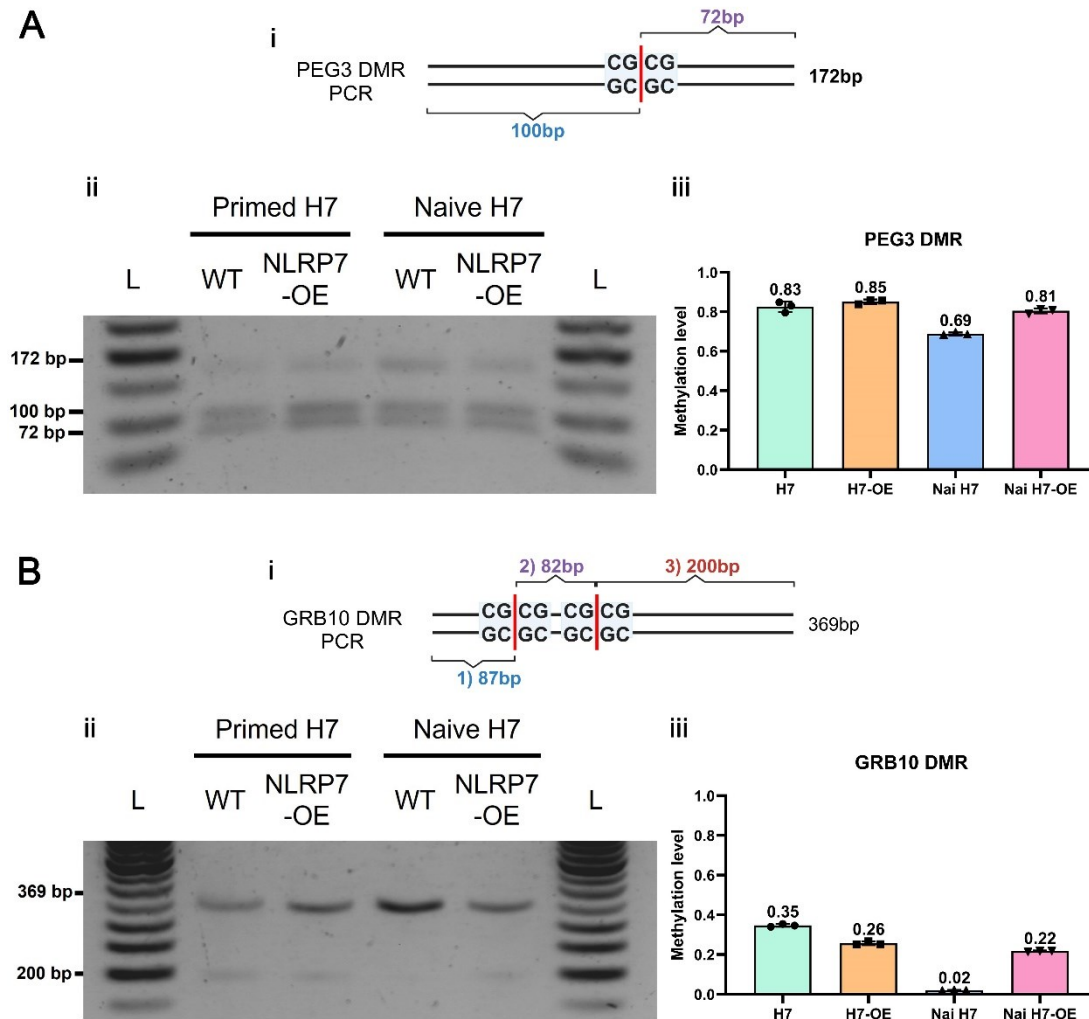


Figure 5.9 Methylation profiles of DMRs at PEG3 and GRB10 in wide-type and NLRP7-OE H7 hESCs before and after naïve conversion.

Methylation levels of DMR sequence were quantified by bisulfite-digestion method for maternally imprinted genes: *PEG3* (**A**), *GRB10* (**B**) in wide-type and NLRP7-OE H7 hESCs before and after naïve conversion. **i**: Schematic illustrating the size of PCR-amplified DMRs of the imprinted genes and the resulting digested fragments, with cutting sites labelled. Each colour represents a different DNA fragment. **ii**: Representative gel images displaying BstUI-digested PCR fragments of DMRs, amplified using bisulfite-converted gDNA from wide-type and NLRP7-OE H7 hESCs before and after naïve conversion. **iii**: quantitative analyses of methylation levels based on the intensity of each band in the representative gel images. Each experiment was measured three times to ensure the accuracy of quantification

Table 5.2 Summary of quantified methylation levels on DMRs in this project.

Cell lines	Wild-type H7		NLRP7-OE H7	
	Primed	Naive	Primed	Naive
KCNQ1OT1	40%	35%	44%	49%
PLAGL1	37%	25%	38%	43%
L3MBTL1	38%	31%	42%	48%
PEG3	83%	69%	85%	81%
GRB10	35%	2%	26%	22%

5.2.7. NLRP7 proteins are localised in close proximity with genomic DNA during cell mitosis

Our findings indicate that NLRP7 may play a crucial role in preserving methylation on maternally imprinted DMRs, thereby protecting maternally imprinted genes from global demethylation. However, it remains unclear how NLRP7 regulates maternal imprinting. I have shown that a small amount of NLRP7 can be detected in the nucleus in naïve hESCs when NLRP7 protein is abundant (Fig 4.6Bii), suggesting that NLRP7 could reside in the nucleus. However, it is also unclear whether this small amount of nuclear NLRP7 could protect the methylated DMR from global demethylation. Nevertheless, my data showed that ectopic expression of NLRP7 alleviates the loss of methylation on maternally imprinted DMRs in the naïve conversion, which is likely to occur at the early stage of naïve conversion (Fig 4.5C). Therefore, I was interested in further investigating the localisation of NLRP7 proteins using primed NLRP7-OE hESCs.

Immunostaining with the antibody against NLRP7 protein on H1 hESCs showed that although no clear signals were visible in WT hESCs as previously shown (Fig 5.10A), strong signals were detected in NLRP7-OE hESCs (Fig 5.10B). Similar to naïve hESCs, NLRP7 protein signals were predominantly localised in the subcortical areas as previously reported, with a small amount in the nucleus (Fig 5.10Bi), confirming that NLRP7 can translocate into the nucleus when the protein levels are sufficiently high. Remarkably, strong condensed NLRP7 signals were detected in a dividing cell at late

anaphase, between sister chromatids and overlapping with chromosomes (Fig 5.10Bii). This observation introduces the possibility that NLRP7 may perform its function on DNA during cell division, potentially interacting directly with DNA sequences or regulating maternal imprinting through other proteins. However, since this dividing cell was accidentally captured, further experiments are needed to substantiate this claim.

