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**EPIGENETIC AND CHROMATIN
REPROGRAMMING IN MOUSE DEVELOPMENT
AND EMBRYONIC STEM CELLS**

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Ph.D.

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CONTENTS

Contents	a-j
List of Tables	h-i
List of Figures	j-l
Declaration	i
Abstract	ii
Publications and communication arising from this thesis	iv
Acknowledgements	vi
Abbreviation	ix
<u>Chapter1 General Background</u>	
1.1. Chromatin Structure	2
1.1.1. Heterochromatin and Euchromatin	4
1.2. Epigenetics – Definitions	9
1.3 Histone Modifications and Epigenetics	10
1.3.1 Histone Acetylation	10
1.3.2 Histone Phosphorylation	11
1.3.3 Histone Ubiquitination	11
1.3.4 Histone Sumoylation	12
1.3.5 Histone Methylation	13
1.3.6 Histone Variants	14
1.3.7 Reading the Histone code	19
1.3.8 DNA Methylation	21
1.3.9 Polycomb and Trithorax proteins	22
1.3.10 Heterochromatin Protein 1 (HP1)	23
1.4 Chromatin Modification in Mouse Development	24
1.4.1 Chromatin Modification in Early Germ Cell Development	24
1.4.2 Chromatin Modification in Oogenesis	25
1.4.3 Chromatin Modification in Spermatogenesis	26
1.4.4 Chromatin after Fertilization	31
1.4.5 Chromatin at Peri-implantation	34

Contents	Page
1.5 Genomic Imprinting Controlled by Epigenetics	36
1.6 Epigenetic Alterations by Reproductive Biotechnology	39
1.7 Epigenetic Control of Pluripotency and Differentiation	41
1.8 Manipulation of Epigenetic Reprogramming	45
1.9 Objectives of the Project	48
<u>Chapter 2</u> General Materials and Methods	
2.1 Animal and embryo manipulation	51
2.1.1 Animals	51
2.1.2 Immature oocyte recovery and <i>in vitro</i> maturation	51
2.1.3 Zygote collection and culture	52
2.1.4 Peri/Postimplantation embryo collection	52
2.2 Cell culture	54
2.2.1 Primary mouse adult fibroblast culture	54
2.2.2 Primary mouse embryonic fibroblast (MEF) culture	54
2.2.3 Embryonic stem cell culture	54
2.2.4 Isolation of ES-like cell from blastocysts	55
2.2.5 <i>In vitro</i> differentiation of ES cells	55
2.2.6 <i>In vitro</i> implantation and blastocyst outgrowth	56
2.2.7 Cell cryopreservation	56
2.3 Detection of global epigenetic and chromatin modifications.	57
2.3.1 Immunofluorescence	57
2.3.1.1 Immunofluorescence for cell culture and whole mount embryo	57
2.3.1.2 Immunohistofluorescence for postimplantation embryo	58
2.3.1.3 Antigen retrieval	59
2.3.1.4 Image Analysis and Software	59
2.3.1.5 Flow cytometry analysis	60
2.3.1.6 Positive and negative control for immunofluorescence	61
2.3.2 Protein electrophoresis	61
2.3.2.1 Histone extraction (Acid extraction)	61
2.3.2.2 Whole cell extraction	62

Contents	Page
2.3.2.3 Protein concentration measurement	62
2.3.2.4 Western blot analysis	63
2.4 Gene expression analysis	66
2.4.1 Sample preparation for total RNA extraction	66
2.4.2 Total RNA extraction by RNeasy kit (Qiagen)	66
2.4.3 Precipitation of embryo mRNA using Pellet Paint® Co-Precipitant	67
2.4.4 cDNA synthesis	67
2.4.5 Polymerase Chain Reaction (PCR)	68
2.4.6 Agarose Gels for DNA Electrophoresis	69
2.4.7 Real time Quantitative Polymerase Chain Reaction (QPCR)	69
2.4.8 Quantification strategy in QRT-PCR	70
2.5 Gene knock-down by siRNA	
2.5.1 Transfection of siRNA into mouse embryonic fibroblast (MEF) cells	74
2.5.2 Transfection siRNA into ES cells	74
<u>Chapter 3</u> Dynamic of histone lysine methylations, modifiers and satellite sequences in mouse preimplantation embryos	
3.1 Introduction	
3.1.1 Genetic reprogramming in preimplantation embryos	77
3.1.2 Protein synthesis in preimplantation embryos	78
3.1.3 Histone methylation in preimplantation embryos	78
3.1.4 Non-coding RNA and transcript from repetitive element in preimplantation embryos.	79
3.15 Objectives.	81
3.2 Materials and methods	81
3.2.1 Experimental design	81
3.2.2 Comparative and Relative gene expression	81
3.2.3 Statistics	86
3.3 Results	86
3.3.1 Asymmetry of histone methylation at zygote	86
3.3.2 Similarly dynamic pattern of H3K9me2 and H3K9me3 but not H3K9me1	87

Contents	Page
3.3.3 Distinct pattern of H4K20me1, H4K20me2 and H4K20me3	87
3.3.4 H3K4me3 and H3K27me3	88
3.3.5 Most histone demethylases but not methyltransferases were detected through preimplantation development	89
3.3.6 Histone demethylation of H3K9 at two-cell stage	89
3.3.7 Suv4-20h was detected at late preimplantation embryos	90
3.3.8 The differential expression of histone modifier blastocyst compare to ES cell	90
3.3.9 The expression profile of major satellite and minor satellite	91
3.4 Discussion	91
3.4.1 Reprogramming of histone lysine methylation	92
3.4.2 Repressive histone methylation peaked at the eight cell embryo stage	93
3.4.3 Function of satellite transcripts in oocytes	93

Chapter 4 Heterochromatin maturation in mouse development and ES cells

4.1 Introduction	114
4.1.1 Epigenetic regulate heterochromatin formation	114
4.1.2 Function of heterochromatin markers H4K20me3 and HP1 α	115
4.1.3 Hypothesis	116
4.2 Materials and Methods	117
4.2.1 Samples	117
4.2.2 Experimental design	117
4.3 Results	118
4.3.1 Epigenetic signature of mouse somatic heterochromatin	118
4.3.2 Immature chromatin in preimplantation embryo	118
4.3.3 Heterochromatin matures post implantation	119
4.3.4 Dynamic of H4K20me3 and HP1 α in ES cell	122
4.3.5 H4K20me3 was higher associated with cell differentiation <i>in vitro</i> implantation than ES cell differentiation	123
4.3.6 Global heterochromatin shows plasticity in early differentiation	124
4.3.7 <i>In vitro</i> culture induce maturation of constitutive heterochromatin	124