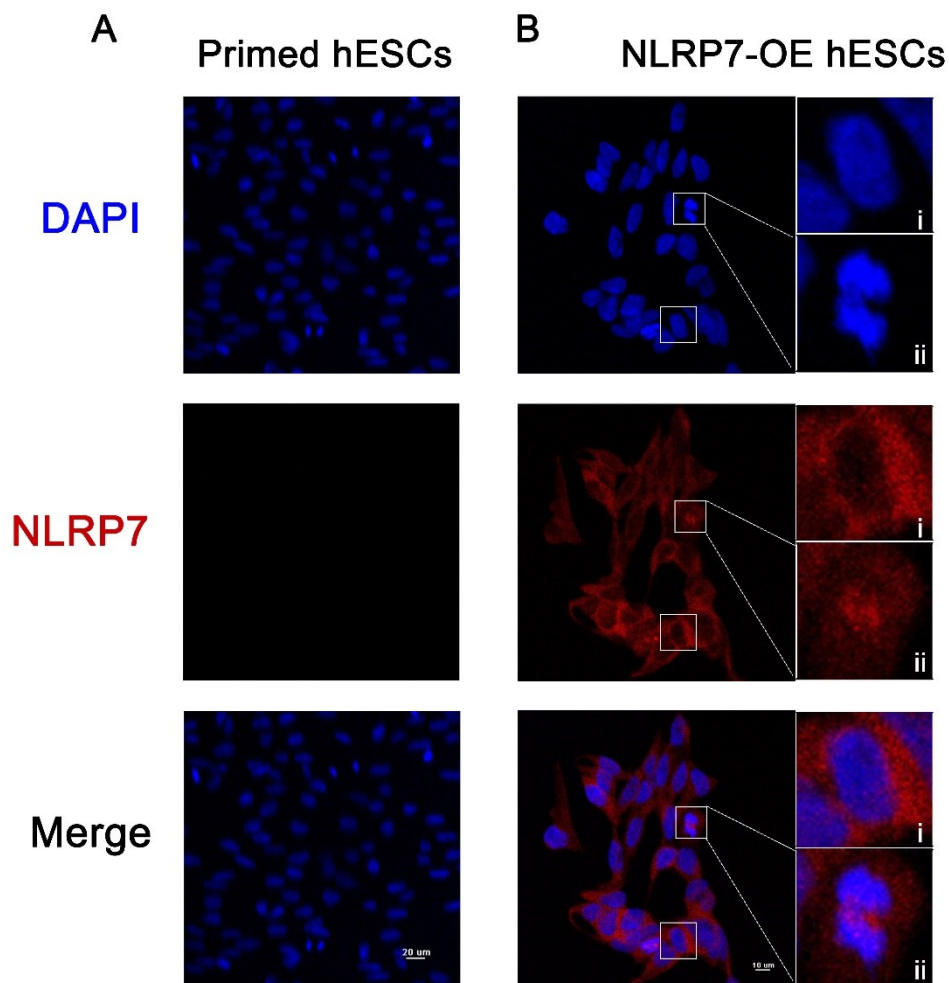


Figure 5.10 The NLRP7 protein localisation in wide-type and NLRP7-OE H1 hESCs.

The immunostaining of NLRP7 in H1 (A) and H1-OE (B) hESCs including the close inspection images which were the larger image of selected block region (i & ii). Blue colour is DAPI representing nucleus; Red colour is NLRP7 protein.

To further investigate the localisation of NLRP7 during cell mitosis in hESCs when the nuclear envelope is disrupted, I synchronised NLRP7-OE hESCs to enrich the

proportion of mitotic dividing hESCs. Various chemicals can arrest cells at specific phases of the cell cycle, but they often cause undesirable side effects, for example, prevention of microtubule assembly by colchicine in cells (Wang and Wang, 2022). Given that NLRP7 localisation has been shown to be associated with microtubules in blood cells (Messaed et al., 2011), the aberrant microtubule assembly may affect the localisation of NLRP7 in hESCs after colchicine treatment. The double thymidine block is a newly developed method to synchronise cells at the G1/S boundary without any side effects. By releasing cells from the double thymidine block at different time intervals, they can be arrested at various stages of mitosis (Wang and Wang, 2022).

Therefore, this experiment was conducted using H7 WT and NLRP7-OE hESCs. The hESCs were cultured in MEF-CM supplemented with thymidine to arrest cells at the G1/S boundary and then cells were released into a routine culture medium for ten hours to enrich mitotic dividing hESCs at metaphase and anaphase. To distinguish hESCs at different stages of cell mitosis, α -tubulin, a key component of spindle fibre, is commonly used as an indicator in different studies (Aizer et al., 2013, Akoury et al., 2015). However, since both NLRP7 and α -tubulin antibodies in our lab are derived from mouse species, I would like to use HA antibody to show ectopic HA-tagged NLRP7 expression. Thus, I first checked the HA antibody to ensure it specifically recognises NLRP7 proteins by performing double staining with HA and NLRP7 antibodies. Immunostaining showed no signals detected by HA and NLRP7 in H7 hESCs, as expected (Fig 5.11A), while HA signals were exclusively and completely co-localised with NLRP7 signals, indicating that the HA antibody specifically and efficiently captured NLRP7 proteins (Fig 5.11B). Interestingly, HA-NLRP7 proteins were exclusively detected in the cytoplasm, with no obvious signals in the nucleus (Fig 5.11Bi), contradicting results from H1-OE hESCs and naïve H7 hESCs (Fig 5.10Bi and Fig 4.6ii, respectively). This might be due to their different expression levels: H1 NLRP7-OE and naïve hESCs express higher levels of NLRP7 than H7 NLRP7-OE hESCs, which

suggest that NLRP7 proteins may only be able to translocate into the nucleus when their expression is above a threshold level through an unknown mechanism.

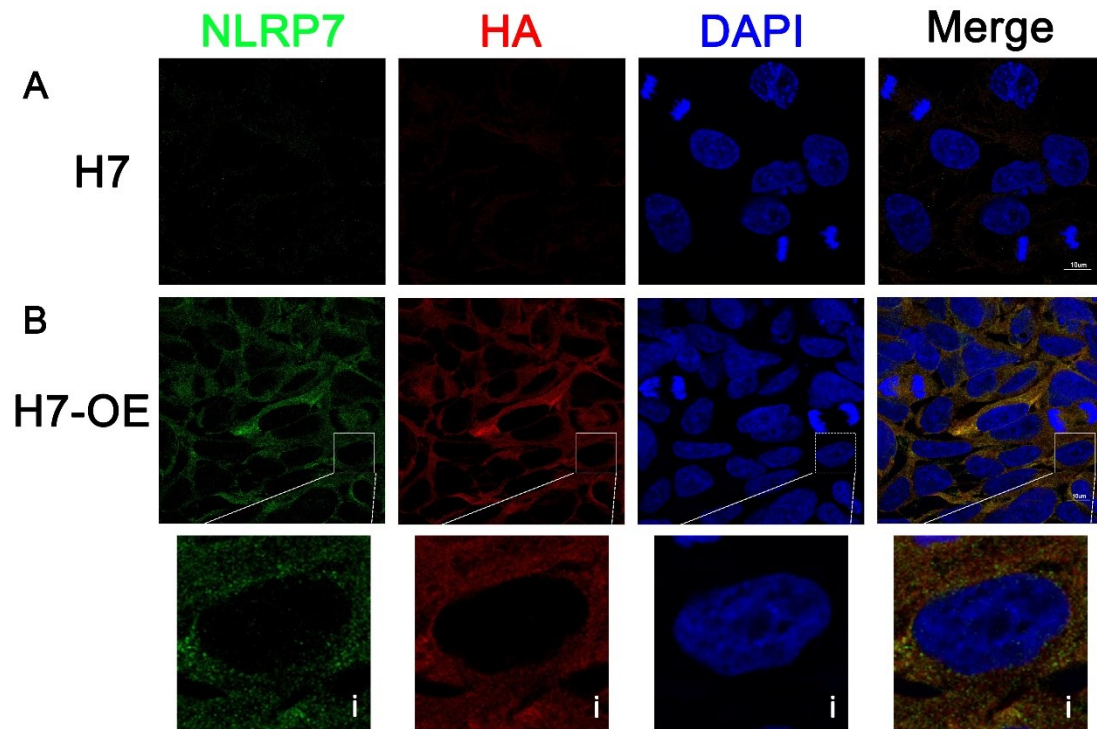


Figure 5.11 The NLRP7 protein localisation in wide-type and NLRP7-OE H7 hESCs.

(A) Immunostaining of NLRP7 and HA in wide-type (A) and NLRP7-OE (B) H7 hESCs. The close inspection image which is the larger image of selected block region (i). Blue colour is DAPI representing nucleus; Green colour is NLRP7; Red colour is HA protein.

When performing double staining with HA and α -tubulin antibodies in H7 WT and NLRP7-OE hESCs, Immunostaining showed some false-positive HA signals in WT H7 hESCs (Fig 5.12A). Since the HA-tag is a nine amino acids long peptide, the HA antibody could easily bind to other proteins and give a false-positive signal. Despite these signals, the immunostaining patterns still showed differences in NLRP7 localization between H7 and H7-OE hESCs. In H7 NLRP7-OE hESCs, HA signals were homogeneously distributed and clearly revealed in a mitotic cell with higher intensities between the two chromatids and this cell was at the anaphase stage by the localisation of α -tubulin signals (Fig 5.12B). This result was consistent with the observation in H1 NLRP7-OE hESCs (Fig 5.10Bii), indicating that NLRP7 proteins can be localised near nuclear DNA at a good level during anaphase of mitosis. These data clearly

demonstrate that although NLRP7 proteins predominantly localise in the cytoplasm of hESCs, particularly at the subcortical areas away from direct contact with genomic DNA, they can be in close contact with nuclear DNA during cell division when the nuclear envelope breaks down. Therefore, NLRP7 may regulate maternally imprinted DMRs during cell division to ensure that newly synthesised DMR of maternally imprinted genes retain the methylation.

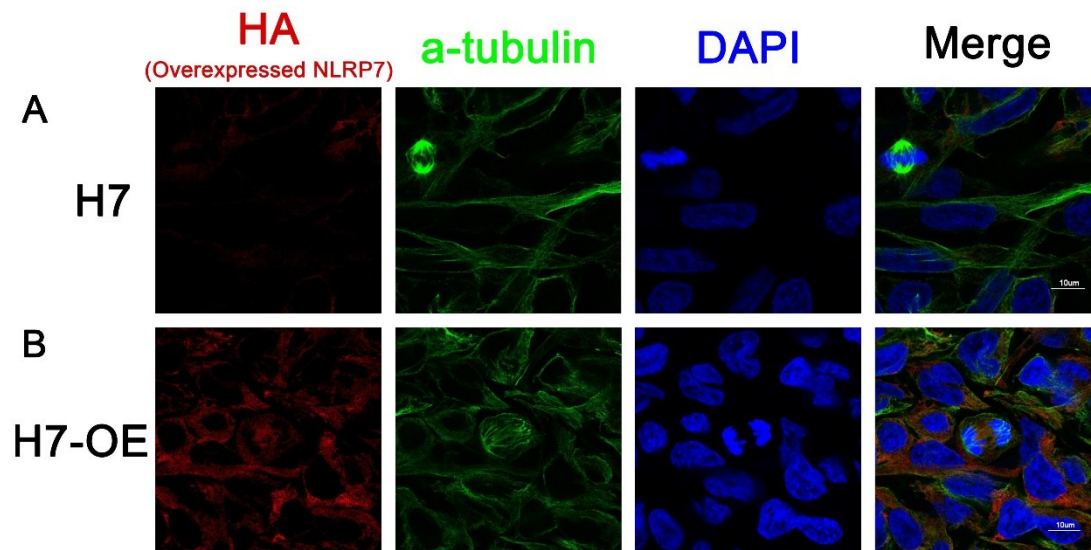


Figure 5.12 NLRP7 is localized in proximity with nuclear DNA at anaphase of mitosis.

Immunostaining of HA and α -tubulin in WT (**A**) and NLRP7-OE H7 hESCs (**B**). Blue colour is DAPI representing nucleus; Green colour is α -tubulin representing spindle fibre during cell mitosis; Red colour is HA protein.

5.3 DISCUSSION

In this chapter, I overexpressed NLRP7 into both H1 and H7 hESCs to establish constitutive NLRP7-expressing hESC lines. In the H7 hESC line, it showed partial rescue phenotype on maternal imprinting in naïve conversion, from complete biallelic expression, manifested by equal SNP peaks, in WT naïve hESC lines to differential allelic expression in NLRP7-OE naïve hESCs, with much higher paternal allele expression (Fig 5.4A). Interestingly, the expression of paternally imprinted gene *MEG3* was not affected by NLRP7-OE, still showing equal biallelic expression following naïve conversion (Fig 5.4C). These results indicate that NLRP7 could mitigate the loss of maternal imprinting from global DNA demethylation during naïve conversion of hESCs.

Given that genomic imprinting is predominately regulated by DNA methylation on DMRs, it is reasonable to assume if NLRP7 has a function to protect maternally imprinted genes from global DNA demethylation, it may protect maternal DMRs from naïve conversion. To date, multiple techniques have been developed to investigate DNA methylation. Whole-genome or single-cell bisulphite sequencing are effective methods for high throughput methylation detection. It can give single-base resolution, providing a detailed methylation map of the entire genome. However, this technique is very expensive and a massive amount of data is difficult to analyse, requiring special bioinformatics experts (Sun and Zhu, 2022). Since I only wanted to measure methylation levels for imprinting DMRs, I thought to use more targeted methods. Bisulphite-PCR sequencing and bisulphite-digestion are good approaches for measuring methylation levels at interested regions (Reed et al., 2010). In comparison between these two techniques, bisulphite-PCR sequencing could give single CpG resolution within the region of interest and provide quantitative analysis on methylation levels (Reed et al., 2010). However, plasmid cloning bias and colony-picking stochasticity are difficult to avoid, which may give an inconsistent result (Fig 5.5B) (Grunau et al., 2001). Thus, I developed a bisulphite-digestion method which uses

restriction enzyme to distinguish methylated and unmethylated DNA sequences, rather than plasmid cloning followed by Sanger sequencing. However, the accuracy of this method is heavily relied on the DNA sequence at the enzyme cutting sites. Constantly methylated CpGs within the CGCG cutting site can affect the digestion results, leading to variations in the intensity of cut fragments, thereby influencing the methylation levels. For instance, in the *PEG3* DMR, even the lowest methylation level in naïve H7 hESCs was around 70% (Fig 5.9Aiii). This could be due to both CpGs within the CGCG cutting site being consistently methylated on paternal and/or maternal alleles in some cells, resulting in higher intensities of methylated fragments. The exact reason for this constant methylation of these CpGs remains unclear. Given that the primary goal of this experiment is to distinguish the methylation levels in hESCs before and after naïve conversion, it is not necessary to achieve single CpG resolution as long as the method can reveal differences in methylation levels between two cell lines. Thus, the bisulfite-digestion technique is suitable for this purpose.