Contents	Page
4.4 Discussion	126
4.4.1 The difference between ICM and ES cells	127
4.4.2 Mechanism of heterochromatin maturation	127
4.4.3 Chromatin and cell differentiation.	128
4.4.4 Heterochromatin, tissue maturation and aging	129
<u>Chapter 5</u> Role of histone demethylases in mouse preimplantation development	
5.1 Introduction	155
5.1.1 Histone demethylases	156
5.1.2 Objectives of the study	156
5.2 Materials and Methods	158
5.2.1 Relative gene expression	158
5.2.2 Isolation of inner cell mass (ICM) and trophectoderm (TE)	158
5.3 Results	159
5.3.1 Jmjd2c regulates cell fate during the morula to blastocyst transition	159
5.3.2 Jmjd2a essential for very early development	160
5.3.3 Jmjd1a is essential for early development and specific at TE	160
5.3.4 Preliminary studies of histone methylation in jmjd1a and jmjd2a deficient embryos	161
5.3.5 Zygotic gene expression in Jmjd1a and Jmjd2a deficient embryo	161
5.4 Discussion	162
5.4.1 Jmjd2 might control epigenetic asymmetry of parental genome	163
5.4.2 Histone demethylases and zygotic gene activation	163
5.4.3 Histone demethylases is important for blastocyst formation	164
5.4.4 Redundancy or functional difference of Jmjd1 and Jmjd2 family	164
<u>Chapter 6</u> General Discussions	
6.1 Overview and discussions	178
6.1.1 The dynamics of heterochromatin markers in preimplantation embryos	178

Contents	Page
6.1.2 Heterochromatin maturation in postimplantation development and in ES cells	179
6.1.3 The function of H4K20me3 in development	180
6.1.4 Global epigenetic different between ES cell and inner cell mass	181
6.1.5 The importance of demethylases in preimplantation development	182
6.2 Conclusions	183
6.3 Directions for future research	183
6.3.1 Chromatin modifications in development embryos	183
6.3.2 Heterochromatin and pluripotency	184
6.3.3 Functions of Histone demethylases in developments	184
6.3.4 Function of histone demethylases in cell reprogramming	185
 <u>Bibliography</u>	 188
 <u>Appendix 1 Detail of Chemicals and Reagents</u>	 216
• Antibodies	216
• Serum and BSA	216
• DNA stain and mounting reagents	217
• Western Blot	217
• PCR	217
• Hormones, growth factors and enzymes	217
• Media	218
• Chemicals	218
• siRNA	218
• Others	219
• Software	219
 <u>Appendix 2 Recipes of Buffer Media and Stock solutions</u>	 220
• Mitomycin C Stock solution	220
• 10x TBE stock	220
• Gelatine for extracellular matrix	220

Contents	Page
• Fibroblast cell medium	220
• Mouse embryonic stem cell medium	221
• Trypsin solution	221
• Phosphate Buffered Saline (PBS)	221
• Histone extraction Buffer	222
• Paraformaldehyde (PFA) 20 % stock solution	222
• Triton X-100 10 % stock solution	222
• Tween 20 (5 % stock solution)	223
• Blocking Solutions	223
• Cryopreservation medium (2x) (10 ml)	223
• RIPA buffer (Radioimmunoprecipitation assay)	223

List of Tables

Table 1.1 Nomenclature of histone modifying enzymes	16
Table 1.2 Histone methylation, methyltransferases, demethylases and their functions in mice	17
Table 1.3 Differences between male and female gametogenesis	28
Table 1.4 Epigenetic modifiers that are critical for mouse development (knock-out mice model)	35
Table 1.5 Imprinted genes that affect embryo and placental development	38
Table 1.6 Pluripotency and differentiation defects are caused by epigenetic alteration	44
Table 2.1 Primary antibodies used in the experiments	64
Table 2.2 Secondary antibodies used in the experiments.	65
Table 2.3 Primer sequences for PCR	72
Table 2.4 Primer sequences for QPCR	73
Table 2.5 siRNA sequences	75
Table 3.1 Mouse strains that are commonly used to study epigenetics in development embryos	86
Table 4.1 The presence and pattern of heterochromatin markers in development and ES cells.	130
Table 5.1 Summary of histone lysine (K) demethylase (KDM) nomenclature and substrate specificity for mice and human	157
Table 5.2 Effect of siRNA knock down at specific genes on preimplantation development.	165
Table 5.3 Relative gene expression of ICM compare to TE.	166
Table 5.4 Non-relative gene expression (no normalisation with reference genes) of ICM compare to TE	166
Table 5.5 Relative gene expression of Jmjd2c depletion embryos compare to normal embryos.	167
Table 5.6 Non-relative gene expression (no normalisation with reference genes) of Jmjd2c depletion embryos compare to normal embryos.	167
Table 5.7 Relative gene expression in Jmjd1a deficient two-cell embryos compare with control	168

Contents	Page
Table 5.8 Non-relative gene expression in Jmjd1a deficient two-cell embryos compare with control	168
Table 5.9 Relative gene expression in Jmjd2a deficient two-cell embryos compare with control	169
Table 5.10 Non-relative gene expression in Jmjd2a deficient two-cell embryos compare with control	169
Table 5.11 Estimated gene expression profile in early implantation embryo from published microarray data and qPCR of the present study.	170

List of Figures

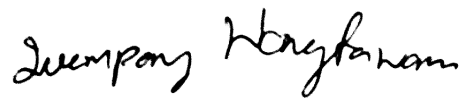
Figure 1.1 Model of nucleosome at 2.8 Å	4
Figure 1.2 The major structures of chromatin compaction	5
Figure 1.3 A map of chromosome territory in a human cell.	6
Figure 1.4 Heterochromatin in mouse fibroblast.	8
Figure 1.5 Summary of histone modifying enzymes	18
Figure 1.6 Summary of histone modification binding domains/proteins	20
Figure 1.7 Diagram of mouse development	29
Figure 1.8 Epigenetic changes in germ cell development	30
Figure 1.9 Dynamics of histone methylation in preimplantation development	33
Figure 2.1 Diagram of embryonic stages collected in this experiment	53
Figure 3.1 Expression of reference gene Gapdh	83
Figure 3.2 Model for calculation of Ct value	84
Figure 3.3 H3K9me1 in preimplantation embryos.	94
Figure 3.4 H3K9me2 in preimplantation embryos	95
Figure 3.5 H3K9me3 in preimplantation embryos	96
Figure 3.6 H4K20me1 in preimplantation embryos	97
Figure 3.7 H4K20me2 in preimplantation embryos	98
Figure 3.8 H4K20me3 in preimplantation embryos	99
Figure 3.9 H3K4me3 in preimplantation embryos.	100
Figure 3.10 H3K27me3 in preimplantation embryos	101
Figure 3.11 Semi-quantification of total nuclear fluorescent intensity of H3K9me1, H3K9me2 and H3K9me3 in preimplantation embryos	102
Figure 3.12 Semi-quantification of total nuclear fluorescent intensity of H4K20me1 and H4K20me2 in preimplantation embryos	103
Figure 3.13 Semi-quantification of total nuclear fluorescent intensity of H3K4me3 and H3K27me3 in preimplantation embryos	104
Figure 3.14 Comparative and relative gene expression of Eset, Suv39h1 and Suv39h2 in preimplantation embryo	105
Figure 3.15 Comparative and relative gene expression of Eset, Ehmt2, Suv39h1 and Suv39h2 in preimplantation embryo	106