Methylation levels of five selected DMRs (*PLAGL1*, *KCNQ1OT1*, *L3MBTL*, *PEG3* and *GRB10*) were analysed in WT H7 and H7-OE hESCs before and after naïve conversion. All selected maternal DMRs showed comparable methylation levels between primed WT H7 and H7-OE hESCs (Fig 5.8 & 5.9), confirming that NLRP7 alone does not change methylation levels on the DMR of imprinted genes. After naïve conversion, the methylation level was reduced in WT H7 hESCs, while H7-OE hESCs exhibited comparable methylation levels to the primed hESCs. These results revealed that ectopic NLRP7 expression preserves the methylation levels of maternally imprinted DMRs during the naïve conversion of hESCs, indicating NLRP7 might protect the DNA methylation of maternal DMRs from global demethylation resulting in a partial rescue of the loss of maternal imprinting.

The fact that NLRP7 overexpression does not completely prevent the loss of maternal imprinting in naïve conversion, which might be attributed to several possibilities. 1)

Ectopic NLRP7 expression was not at a sufficiently high level in the primed hESCs (~130-fold increase) given that endogenous NLRP7 expressions in naïve hESCs was approximately 3000-fold higher than in primed hESCs (Fig 4.5A). Since NLRP7 mRNA is scarcely detectable while the protein is abundant in mature oocyte and early preimplantation embryos (Yan et al., 2013, Petropoulos et al., 2016, Akoury et al., 2015), it is difficult to quantify the functional levels of NLRP7 mRNA from *in vivo* RNA-seq data. Thus, NLRP7 expression may still be insufficient to fully protect maternal imprinting during global demethylation. 2) NLRP7 might require additional factors to more efficiently regulate maternal imprinting. A recent study on *KHDC3L*-deficient human oocytes demonstrates that *KHDC3L* regulates imprinting through *de novo* methylation in oocytes and also via the maintenance of maternally imprinted DMRs during early embryonic development (Demond et al., 2019). 3) NLRP7 and *KHDC3L* are observed to co-localise predominant at the cortical region in growing oocytes and early stages of preimplantation embryos (Akoury et al., 2015). Therefore, NLRP7 and *KHDC3L* may cooperate in regulating maternal imprinting during embryonic development. These results indicate that high levels of NLRP7 can partially rescue the loss of maternal imprinting, manifesting as unequal biallelic expression, during the global DNA demethylation process.

Another finding from my experiments is that the majority of NLRP7 proteins were homogeneously distributed in the cytoplasm of NLRP7-OE hESCs (Fig 5.10Bi & Fig 5.11Bi), consistent with the localization observed in cells from the ICM (Akoury et al., 2015). Additionally, a small amount of NLRP7 protein was detected in the nucleus exclusively in H1-OE hESCs which expressed a higher level of NLRP7 (Fig 5.10Bii). This suggests that NLRP7 can translocate into the nucleus in both primed and naïve hESCs, as long as the expression level is sufficiently high. However, despite the abundance of NLRP7 in preimplantation embryos, nuclear localization has not been observed *in vivo* (Akoury et al., 2015). This discrepancy may be attributed to structural

differences in the cytoskeleton between hESCs and early stages of preimplantation embryos, as NLRP7 localise at the outer cortical region during embryonic development until the blastocyst stage, where it redistributes homogeneously into the cytoplasm, similar to the observation in hESCs (Akoury et al., 2015). Therefore, NLRP7 probably does not regulate maternally imprinted DMRs in the nucleus *in vivo*. Interestingly, NLRP7 was found to be localised near nuclear DNA at anaphase in NLRP7-OE hESCs (Fig 5.10Bii & Fig 5.12B). Recent research has shown that the LRR region of the NLRP1 protein has a high affinity for dsDNA and dsRNA (Bauernfried et al., 2021). Given that NLRP7 also contains an LRR domain with a high sequence identity to that of NLRP1 (data not shown), it is plausible that NLRP7 may bind to dsDNA to directly regulate maternal DMRs. However, further experiments are necessary to test this hypothesis. Therefore, these findings provide a new notion that NLRP7 may regulate maternally imprinted DMRs during cell division and directly interact with DNA sequence or regulate maternal imprinting through some histone proteins.

In summary, this chapter demonstrates that high expression levels of NLRP7 can protect maternally imprinted genes by regulating methylation on maternally imprinted DMRs, thereby partially rescuing maternal imprinting from the global DNA demethylation process. In addition, as a cytoplasmic protein, NLRP7 can translocate to the cell nucleus and localize near nuclear DNA during anaphase of mitosis. Therefore, it is likely that NLRP7 regulates maternally imprinted DMRs during cell division, either through direct interaction with DNA sequences or by influencing maternal imprinting via histone proteins.

Chapter 6

General Discussion

Genomic imprinting is an epigenetic phenomenon, which regulates gene expression based on a parent-of-origin-specific manner (Reik and Walter, 2001). It is established by *de novo* DNA methylation during germ cell development and is preserved through the global DNA demethylation that occurs post-fertilization (Smallwood and Kelsey, 2012, Wang et al., 2014). However, the mechanisms of its establishment in germ cells and maintenance during early embryonic stages remain to be fully understood. Notably, defective *NLRP7* can lead to BiCHM, and a study on these molar tissue samples has shown a lack of methylation on maternal DMRs in the embryonic tissue which results in the loss of maternal imprinting (Sanchez-Delgado et al., 2015). Therefore, the study has linked *NLRP7* with maternal imprinting. However, it still lacks direct evidence yet that *NLRP7* has a function in regulating maternal imprinting. In this project, I have investigated the effect of *NLRP7* on the status of maternal imprinting by genetically manipulating the expression of *NLRP7* in hESCs and using naïve conversion of hESCs as a cell model to recapitulate the process of global DNA demethylation. I have shown that hESCs with low expression of *NLRP7* lose maternal imprinting after naïve conversion and that ectopically overexpressing *NLRP7* in hESCs partially rescued the loss by preserving DNA methylation on maternal DMRs. These findings indicate that *NLRP7* may have a protective role on maternally imprinted genes.

Furthermore, I found the *NLRP7* predominantly resides in the cytoplasm but can translocate into the nucleus when expressed at sufficiently high levels. Importantly, this project revealed that *NLRP7* has a close contact with genomic DNA during cellular mitosis when the nuclear envelope is broken, which implies a possibility that *NLRP7* may perform its protective function on maternally imprinted genes at this stage.

6.1 Naive conversion of hESCs as a cell model for the study of NLRP7 on maternal imprinting during global demethylation in embryonic development

The analysis of methylome in molar tissues from females with *NLRP7* mutations has revealed that defective *NLRP7* can cause loss of methylation on maternally imprinted DMRs which results in the loss of maternal imprinting (Sanchez-Delgado et al., 2015). This aberrant hypomethylation on maternal DMRs could originate from several critical stages during oogenesis and early embryonic development: 1) an abnormality in *de novo* methylation during oogenesis affecting the establishment of maternal imprinting; 2) a defect in the maintenance of methylation on maternal DMRs during postzygotic demethylation (Proudhon et al., 2012). My results showed that NLRP7 positively retain DNA methylation on maternal DMRs during the global DNA demethylation process. Since zygotes contain abundant NLRP7 protein accumulated during oocyte maturation, NLRP7 may have a role in maintaining DNA methylation during postzygotic demethylation (Section 5.2.4).

Mechanistically, DNA methylation patterns can be reset in the genome through passive and active DNA demethylation followed by re-methylation. The active demethylation pathway relies on TET proteins which can oxidase 5mC and eliminate the methyl group by replication-dependent dilution or BER pathway (Wu and Zhang, 2017). Conversely, the passive process involves the dilution of DNA methylation marks by DNA replication, primarily due to the absence of DNMT1 and/or UHRF1 (DNMT1 cofactor) or sequestration from the nucleus (Kagiwada et al., 2013).

During the naïve conversion of hESCs, all TETs transcript levels were significantly upregulated (section 4.2.4). In addition, 5hmC was detected by immunostaining in hESCs before and after t2iLGö mediated naïve conversion, indicating that active demethylation contributes to global DNA demethylation during this process (Takashima

et al., 2014). The increased mRNA expressions of TET1, TET2, and TET3 during naïve conversion suggest that all three proteins may play roles in active DNA demethylation in hESCs. As for passive demethylation during naïve conversion, my result showed that DNMT1 expression was reduced to 70% during the naïve conversion process (section 4.2.4), which could significantly reduce its activity to methylate newly synthesised DNA, leading to demethylation. It has been reported that a 40% reduction in DNMT1 has been shown to be sufficient to induce demethylation on the promoter of CDH1 gene in human hepatocellular carcinoma cells (Fan et al., 2007). Moreover, purified naïve hESCs by 5iLAF exhibited a 50% decrease in DNMT1 at both mRNA and protein levels and a 50% reduction of UHRF1 protein (Pastor et al., 2016). Thus, passive demethylation during naïve conversion of hESCs is likely mediated by downregulation of DNMT1 and UHRF1 expression. Consequently, both active and passive demethylation mechanisms contribute to the global DNA demethylation during naïve conversion of hESCs.

During human preimplantation development, the greatest reduction in DNA demethylation occurred between gametes and zygotes, exhibiting a low 5mC and high 5hmC staining on paternal pronuclei compared to maternal pronuclei (Fulka et al., 2004, Pendina et al., 2011, Fulka et al., 2008). This result provides evidence that active DNA demethylation contributes to the demethylation process, and it is more robust in the paternal genome. *TET3* transcript levels are much higher than those of *TET1* and *TET2* in human oocytes and zygotes (Okabe et al., 2014, Kobayashi et al., 2012, Yan et al., 2013), suggesting that TET3 is primarily responsible for active DNA demethylation in preimplantation embryos. During cleavage divisions, DNA demethylation possibly takes place via passive replication-dependent mechanisms. This is evidenced by the 5mC staining of metaphase chromosomes, which showed decreasing proportions of asymmetric 5mC staining of sister chromatids in both parental chromosomes from the 2-cell to morula stage (Pendina et al., 2011, Efimova

et al., 2015). However, both DNMT1 and UHRF1 were abundant at the mRNA and protein levels (Yan et al., 2013, Petrusa et al., 2014), which is different from the naïve conversion process. The exact mechanism of passive demethylation in human preimplantation is still unknown, but despite the downregulation of Dnmt1 after 2-cell stage in mouse preimplantation embryos (Uysal et al., 2017), the exclusion of Dnmt1 from the nucleus is another possible factor to induce passive DNA demethylation (Branco et al., 2008, Kurihara et al., 2008). Therefore, both passive and active demethylation contribute to the DNA demethylation process in preimplantation embryonic development.

In hPGC development, active demethylation is involved in the erasure of DNA methylation. The 5hmC staining shows the presence of 5hmC in female hPGCs in week 4 but depletion from week 7 to week 10 (Guo et al., 2015, Tang et al., 2015), indicating that active demethylation plays a role in the initial stages of hPGC development. TET1 protein is highly expressed, while TET2 is modestly expressed, and TET3 is absent in female hPGCs from week 4 to week 9 after gestation when they migrate to the congenital ridge (Tang et al., 2015, Gkoutela et al., 2015, Guo et al., 2015), suggesting active demethylation in hPGC development is predominantly mediated by TET1. Passive demethylation probably contributes to this process as well. DNMT1 and UHRF1 are extremely low in hPGCs between week 4 to week 9, probably being responsible for the passive demethylation in hPGC development (Tang et al., 2015, Gkoutela et al., 2015, Guo et al., 2015). Additionally, passive demethylation also facilitates the dilution of 5mC oxidative products by TET proteins. Passive dilution of 5mC and 5hmC is supported by the absence of 5hmC further oxidative products, like 5fC and 5caC, as well as BER pathway member, TDG gene, in hPGCs from week 7 to week 9 (Tang et al., 2015).

Taken together, the demethylation processes in naïve conversion of hESCs, preimplantation embryonic development and hPGCs development involve both active

and passive mechanisms. During the naïve conversion process, the passive mechanism is induced by the downregulation of DNMT1, similar to hPGC development. Active demethylation is mediated by increased expressions of TET1/2/3, resembling a combination of TET1/2 upregulation in hPGCs and high TET3 expression in preimplantation development. The dynamic gene expressions related to DNA demethylation during naïve conversion share characteristics with both hPGC development and preimplantation development. Thus, the DNA demethylation machinery in naïve conversion appears to be a combination of these two demethylation waves. Consequently, the naïve conversion of hESCs serves as a good cell model for mimicking global DNA demethylation *in vivo*.

Given the similarities between the DNA demethylation machinery during naïve conversion and the two waves of demethylation in human embryogenesis, NLRP7 might be able to perform the function to preserve DNA methylation on maternal DMRs *in vivo* as well. However, the precise mechanisms of DNA demethylation on maternal DMRs during naïve conversion remain unclear.

During naïve conversion, since passive demethylation is a DNA replication-mediated dilution process, DNA methylation on maternal DMRs can be gradually erased after each DNA replication due to the low activity of methylation maintenance machinery resulted by the reduced levels of DNMT1 during naïve conversion. Recent evidence has shown that transgenic mESCs with defective DNMT1 could induce hypomethylated maternal DMRs, such as *Peg10*, *Peg3* and *Grb10* DMRs (Choudhury et al., 2024). Additionally, both Tet1 and Tet3, but not Tet2, have shown the regulatory function on maternal DMRs from mouse studies. Fusion of mouse embryonic germ cells (mEGCs) with human B cells can result in DNA demethylation including genomic imprinting within the somatic genome from human B cells. In this system, *Tet1* depletion can induce a loss of 5hmC accumulation at parentally imprinted DMRs, such as *H19*, *SNRPN/SNURF*, and *PEG3* ICRs. Whereas *Tet2* depletion in mEGCs has no

effect on genomic imprinting (Piccolo et al., 2013). Moreover, Tet3 may regulate some certain imprinted DMRs. In comparison with Tet1/2 double knock-out mESCs, Tet1/2/3 triple knock-out mESCs have higher methylation levels on *Snrpn* DMR and *Peg1* DMR, while *Peg3* DMR and *H19* DMR are not significantly different (Liu et al., 2015). Therefore, both passive and active mechanisms probably are involved in inducing the erasure of DNA methylation on maternal DMRs during naïve conversion of hESCs.