Contents	Page
Figure 3.16 Comparative and relative gene expression of Jmjd2a and Jmjd2c in preimplantation embryo	107
Figure 3.17 Comparative and relative gene expression of Jmjd1a in preimplantation embryo	108
Figure 3.18 Comparative and relative gene expression of minor and major satellite in preimplantation embryo	109
Figure 3.19 Comparative and relative gene expression of minor and major satellite in preimplantation embryo	110
Figure 3.20 Relative gene expressions of Eset, Suv39h1 and Suv39h2 compared between blastocyst and ES cell.	111
Figure 3.21 Relative gene expressions of Jmjd1a, Jmjd2a and Jmjd2c compared between blastocyst and ES cell.	112
Figure 4.1 Model for heterochromatin spreading and gene relocation affecting gene expression during development and differentiation	131
Figure 4.2 Experimental designs of the study	132
Figure 4.3 Heterochromatin markers in somatic cells	133
Figure 4.4 Immunofluorescence microscopy images of heterochromatin markers in pre-implantation embryo nuclei	134
Figure 4.5 The mRNA level of <i>suv4-20h1</i> and <i>suv4-20h</i>	135
Figure 4.6 Immunofluorescence confocal microscopy images of <i>in vitro</i> implantation embryos	136
Figure 4.7 Effect of culturing <i>In vitro</i> implantation embryos cultured for 4 days	137
Figure 4.8 Immunofluorescence confocal microscopy images of <i>in vivo</i> pre-implantation and post-implantation embryos	138
Figure 4.9 H3K9me3, not H4K20me3 and HP1 α , was found in early postimplantation embryos	139
Figure 4.10 Immunofluorescence images of post-implantation embryo (E8.5) from paraffin section	140
Figure 4.11 Heterochromatin marker H4K20me3 and HP1 α was not detected in E8.5 embryos	141
Figure 4.12 Immunofluorescence microscopy images (H4K20me3) of post-implantation embryos	142

Contents	Page
Figure 4.13 Immunofluorescence microscopy images (HP1 α) of post-implantation embryos	143
Figure 4.14 Western blots of post-implantation embryos extract SDS-PAGE	144
Figure 4.15 Dynamic of H4K20me3 and HP1 α in ES cells	145
Figure 4.16 Correlation between H4K20me3 and Oct4 of <i>in vitro</i> postimplantation embryo	146
Figure 4.17 Correlation between H4K20me3 and Oct4of <i>in vitro</i> ES cell differentiation	147
Figure 4.18 Proteomic profile of epigenetic marker during ES cell differentiation	148
Figure 4.19 Heterogeneous populations of ES cells	149
Figure 4.20 ES-like cells (noo01)	150
Figure 4.21 Immunofluorescence microscopy images of ES cells	151
Figure 4.22 Western blot of ES cell cultured in medium contained serum compare with serum free medium	152
Figure 4.23 Model for immature heterochromatin in early development	153
Figure 5.1 Validation siRNA by transfection into ES cells	171
Figure 5.2 <i>In vitro</i> development of siRNA-injected embryos	172
Figure 5.3 Loss of H3K9me2 asymmetry in Jmjd1a deficient zygotes	173
Figure 5.4 Loss of H3K9me3 asymmetry in jmjd2a deficient zygotes	174
Figure 5.5 Loss of H3K9me2 asymmetry in jmjd1a deficient two-cell embryos	175
Figure 5.6 Loss of H3K9me3 asymmetry in jmjd2a deficient two-cell embryos	176
Figure 6.1 Model for removal of heterochromatin markers	186
Figure 6.2 Summary of the differences between inner cell mass (ICM) and ES cells	187

DECLARATION

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies

A handwritten signature in black ink, reading "Tuempong Wongtawan". The signature is written in a cursive style with a large initial 'T'.

Tuempong Wongtawan

ABSTRACT

It is well established that epigenetics and chromatin modifications are important factors that can govern gene activity and nuclear architecture. They are also proven to be essential for normal embryonic development and cell differentiation. One important event during mouse development is the establishment of epigenetic reprogramming which is believed to be essential for normal growth and development, however; the mechanism is still poorly understood. The general objective of this PhD study was to investigate the profiles and mechanisms of epigenetic and chromatin modifications during normal mouse development and in embryonic stem cells. Mouse pre- and post-implantation embryos and ES cells were used in experiments employing a range of different methodologies. The dynamics of epigenetic DNA and histone methylation were captured using laser confocal immunofluorescent microscopy and western blotting. The activity of epigenetic modifiers was monitored by real-time PCR and candidate genes were validated using siRNA technology. The present studies demonstrate that heterochromatin markers H3K9me3, H3K9me2, H4K20me2, H4K20me3, HP1 α and HP1 β are reprogrammed during early development. Demethylation of H3K9me2, H3K9me3 and H4K20me3 took place at two-cell stage and remethylation occurred at four-cell stage except for H4K20me3. The reestablishment of H4K20me3 was initially observed in early postimplantation embryos in extraembryonic tissue, specifically in the mural trophoctoderm. In embryonic tissue, H4K20me3 was not clearly detected until in mid to late postimplantation development. The mechanism of H3K9me2 and H3K9me3 demethylation might be due to either an imbalance of epigenetic modifiers or the presence of Jmjd2a and Jmjd1a histone demethylase postfertilisation. We have also report evidence that HP1 α and Suv4-20h are required in heterochromatin before the recruitment of H4K20me3 during mouse development and in ES cells. Therefore H4K20me3 removal was believed to involve the lack of prerequisite heterochromatin complexes such as HP1 α and Suv4-20h enzymes. Furthermore, the presence and levels of H4K20me3 and HP1 α might be strongly associated with cell differentiation and tissue maturation in mouse *in vivo* development but not *in vitro* early differentiated ES cells. Surprisingly, the results showed that chromatin modifications and their modifiers in ES cells are different from

ICM and epiblast. Chromatin modifications H4K20me3 and HP1 α were absent from ICM and epiblast, but were detected in ES cells. Notably, H4K20me3 and HP1 α were established after early incubation of ICM into ES cell medium, but this change was not dependent on the presence of serum and leukaemia inhibiting factor. Epigenetic modifier Jmjd2a but not Jmjd1a was found in ICM. Conversely, Jmjd1a is highly expressed in ES cells while Jmjd2a was inactivated. In addition, the present studies revealed the substantial role of histone demethylases in development, as it may be important for epigenetic reprogramming. The results demonstrated that inhibition of demethylase Jmjd2a and Jmjd1a caused preimplantation embryos to arrest at the two-cell stage while Jmjd2c deficient embryos failed to reach blastocyst. Thus it is possible that Jmjd2a and Jmjd1a were essential for epigenetic reprogramming while Jmjd2c is critical for cell fate establishment during blastocyst formation. In conclusion, the global chromatin signature in ES cells differs from ICM and epiblast; heterochromatin reprogramming occurs at two-cell stage; maturation of heterochromatin occurs at postimplantation; and histone demethylases Jmjd1a, Jmjd2a and Jmjd2c are important in preimplantation development. Results from the present studies could provide crucial information for developmental biology and stem cell research, and provide as a model for improvement of reproductive biotechnologies such as somatic cell reprogramming, and diagnosis of epigenetic abnormalities in early development.