During preimplantation embryonic development, DNA methylation in the maternal genome gradually declines, likely due to a passive demethylation mechanism (Zhu et al., 2018, Guo et al., 2014, White et al., 2016). Although active demethylation mediated by Tet3 also occurs in the maternal genome of mouse zygotes, it does not show specificity on maternal DMRs but is widespread throughout the genome to a lesser extent when compared to the paternal genome (Shen et al., 2014). Therefore, DNA methylation on maternal DMRs is most likely affected by passive demethylation. Given that both passive and active mechanisms mediate the loss of DNA methylation on maternal DMRs during naïve conversion, the maternal genome might undergo a more robust demethylation process compared to preimplantation embryonic development. Therefore, the naïve conversion of hESCs could be used to serve as a more robust *in vitro* model for studying DNA demethylation on maternal DMRs during preimplantation embryonic development. Since ectopic NLRP7 can preserve DNA methylation on maternal DMRs and partially rescue the loss of maternal imprinting during naïve conversion, NLRP7 may perform a similar function in human embryos, and facilitate the maintenance of differential methylation profiles on maternal DMRs during preimplantation development.

6.2 Role of NLRP7 in protecting/remethylating maternal imprinted DMRs

To date, most research on *NLRP7* is mainly case reports, with few studies validating the relationship between *NLRP7* and maternal imprinting. In this project, I have demonstrated that *NLRP7* may have a function to maintain DNA methylation on maternally imprinted DMRs during global DNA demethylation. However, the precise role of *NLRP7* in regulating maternal DMRs remains unclear. There are two potential mechanisms for this function: 1) protecting maternal DMRs from global demethylation machinery; 2) remethylating the maternal DMRs after demethylation to retain their methylated status. Therefore, it is interesting to explore the potential mechanism of *NLRP7* in regulating DNA methylation on maternal DMRs.

Based on the discussion from the last section, both passive and active demethylation are possibly involved in inducing the erasure of DNA methylation on maternal DMRs. Regardless of the mechanism by which DNA demethylation occurs, genomic DNA is located in the cell nucleus, while *NLRP7* proteins are predominantly localised in the cytoplasm of hESCs, particularly at the subcortical areas, away from direct contact with genomic DNA. Although limited nuclear *NLRP7* signals were observed in hESCs when its expression is very high (Section 5.2.7), it requires more experiments to test whether the small amount of nuclear *NLRP7* is able to prevent DNA demethylation on maternal DMRs. On the other hand, hESCs with detectable expression of *NLRP7* protein that is localised in cytoplasm without nuclear signal observed, have shown alleviated DNA demethylation on maternal DMRs in naïve conversion, comparable to the hESCs with very high *NLRP7* expression (Section 5.2.6). These results suggest that the cytoplasmic *NLRP7* is the main contributor to retaining DNA methylation on maternal DMRs but how could the cytoplasmic protein regulate maternal genomes which reside in the nucleus? Notably, my result revealed that *NLRP7* can be in close contact with nuclear DNA during cell division when the nuclear envelope breaks down (Section

5.2.7). This result provides a new idea that NLRP7 may regulate maternal DMRs during the mitotic phase.

The other way to retain maternal DMRs is to remethylate DMRs after they are demethylated either through active or passive demethylation. The expression levels of DNMTs are changed divergently during naïve conversion of hESCs. The expression levels of *de novo* methyltransferases DNMT3A/3B/3L were generally elevated during naïve conversion, but their dynamic trends were variable. DNMT3A showed a progressive increase to around 2-fold, while DNMT3B exhibited a transient spike followed by a drop to the initial level. Interestingly, DNMT3L exhibited a continuous upregulation with a surge and reached to approximately 1400-fold increase (section 4.2.4). However, Dnmt3b is not shown to be involved in *de novo* methylation on maternal genomes in mice, as offsprings from females with defective *Dnmt3b* showed no apparent phenotype and methylation status of maternally imprinted genes were not affected (Kaneda et al., 2004). By contrast, offspring from mutant *Dnmt3a* or *Dnmt3l* females exhibited erasure of methylation at maternal DMRs and a loss of allelic-specific expression in maternally imprinted genes (Hata et al., 2002, Kaneda et al., 2004), indicating these two genes are responsible for methylating maternal DMRs. Due to the lack of a catalytic domain in Dnmt3l, the individual Dnmt3l could not perform *de novo* methylation (Chédin et al., 2002). Notably, research found that Dnmt3a could methylate maternal DMRs without Dnmt3l but it was strikingly stimulated by interaction with Dnmt3l (Chédin et al., 2002). Given that DNMT3A can methylate maternal DMRs solely and hESCs do not express DNMT3L in oocytes and early embryos, NLRP7 may facilitate DNMT3A to remethylate hypomethylated maternal DMRs after DNA demethylation.

Interestingly, DNMT1 is found to be localised to replication foci and constitutive heterochromatin to perform methylation maintenance on parental genomes from late S throughout the G2 and M phases (Easwaran et al., 2004, Hervouet et al., 2012). This

enrichment of DNMT1 in the M phase provides a possibility that NLRP7 might be able to facilitate DNMT1 to regulate maternal DMRs, as NLRP7 could close contact with nuclear DNA at the M phase when the nuclear envelope breaks down (Section 5.2.7). Notable, a recent study in mESCs showed the DNA methylation at DMRs was erased by the absence of *Dnmt1* or *Uhrf1*, indicating that both DNMT1 and UHRF1 are necessary for the maintenance of DNA methylation at DMRs (Liu et al., 2022). During the naïve conversion process, DNMT1 was reduced to 70% (around 0.021% of β -actin) but later returned to the initial level in hESCs (section 4.2.4). Therefore, the remaining DNMT1 may still be able to maintain DNA methylation on maternal DMRs. Consequently, NLRP7 may preserve DNA methylation on maternal DMRs by facilitating DNMT1 and UHRF1 to remethylate the hypomethylated DMRs after global DNA demethylation.

6.3 Potential molecular mechanisms by which NLRP7 may be involved in regulating maternal imprinting.

During the DNA demethylation process in naïve conversion, my results showed overexpressing NLRP7 can preserve DNA methylation on maternal DMRs, partially rescuing the loss of maternal imprinting. This finding raises two questions: First, how does NLRP7 exclusively recognize maternal DMRs but not paternal DMRs? Second, why does ectopic NLRP7 only partially rescue maternal imprinting?