PUBLICATIONS AND COMMUNICATIONS ARISING FROM THIS THESIS

Published abstract

Wongtawan T, Taylor J, Wilmot I, Pennings S. Heterochromatin reprogramming in mouse development. *Reproduction, Fertility and Development*, Vol. 21 No. 1 Pages 149 - 149, Published 9 December 2008.

Manuscripts

Wongtawan T, Taylor J, Lawson K, Wilmot I, Pennings S. Heterochromatin markers H4K20me3 and HP1 α reveal epigenetic difference between developing embryo and ES cells. Submitted to *Journal of Cell Biology*.

Oral presentation

Wongtawan T, Taylor J, Wilmot I, Pennings S. Global chromatin and epigenetic profiles reveal differences between mouse embryonic stem cells, inner cell mass and epiblast. International Society for Stem Cell Research (ISSCR) 7th Annual Meeting, July 8-11, 2009 at Centre Convencions Internacional, Barcelona, Spain.

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“PhD Graduation is not the end of study; it's just the beginning of the next real life course. Everything in your life is the experiment and you have to test the hypothesis and investigate them everyday”



Tuempong Wongtawan

(writing at 85 Iona street, Edinburgh, 10 Dec 2009)

ABBREVIATIONS

5MeC	5-Methyl Cytocine
BSA	Bovine Serum Albumin
Cdx	Caudal type homeo box
CI	Confidential interval
cDNA	Complementary DNA
COCs	Cumulus oocyte complexes
DAPI	4',6-diamidino-2-phenylindole
DS	Donkey Serum
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
ES cell	Embryonic stem cell
Ehmt	Euchromatic histone lysine N-methyltransferase
ESET	ERG-associated protein
ERG	Erythroblastosis virus E26 oncogene
Ezh	Enhancer of zeste
EGF	Epidermal growth factor
FITC	Fluorescein isothiocyanate
GV	Germinal Vesicle
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
H	Histone
HCG	Human Chlorionic Gonadotrophin
H3K9me1	Monomethylated Histone 3 Lysine 9
H3K9me2	Dimethylated Histone 3 Lysine 9
H3K9me3	Trimethylated Histone 3 Lysine 9
H4K20me1	Monomethylated Histone 4 Lysine 20
H4K20me2	Dimethylated Histone 4 Lysine 20
H4K20me3	Trimethylated Histone 4 Lysine 20
H3K27me3	Trimethylated Histone 3 Lysine 27
H3K4me3	Trimethylated Histone 4 Lysine 3
HP1	Heterochromatin protien 1
HMT	Histone methyltransferase
Hprt	Hypoxanthine guanine phosphoribosyl transferase
Hox	Homeobox gene

ICM	Inner cell mass
Jmjd	Jumonji domain protien
KDM	Lysine (K)-specific demethylase 4A
K	Lysine
KSOM	Potassium Simplex Optimised Medium
LINE	Long interspersed repetitive
LIF	Leukaemia inhibition factor
MII	Methaphase II
MuERV	Murine endoretrovirus
MET	Maternal to Embryo transition
mRNA	Messenger RNA
MEM	Minimum Essential Medium
MEF	Mouse embryonic fibroblast
Nanog	Nanog homeobox
NTFI	Nuclear total fluorescent intensity
Oct4	Octamer-4
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
PMSG	Pregant Mare Serum Gonadotrophin
PCR	Polymerase chain reaction
Pol	Polymerase
PcG	Polycomb group protiens
QPCR	Quantitative polymerase chain reaction
REF	Reference gene
RT	Reverse transcriptase
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interference RNA
Sox2	SRY (sex determining region Y)-box 2
Suv	Suppressor of variegation
SINE	Short interspersed repetitive
SUMO	Small ubiquitin-related modifier
TE	Trophectoderm
TRG	Targeted gene

TrxG	Trithorax group proteins
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
TDMs	Tissue-specific differentially methylated regions
Xi	Inactive X-chromosome
XIST	X-inactive specific transcript
ZGA	Zygotic gene activation

CHAPTER 1

General Background

CHAPTER 1

General Background

1.1 Chromatin Structure

DNA and histones are the major components of chromatin in the eukaryotic nucleus. The DNA helix is wrapped around core histones to form a “beads-on-a-string” structure of around 11 nm thickness, of which the basic subunit is called the nucleosome (Figure 1.1) (Felsenfeld and Groudine, 2003, Lewin, 2004). The histone core contains two types of complexes, a tetramer formed by two pairs of histone 3 and histone 4 (H3₂-H4₂) and two dimers of histone 2A and 2B (Figure 1.1). Chromatin also contains various non-histone proteins that are required for its assembly and packaging, DNA replication, chromatin modification, gene transcription, and DNA repair and recombination (Felsenfeld and Groudine, 2003, Lewin, 2004, Tremethick, 2007). The secondary structure of chromatin organisation involves a coiling of the array of nucleosomes into a superhelical type of fibre of 30 nm thickness (Figure 1.2). Each nucleosome is connected to its neighbours by a short segment of linker DNA (approximately 10–80 bp in length) associated with linker histone H1 (Figure 1.2). Finally, the 30 nm chromatin fibre undergoes further levels of packing to form an entire chromosome (Figure 1.2) (Felsenfeld and Groudine, 2003, Lewin, 2004, Tremethick, 2007).

The largest unit of organisation of eukaryotic chromatin is the chromosome. Each chromosome maintains itself individually during the cell cycle and occupies a spatially limited volume, known as a chromosome territory (Bolzer et al., 2005). Each chromosome has a preferred position relative to the centre or periphery of the nucleus, (Figure 1.3) (Fraser and Bickmore, 2007, Bolzer et al., 2005). Chromosome territories are not random, they vary between cell types, and this has an effect on chromosome interactions in *trans* with other parts of the genome (Meaburn and Misteli, 2007, Bolzer et al., 2005). The positional organisation of chromosomes within the nucleus could influence other aspects of genome function, in particular the regulation of gene expression (Fraser and Bickmore, 2007, Meaburn and Misteli, 2007). Concurrently with

transcriptional activation, some genes can relocate a substantial distance outside of their chromosome territory, for example the *Hox* gene cluster (Mahy et al., 2002, Chambeyron and Bickmore, 2004). Conversely, gene repression in the inactivated X-chromosome happens within a chromosome territory; this phenomenon is associated with *Xist* RNA that coats the chromosome which will be inactive excluding Pol II from the compartment that comprises the X-chromosome territory (Chaumeil et al., 2006).

The nucleosome exhibits at least three dynamic properties *in vivo*: composition alteration, covalent modification and translational repositioning. First, the composition alteration is achieved by the exchange between histone variants and canonical histones. For example, remodelling complexes of the SWR1 family can remove the canonical H2A-H2B dimers and replace them with histone variant H2A.Z-H2B dimers, forming a nucleosome with unique tails that might bind particular regulatory proteins to promote gene activation (Saha et al., 2006, Henikoff and Ahmad, 2005). Secondly, nucleosomes can be covalently modified, for example by acetylation on lysine residues in their N-amino-terminal tails that extend from the octamer core (Saha et al., 2006, Cheung et al., 2000). The acetylation of histones removes the positive charge that resides on the NH⁺ group; this neutralisation of the overall positive charge of protein reduces interactions with DNA phosphates. This may alter chromatin structure and contribute to the DNA becoming more accessible to the transcription machinery (Henikoff, 2005, Wade et al., 1997). Thirdly, nucleosomes can be repositioned or evicted by chromatin remodelers to provide regulated access to DNA sequences (Saha et al., 2006, Li et al., 2007). The repositioning and eviction are important for many chromatin functions: to properly space chromatin during chromatin assembly, to enable the ordered access of transcription factors to specific genes during transcription, and to regulate the access of DNA-repair factors to chromatin lesions (Saha et al., 2006, Li et al., 2007).

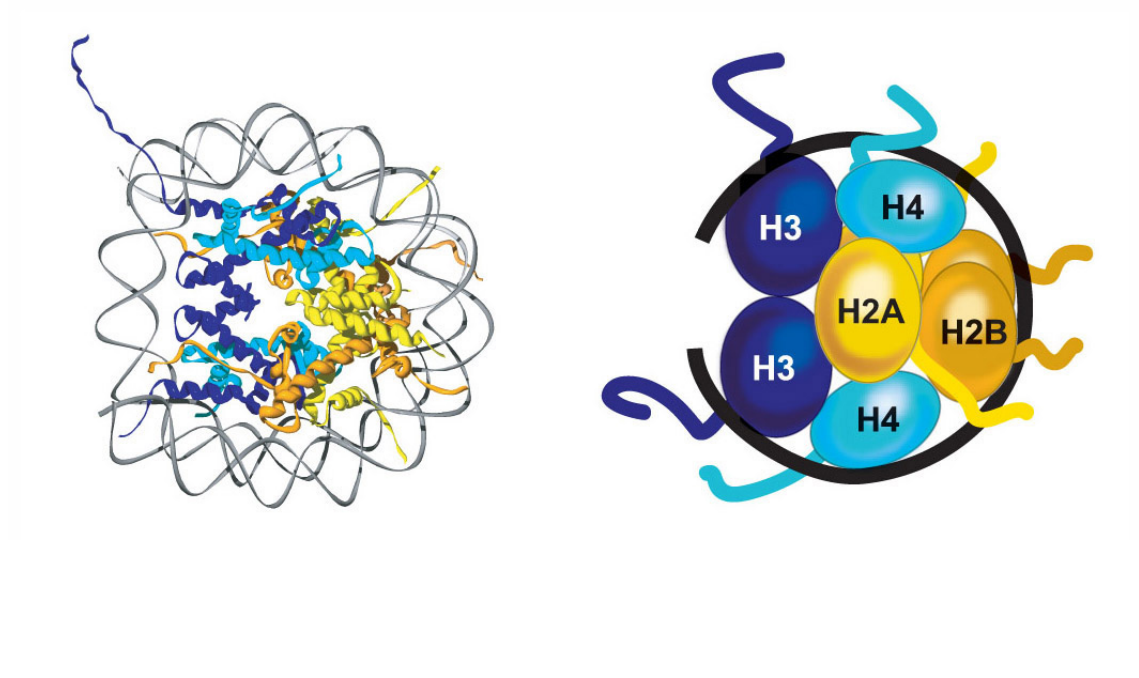


Figure 1.1 Model of nucleosome at 2.8 Å, (Left) the crystal structure and (Right) a schematic image of nucleosome core particle. The nucleosome contains the DNA helix (black line) wrapped around a tetramer of Histone H3 (dark blue) and H4 (light blue) followed by a dimer of Histone H2A (yellow) and a dimer of Histone H2B (gold). (Images are modified from Allis et al., 2006).