In this project, I have shown the ectopic NLRP7 expression could partially rescue the loss of maternal imprinting during global DNA demethylation process, while the methylation on paternal DMR was not affected. This result is consistent with previous research about the methylation level on paternal and maternal DMRs in CHM embryos (Sanchez-Delgado et al., 2015). Therefore, NLRP7 can regulate maternal imprinting specifically, but not paternal imprinting. However, how does NLRP7 specifically recognise maternal DMRs not paternal DMRs remains elusive. To explore the underline mechanism about NLRP7 regulating maternal DMRs, I have shown that NLRP7 proteins are localised near nuclear DNA during metaphase to anaphase of mitosis, it is possible that NLRP7 may have the ability to directly recognise and bind to the DNA sequence of maternal DMRs or through interaction with other proteins containing DNA-binding motif. The LRR domain is a common domain shared between NLRP family members, which has been mainly recognised as a protein recognition motif (Kobe and Kajava, 2001). However, recent research has shown that the LRR region of the NLRP1 protein has a high affinity for dsDNA and dsRNA (Bauernfried et al., 2021), though specific DNA/RNA motifs for LRR recognition have not been identified yet. Given the high sequence homology between the LRR regions of NLRP7 and NLRP1, it is plausible that the LRR in NLRP7 may also be able to directly recognise DNA sequences in maternal DMR. Since overexpressing NLRP7 can specifically rescue the loss of DNA methylation on maternal DMRs but not paternal

DMRs, it is interesting to find whether the binding motif for LRR recognition is highly distributed on maternal DMRs but not paternal DMRs if the LRR region of NLRP7 truly recognise DNA sequence. In addition to the LRR domain, other domains in NLRP7 also exhibit protein-protein interactions, potentially binding to DNA-binding proteins. It has been reported that the YY1 protein interacts directly with the PYD domain of NLRP7 (Mahadevan et al., 2014). YY1 is a well-known transcription factor and a director for histone deacetylases (Bushmeyer et al., 1995, Aoyama et al., 2010). The research found that YY1 can directly bind to histone deacetylase 2 (HDAC2) to repress gene expression via DNA methylation on the promoter region (Aoyama et al., 2010). HDACs can reset the lysine of H3K9ac, allowing H3K9 to be methylated by G9a protein. The methylated H3K9 enables binding of the heterochromatin protein 1 (HP1), which facilitates the formation of heterochromatin. Subsequently, G9a recruits DNMT3A and DNMT3B to catalyse *de novo* DNA methylation at the promoters (Feldman et al., 2006, Epsztejn-Litman et al., 2008). Additionally, HDAC2 has also been shown to bind directly to DNMT1 and DNMT3A (Rountree et al., 2000, Ling et al., 2004). Thus, the YY1-HDAC2 complex may recruit DNMTs to perform DNA methylation. Intriguingly, some studies demonstrate that YY1 can identify some ICRs in a target-specific manner (Kim and Kim, 2008, Kim et al., 2006). The consensus DNA binding motifs recognised by YY1 protein present as CGCCATnTT (n indicates any base at that position), while the shorter core motif GCCAT is essential for the binding of YY1 protein (Kim et al., 2006, Yant et al., 1995). In addition, a recent study has indicated a higher preference for YY1 binding to maternal DMRs (Zink et al., 2018). Therefore, this could explain why NLRP7 only can regulate maternal DMRs but not paternal DMRs. Additionally, the Zinc Finger Protein 57 (ZFP57) protein is another possible interactive protein. ZFP57 can directly bind to the methylated allele of imprinted DMRs, and its binding motifs (TGCCGC) are present within the majority of imprinted gDMRs, including maternal DMRs (*KCNQ1OT1*, *NAP1L5* and *PEG3*) (Anvar et al., 2016, Quenneville et al., 2011). In addition, ZFP57 can recruit KAP1 on the methylated allele of imprinted DMRs and

these two proteins are colocalised with H3K9me3 which further regulates methylation on imprinted DMRs as discussed above (Quenneville et al., 2011).

In addition, although DNMT3L and STELLA have not shown direct or indirect interaction with NLRP7, these proteins are responsible for the establishment and protection of maternal imprinting, respectively. Dnmt3l and Dnmt3a are responsible for *de novo* methylation during oogenesis, particularly on maternal DMRs (Shirane et al., 2013, Bourc'his et al., 2001). However, DNMT3L is absent in human oocytes and early stages of human preimplantation embryos, while it is dramatically upregulated from the morula stage (Yan et al., 2013, Gkountela et al., 2015). Stella has been shown to recognise maternal-specific H3K9me2 to protect the maternal genome from Tet3 and it is also expressed in human oocytes and embryos (Nakamura et al., 2012, Yan et al., 2013). Nevertheless, whether these proteins facilitate NLRP7 in identifying maternal DMRs remains to be determined.

As discussed above, NLRP7 is composed of several domains that are capable of protein-protein interaction. This molecular structure of the NLRP7 protein makes it very likely that NLRP7 may function through multiprotein complexes. This may also explain why exclusively overexpressing NLRP7 is unable to fully rescue the loss of maternal imprinting during naïve conversion of hESCs (section 5.2.4). It is likely that other interacting proteins also contribute to preventing the DNA demethylation on maternal DMRs during global demethylation occurring during naïve conversion, thereby enhancing the protective efficiency of NLRP7.

Given the main subcortical localisation and maternal effect nature of NLRP7, it has been proposed that NLRP7 might be a member of the subcortical maternal complex (SCMC) (Monk et al., 2017). SCMC is a protein complex consisting of maternal effect proteins NLRP5, KHDC3L, OOEP and TLE6, though the combined molecular weight is much less than the total SCMC, suggesting that more proteins involved in this complex have not been identified (Zhu et al., 2015). Interestingly, maternal deficiency

in some SCMC members, like NLRP5 and KHDC3L, can cause abnormal epigenetic profiles at imprinted loci in embryos (Docherty et al., 2015, Demond et al., 2019). It is possible that NLRP7 performs a protective role on maternal DMRs via a mechanism involving the SCMC complex. The extremely low expression levels of these SCMC members in primed hESCs might explain why maternal imprinting is not fully rescued by ectopic NLRP7 after naïve conversion (Pastor et al., 2016, Bi et al., 2022).

To test this idea that possible interaction of LRR region with other proteins or DNA sequence, the LRR region could be singly expressed in cells by transformation of plasmid with LRR sequence and collected for immunoprecipitation and mass spectrometry to identify possible interactive proteins and ChIP-PCR for possible DNA sequence motifs. For these known genomic imprinting related proteins or NLRP7 possible interactive proteins, the yeast two hybrid assay could simply to test whether two protein interacts or not. If two proteins could interaction, it is interesting to study the localisation of these two proteins through immunostaining to identify whether this interaction occurs in the cell nucleus during cell division (Figure 5.12), since the NLRP7 was found to close to the chromosomes during cell division.

Taken together, the exact molecular mechanisms by which NLRP7 regulates maternal imprinting remain elusive, but all the recent progress in this field has facilitated future investigation.