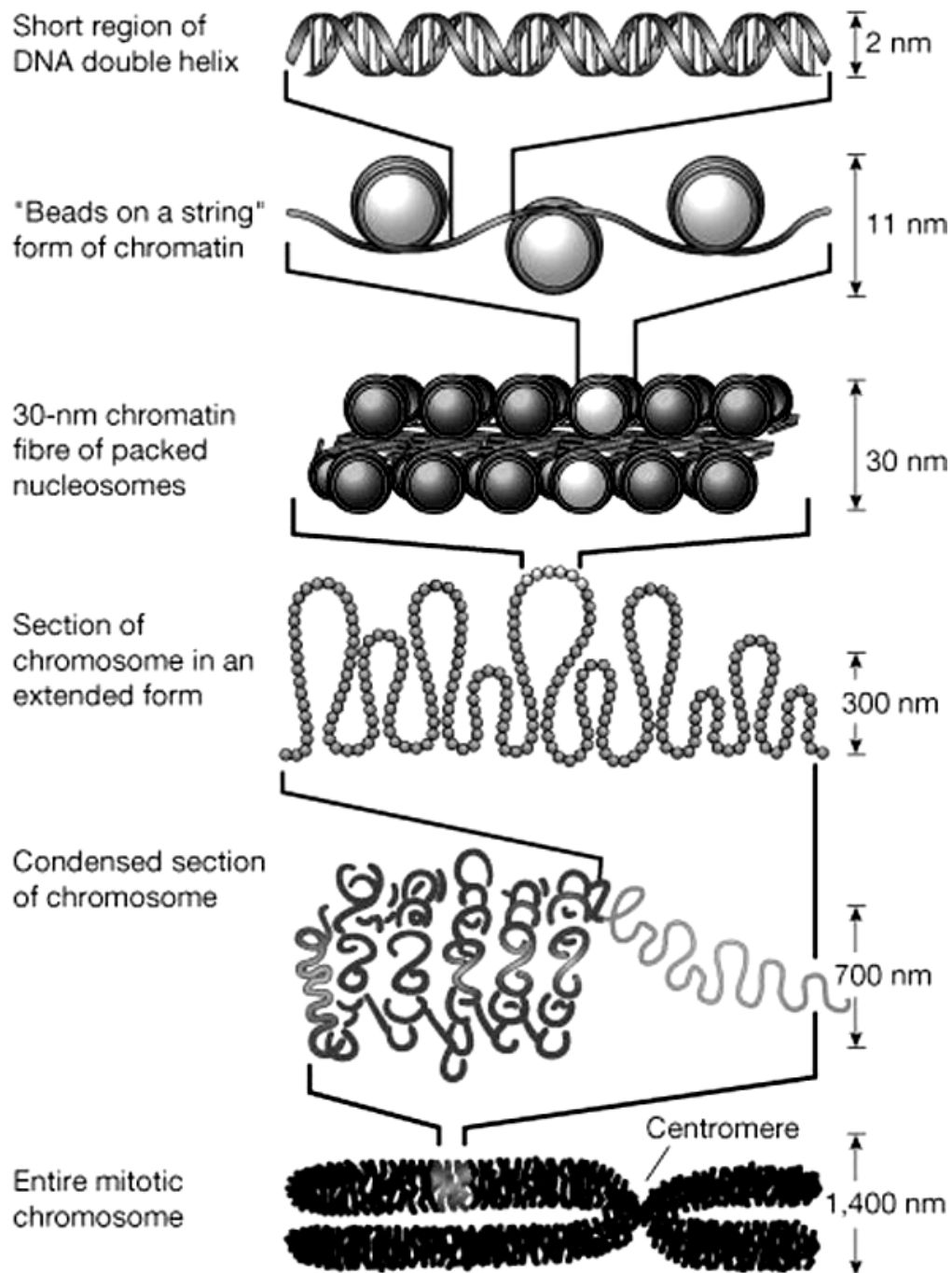


Figure 1.2 The major structures of chromatin compaction. DNA, the nucleosome, the 10nm "beads-on-a-string" fibre, the 30nm fibre and the metaphase chromosome (Images are modified from Felsenfeld and Groudine, 2003)

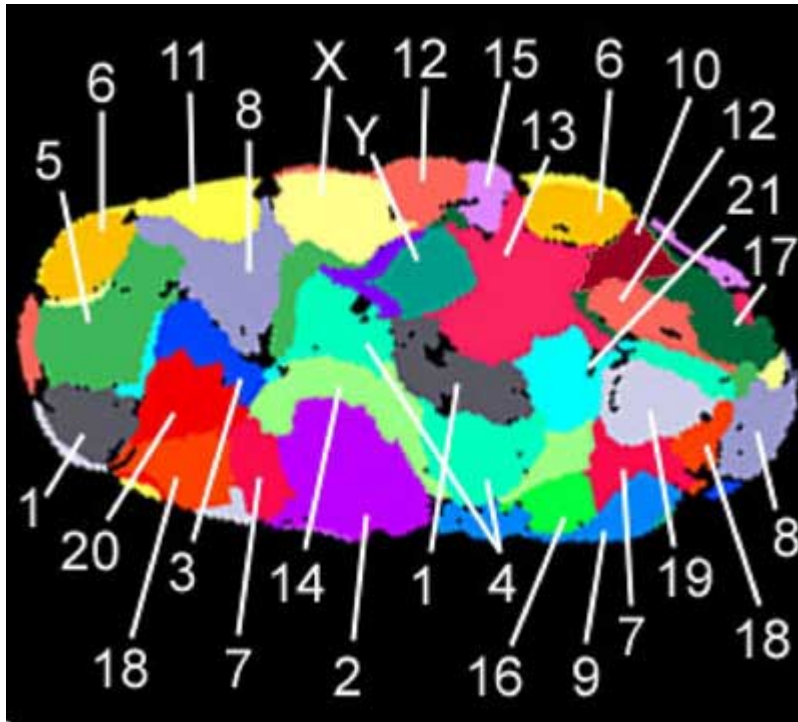


Figure 1.3 A map of chromosome territories in a human cell nucleus. Human cells have 24 chromosome pairs and each chromosome has its own nuclear location represented by a different colour. Numbers 1-22, X and Y are chromosome names.(1-21, X and Y) (Images are modified from Bolzer et al., 2005)

1.1.1 Heterochromatin and Euchromatin

Generally, chromatin is not uniform with regards to gene distribution, architecture, replication timing and transcriptional activity; instead it is divided into two distinct domains, heterochromatin and euchromatin (Figure 1.4) (Grewal and Jia, 2007, Dillon, 2004). Euchromatin describes regions within the weakly stained nuclear area in which chromatin is decondensed during interphase and contains actively transcribed genes (Grewal and Jia, 2007, Dillon, 2004). Heterochromatin is defined as regions that remain densely stained nuclear area and highly condensed throughout the cell cycle and largely consists of transcriptionally silenced elements. Heterochromatin can be specifically subdivided into constitutive and facultative heterochromatin (Grewal and Jia, 2007, Dillon, 2004).

Constitutive heterochromatin describes the highly condensed regions of the genome that are visible as bright nuclear speckles after staining with DNA dyes such as 4',6-diamidino-2-phenylindole (DAPI) or Hoechst (Figure 1.4A) (Kobayakawa et al., 2007, Martin et al., 2006). These regions often comprise repetitive DNA (such as satellite sequences surrounding centromeres) and non-coding regions. Such areas can exert a strong repressive effect on gene transcription, particularly when a gene is inserted within or close to heterochromatin (Lewin, 2004, Grewal and Jia, 2007).

Facultative heterochromatin describes a previously permissive chromatin that is transformed to transcriptional silent chromatin (Lewin, 2004). However, the precise property of facultative heterochromatin has not been fully defined, and different genes might employ a variety of silencing mechanisms. Unlike constitutive heterochromatin, facultative heterochromatin often can not be visualised. One notable exception is the formation of the Barr body on the inactivated X-chromosome in female mammalian cells, which is visible as a distinct DNA-dense structure often located close to the nuclear periphery (Lucchesi et al., 2005).

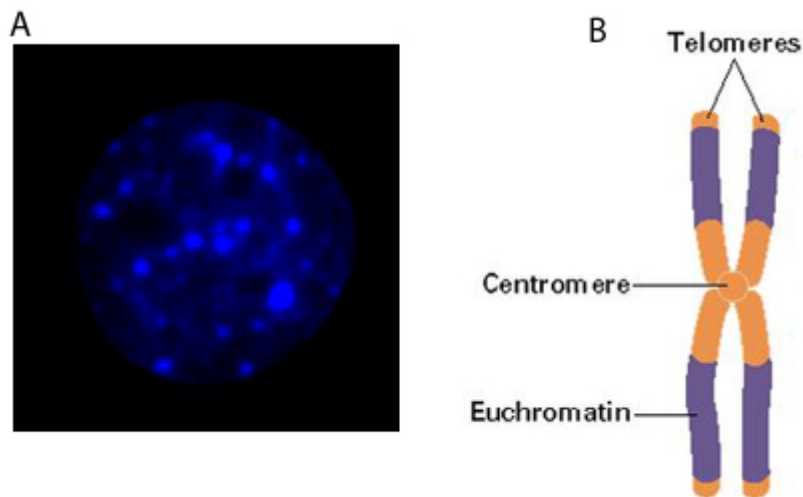


Figure 1.4 Heterochromatin in a mouse fibroblast cell. (A) The nucleus of a mouse fibroblast is stained with DAPI (blue), bright blue dense speckles represent constitutive heterochromatin (Original image by Tuempong Wongtawan). (B) Model of a chromosome showing the separation into heterochromatin (orange) and euchromatin (blue) (Image is modified from original image on David Ussery's website <http://www.cbs.dtu.dk/dtucourse/cookbooks/dave/Chromosome-anat.html>).

1.2 Epigenetics - Definitions

Historically, the word “epigenetics” was coined by C. H. Waddington in 1942 as a portmanteau of the words “genetics” and “epigenesis”. Originally the term epigenetics was defined as “the interactions of genes with their environment that bring the phenotype into being” (Waddington, 1942). Epigenetics could be regarded as Waddington's model of how genes within a multicellular organism interact with their environment to produce a phenotype, because all cells within an organism inherit the same DNA sequences. However at the time when Waddington first used the term “epigenetics”, the physical nature of genes and their role in heredity was unknown (Waddington, 1942, Goldberg et al., 2007).

Presently, epigenetics generally means “the study of heritable changes in genome function that occur without an alteration in DNA sequence” (Goldberg et al., 2007, Bird, 2007). This may include the study of inherited gene expression from one cell to its descendants, alteration of gene expression during cell differentiation, and how environmental factors can induce the change of gene activities (The-Epigenome-Network-of-Excellence, 2009). There are many implications of epigenetic research for agriculture and for human biology and disease, which bear on our understanding of stem cells, cancer, development and ageing (Laird, 2005, Roloff and Nuber, 2005, Srebro, 1993).

Epigenetic modifications can be subdivided into four major categories, most of which are interdependent. First is the modification of DNA called DNA methylation which occurs at cytosine residues in CpG dinucleotides. It is usually associated with gene regulation by transcriptional silencing (Bird, 2002). Second are the modifications of core histones, in particular methylation of specific residues in the histone tails. This modification can have either an active or repressive effect on transcription (Kimura et al., 2005, Shilatifard, 2006). Third, variant histones such as protamines or macro-H2A have been shown to correlate with epigenetic states in the mouse. The dynamic exchange of histones and histone variants in chromatin may correlate with the transcriptional status of a given locus (Henikoff and Ahmad, 2005). Finally, the Polycomb (PcG) and Trithorax (TrxG) group of proteins is considered to be one of the

classical epigenetic systems (Bird, 2007), as they work by maintaining the repression or activation of developmentally important genes (Ringrose and Paro, 2004).

1.3 Histone Modifications and Epigenetics

Histone N-terminal tails and to lesser extent C-terminal tails and globular domains can be modified at many sites; there are more than 60 different residues on histone tails where modifications have been detected (Kouzarides, 2007). Histones are diversely modified by acetylation, methylation, phosphorylation, sumoylation and ubiquitination (Kouzarides, 2007). Histone modifications are summarised in Figure 1.5. The new and former nomenclature of histone modifying enzymes is shown in Table 1.5.

1.3.1 Histone Acetylation

Acetylation occurs at lysine residues in the amino-terminal tails of the histones, which results in neutralisation of the positive charge of the histones decreasing their affinity for DNA (Bhaumik et al., 2007). The acetylation may alter the nucleosomal conformation and lead to a chromatin architectural change renders chromosomal domains more accessible. In addition, histone acetylation acts to recruit regulatory proteins that carry bromodomains, which bind histone acetyl groups. Consequently, the transcription machinery including RNA polymerase II, SRC1, GCN5/PCAF and CBP/p300 may be attracted and be able to access promoters and hence initiate gene transcription (Bhaumik et al., 2007).

Histone acetylation is very dynamic, and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Acetylation of histones induces transcriptional activation whereas deacetylation leads to transcriptional repression. HATs may work as co-activators of transcription while HDACs work as repressors and can be associated with DNA methylation and repressive histone methylation (Kimura et al., 2005). Moreover, histone acetylation is not only at the core of transcriptional regulation but also seems to influence other processes such as chromatin replication, site-specific recombination and DNA repair (Wolffe and Hayes, 1999, Roth et al., 2001)

Histone acetylation may also act as a biological switch allowing inter-conversion between permissive and repressive chromatin structures and domains. Although both global and local histone acetylations are necessary for transcription, they are insufficient to establish fully accessible chromatin (Eberharter and Becker, 2002). The switch to a permissive chromatin structure requires the recruitment of nucleosome remodelling complexes of the SWI2/SNF2 family that contain ATPase, which can weaken histone–DNA interactions resulting in histone relocation (Fry and Peterson, 2001). On the contrary, the switchback to repressive chromatin involves histone deacetylation, which promotes the condensation of the nucleosomal fibre and invites repressive factors such as histone methylation, DNA methylation and heterochromatin associated proteins (Eberharter and Becker, 2002).

1.3.2 Histone Phosphorylation

Phosphorylation of histones often occurs at specific sites during cell division and follows spatial and temporal patterns. For example, histone H3 phosphorylation in different organisms is commonly found in metaphase chromosomes, but after exit of mitosis/meiosis a global dephosphorylation of H3 is observed (Barber et al., 2004, Hans and Dimitrov, 2001). Moreover phosphorylation of serine 10 in histone H3 has been shown to correlate with gene activation in mammalian cells of for example *c-fos* and *c-jun* (Nowak and Corces, 2004), but the mechanism by which phosphorylation contributes to transcriptional activation remains uncertain. One simple explanation might be that addition of negatively charged phosphate groups to histone tails probably neutralises their basic charge and reduces their affinity for DNA (Grant, 2001). Furthermore, phosphorylation of histone H2A is also known to occur after activation of DNA-damage signalling pathways; this suggests that phosphorylation may mediate an alteration of chromatin structure, which in turn facilitates DNA repair (Foster and Downs, 2005).

1.3.3 Histone Ubiquitination

Ubiquitin (Ub) is a 76-amino acid protein that is ubiquitously distributed and highly conserved amongst eukaryotic organisms. Ubiquitination can be mono or poly-Ub on

lysine residues (Bhaumik et al., 2007, Weake and Workman, 2008). It has been found to be associated with numerous processes including protein degradation, DNA repair, cell-cycle control, stress response, cell differentiation, ribosome biogenesis, peroxisome biogenesis, viral infection, neural/muscular degeneration and transcription (Jason et al., 2002, Finley and Chau, 1991, Jennissen, 1995)

Histone ubiquitination is reversible; the levels are determined by the availability of free ubiquitin and enzymatic activities involved in adding or removing the ubiquitin moiety from histones. Addition of a ubiquitin moiety to a protein involves the sequential action of E1, E2, and E3 enzymes whereas deubiquitination is performed by a class of thiol proteases known as ubiquitin-specific proteases (USPs in mammals) (Weake and Workman, 2008, Nijman et al., 2005). Ubiquitinated H2A and H2B (ubH2A and ubH2B) are the most abundant ubiquitin conjugates in higher eukaryotes (Jason et al., 2002). In mammals, the H2A is conjugated to only a single ubiquitin (rather than being polyubiquitinated) at lysine 119 and H2B at lysine 120 in mammals (Osley, 2006). H2B ubiquitination is involved in the DNA repair and protein degradation pathways, and gene regulation; the mechanisms for these may be induced by the cross-talk with other histone modifications (Weake and Workman, 2008, Bhaumik et al., 2007, Jason et al., 2002). In humans, H2A ubiquitination is mediated by at least two different E3 ubiquitin ligases, Ring1B and 2A-HUB, both of which are associated with transcriptional silencing (Wang et al., 2004, Zhou et al., 2008b, Cao et al., 2005) and DNA damage (Zhou et al., 2008a). Recent work in mammalian cells indicates that H2A.Z is mono-ubiquitinated (ubH2A.Z) at lysine 120 or lysine 121, and enriched on the inactive X chromosome (Xi) in female cells, but unmodified H2A.Z is excluded from this chromosome (Sarcinella et al., 2007)