6.4 FUTURE WORK

6.4.1. Validation of current findings

This project still has immediate future work to validate current findings. As mentioned in Chapter 4, the dynamic changes of global DNA methylation level during naïve conversion should be analysed by immunostaining or DNA dot blot with an antibody against 5mC to monitor the dynamic changes in DNA methylation levels during naïve conversion and its relationship with NLRP7 expression. This experiment could provide additional information to illustrate the relationship between DNA methylation and NLRP7 expression and provide strong evidence that the dramatic upregulation of NLRP7 expression is caused by genomic DNA demethylation. Therefore, the loss of maternal imprinting in naïve conversion could be attributed to the low level of NLRP7 in primed hESCs. In addition, even though the naïve conversion of NLRP7-OE hESCs has been down twice in H7 hESC lines by different people, and both experiments showed consistent results that the ectopic NLRP7 can partially rescue the loss of maternal imprinting in naïve conversion, only one cell line is still limited and cannot eliminate the bias of cell identity. Therefore, these experiments need to be repeated in more cell lines to further validate this result. Furthermore, many reoccurring NLRP7 mutations were reported and discussed in the introduction section, and it would be interesting to investigate that how these mutations affect the maternal imprinting in hESCs during their naïve conversion. Given that most mutation sites are located at the LRR region and that one frequent mutation site found from patients is NLRP7-E570del which expresses NLRP7 protein without LRR region, we could generate a truncated NLRP7 expression vector without LRR region and express this truncated NLRP7 into hESCs and investigate how this affects maternal imprinting upon naïve conversion. If loss of maternal imprinting is not improved by the truncated NLRP7 as the wild-type NLRP7 after naïve conversion, it would further validate that NLRP7 indeed play a role in maintaining maternal imprinting during global DNA demethylation process. This

experiment may provide more evidence to support the function of NLRP7 on maintaining maternal imprinting during global DNA demethylation process. Explore whether NLRP7 could influence the establishment of oocyte-specific methylome during oogenesis

In this project, my results have indicated that NLRP7 play a role in maintaining maternal imprinting during global DNA demethylation. Since deficient KHDC3L can also result in molar pregnancy and recent study has shown KHDC3L not only preserves maternal imprinting in preimplantation embryos but also facilitates to establishment of genome-wide methylation patterns in oogenesis (Demond et al., 2019). It is interesting to investigate whether NLRP7 has a similar function in the establishment of oocyte methylome during oogenesis. The genome-wide methylation analysis could be performed in oocytes from a patient with *NLRP7*-mutation to investigate whether DNA methylation in oocytes is affected by defective *NLRP7* in human oocytes. A similar experiment was conducted in oocytes from a patient with *KHDC3L* mutation (Demond et al., 2019), indicating this method is feasible to achieve this purpose. However, this approach requires the identification of patients and ethical approval.

Due to ethical concerns, it is very difficult to test this hypothesis in vivo. The generation of NLRP7-defective hESCs could be a good in vitro model for such a study. The research found that naïve hESCs can also be converted to the primed state, a process called capacitation (Rostovskaya et al., 2019). During this process, the genome-wide DNA methylation was reestablished, but genomic imprinting was not rescued (Pastor et al., 2016). Although allele-specific methylation cannot be restored during the capacitation, this cell model could be used to investigate the potential function of NLRP7 in overall *de novo* DNA methylation as well as other epigenetic changes (Agostinho de Sousa et al., 2023).

An alternative method is to develop an *in vitro* model mimicking the oogenesis (Saitou and Hayashi, 2021). A recent study has established PGC-like cells (PGCLCs) in

cynomolgus monkeys (cy) from cyESCs. The resulting cyPGCLCs are further differentiated into early oocyte-like cells upon aggregation with mouse embryonic ovarian somatic cells (Gyobu-Motani et al., 2023). Since *NLRP7* is also expressed in primates (Tian et al., 2009), *NLRP7* could be knocked out in cyESCs and perform *in vitro* oogenesis to mimic the *de novo* DNA methylation *in vivo* to investigate whether defective *NLRP7* affect the establishment of methylation pattern during oogenesis. In addition, human PGCLCs have been developed as well, which share similar gene expression profiles with human PGCs (Yamashiro et al., 2018), suggesting that human PGCLCs have the potential to perform *in vitro* oogenesis. Therefore, this idea could also be achieved when human *in vitro* oogenesis becomes fully developed and relative ethical regulation has been established.

6.4.2. Explore the possible mechanisms by which NLRP7 regulates maternal imprinting

Up to now, the mechanistic function of *NLRP7* is still less understood, because only primates and humans express the *NLRP7* gene but limited human materials are available for research. Since *NLRP7* and most of its putative partners discussed in the previous section are highly expressed in naïve hESCs (Pastor et al., 2016), the naïve hESCs could be used as an unlimited resource to investigate the interaction and localisation of *NLRP7* with other proteins. The interaction of *NLRP7* and other putative interactive proteins could be studied, for example, YY1 and KHDC3L, through *NLRP7* immunoprecipitation, mass spectrometry analysis and further validation via Co-IP, co-immunostaining and proximity ligation assay. A new finding from this project showed that *NLRP7* can be in close contact with nuclear DNA during cell division and a recent publication showed that the LRR region of the *NLRP1* protein has a high affinity for dsDNA and dsRNA (Bauernfried et al., 2021). These results provide a new idea that *NLRP7* may have the ability to bind maternal DMRs directly. Therefore, the ChIP-seq

technique can be performed using cell lysate from naïve hESCs to investigate whether some fragments of maternal DMRs are recognised by NLRP7 protein.

6.5 SUMMARY

Genomic imprinting is an epigenetic phenomenon that regulates gene expression in a parent-of-origin-specific manner (Reik and Walter, 2001). It is established by *de novo* DNA methylation during germ cell development and is preserved through global DNA demethylation after fertilization (Smallwood and Kelsey, 2012, Wang et al., 2014). However, the underline mechanism of regulation in DNA methylation is not fully understood yet. Interestingly, the defective NLRP7 has shown to affect the methylation on maternal DMRs in the embryonic tissue from BiCHM, leading to the loss of maternal imprinting and BiCHM (Sanchez-Delgado et al., 2015). However, it still lacks direct evidence to support this function of NLRP7. In this project, I investigated the effect of NLRP7 on maternal imprinting by manipulating NLRP7 expression in hESCs and using naïve conversion of hESCs as a model to mimic the process of global DNA demethylation. Although embryonic tissues from BiCHM with defective NLRP7 have already shown the loss of maternal imprinting, this project is the first time to provide direct evidence from molecular level to verify that hESCs with low expression of NLRP7 lose maternal imprinting after naïve conversion and that ectopically overexpressing NLRP7 in hESCs could partially rescue this loss by preserving DNA methylation on maternal DMRs. These findings indicate that NLRP7 may have a protective role on maternally imprinted genes. Furthermore, NLRP7 has been found for the first time being able to translocate into the nucleus when expressed at sufficiently high levels although majority of the proteins reside in the cytoplasm as previously reported. In addition, this project revealed that NLRP7 has a close contact with genomic DNA during cellular mitosis when the nuclear envelope disappears, which is an important finding, implying a possibility that NLRP7 may perform its protective function on maternally imprinted genes at this stage. This finding may give more information and suggestion to explore the function of NLRP7 on maternal imprinting.

There are still some unanswered questions to be explored in future work. Firstly, both embryonic tissues of BiCHM and our molecular experiment results showed that NLRP7 specifically played a role on maternal imprinting, while paternal imprinting was not affected by absent or defective NLRP7. Why does NLRP7 have no effect on paternal imprinting and how could NLRP7 differentially recognise maternal and paternal alleles? Secondly, in this project, I only focused on the function of NLRP7 during the global DNA demethylation process of primed to naïve conversion of hESCs. However, whether NLRP7 play a role in the establishment of DNA methylation on maternally imprinted genes is still to be addressed. Finally, the underlying mechanisms of NLRP7 in the regulation of maternal imprinting is still largely unknown. It remains unclear whether NLRP7 can directly recognise maternal DMRs or it needs to bind other proteins to preserve DNA methylation on maternal DMRs. These questions are still waiting for more experiments to find answers in future.

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