1.3.4 Histone Sumoylation

Small ubiquitin-related modifier (SUMO) is a member of the ubiquitin-like protein family involved in post-translational modifications. In mammals, there are three members of the SUMO protein family: SUMO-1, SUMO-2 (SMT3a), and SUMO-3 (SMT3b) (Melchior, 2000). SUMO is covalently attached to other proteins through the activities of an enzyme cascade (E1–E2–E3) similar to ubiquitination (Nathan et al.,

2003) and involves protein-protein interactions, inhibition of ubiquitin-mediated degradation and transcriptional repression (Park-Sarge and Sarge, 2009, Shiio and Eisenman, 2003). Moreover, protein sumoylation has also been particularly well characterised as a regulator of many nuclear processes and nuclear structure (Park-Sarge and Sarge, 2009).

Amongst the four major core histones, only histone H4 is currently found to be efficiently modified by both SUMO-1 or SUMO-3, and the N-terminal tail is a target for SUMO moieties (Shiio and Eisenman, 2003). This modified region is the same as that targeted by other histone modifications, suggesting that cross-talk between sumoylation and other histone modifications may occur. The mediation of transcriptional repression by sumoylation of H4 has been found to occur through recruitment of histone deacetylases and heterochromatin protein 1 (HP1) (Shiio and Eisenman, 2003).

1.3.5 Histone Methylation

Unlike other histone modifications that are dynamic, histone methylations appear to be more stable modifications. They are maintained through cell division and only some demethylases have been discovered, which qualifies histone methylation as a true epigenetic mark. Methylation of histones can occur on lysine (K) or arginine (R) residues by histone methyltransferases (HMTs). Conversely, demethylases work in the opposite way, as they remove methyl groups from these residues. A summary of histone methylation and the enzymes involved is shown in Table 1.1.

Histone lysine residues can be mono-(me), di-(me₂), or tri-methylated (me₃), and this has a decisive influence on gene and chromatin functions (Li et al., 2007). For instance, H3K9me₃ represses gene transcription and can form constitutive heterochromatin while H3K9me₂ represses genes in euchromatin and forms facultative heterochromatin (Lachner and Jenuwein, 2002). Modifications of histone lysine can act as either repressive or active marks. For example, methylations of H3K4, H3K36 and H3K79 have been highly correlated with transcriptional activation, whereas methylations of H3K9, H3K27 and H4K20 are associated with repressive chromatin states (Vakoc et al., 2006, Sims et al., 2006, Kourmouli et al., 2005, Schotta et al., 2004, Peters et al., 2003,

Brinkman et al., 2006). A combination of histone lysine modifications can occur; this system can separate chromatin or the chromosome into different areas. For instance, H3K27me3 combines with H3K9me2 and H4K20me1 to organise chromatin into facultative heterochromatin, such as the imprinted regions of the inactive X chromosome, whereas H3K9me3, H4K20me3 and H3K27me1 together lead chromatin to form constitutive heterochromatin (Vakoc et al., 2006, Sims et al., 2006, Kourmouli et al., 2005, Schotta et al., 2004, Peters et al., 2003, Brinkman et al., 2006).

Recently, the methylation of lysine 20 on histone H4 (H4K20) has received more attention. Although H4K20me was discovered more than four decades ago, the biological role of this modification has remained a mystery (Murray, 1964). The HMTs responsible for H4K20 have been recently discovered (SetD8 and Suv4-20h), but the demethylase has not been identified (Schotta et al., 2004, Yin et al., 2005). This possibly makes the H4K20 mark more stable than other histone modifications. Previous studies strongly suggest that H4K20 methylation and their HMTs are involved in many important DNA-associated processes such as transcriptional silencing, higher order chromatin structure, cell differentiation, genomic stability, cell division, cancer and imprinted genes (Martens et al., 2005, Schotta et al., 2004, Brinkman et al., 2006, Pogribny et al., 2006, Sanders et al., 2004).

Methylation of histone arginine residues by arginine methyltransferases (PRMTs) has been found to play a significant role in gene regulation. Histone arginine methylation can be transcriptionally activating (Chen et al., 1999) or repressive (Pal et al., 2004) depending on the target residue, and on whether the methylation is symmetric or asymmetric. In mammals, PRMT1- and CARM1-catalysed histone asymmetric histone dimethylation at arginine is involved in gene activation (Chen et al., 1999) while PRMT5-catalyzed symmetric histone dimethylated arginine is associated with gene repression (Pal et al., 2004).

1.3.6 Histone Variants

In the past few years, histone variants and their special associated mechanisms have been shown to play important roles in chromatin organisation and epigenetic

maintenance. Many variant histones can be seen as polymorphisms of the major canonical form and just assembled into chromatin (Henikoff and Ahmad, 2005). However, other variants are distinct from the canonical form, either in mechanism or function (Henikoff and Ahmad, 2005). The centromeric chromatin protein named CENP-A has been found to be an H3 variant, and it is vital for chromosome segregation and centromeric heterochromatin formation (Palmer et al., 1991). Histone H3.3 is similar to canonical H3 and is enriched in active chromatin: it can also be methylated and is correlated with active transcription (McKittrick et al., 2004, Hendzel and Davie, 1990). One study of H2AZ genetically altered mice showed that embryos die during implantation, suggesting that it has a critical role in early development (Faast et al., 2001). Moreover, H2AZ has been found in heterochromatin and it can interact with HP1 α (Fan et al., 2004). H2AX phosphorylation has been demonstrated to have a role in DNA repair, cell cycle checkpoints, regulated gene recombination events, and tumour suppression (Fernandez-Capetillo et al., 2004). Furthermore, MacroH2A is enriched in regions of the mammalian inactive X chromosome that is associated with facultative heterochromatin, Xist RNA and H3K27me3 (Chadwick and Willard, 2004, Chadwick and Willard, 2001).

Table 1.1 Nomenclature of histone modifying enzymes (Allis et al., 2007)

New Name	Old Name
KDM1	LSD1/BHC110
KDM2	
KDM2A	JHDM1a/FBXL11
KDM2B	JHDM1b/FBXL10
KDM3A	JHDM2a
KDM3B	JHDM2b
KDM4	
KDM4A	JMJD2A/JHDM3A
KDM4B	JMJD2B
KDM4C	JMJD2C/GASC1
KDM4D	JMJD2D
KDM5	
KDM5A	JARID1A/RBP2
KDM5B	JARID1B/PLU-1
KDM5C	JARID1C/SMCX
KDM5D	JARID1D/SMCY
KDM6A	UTX
KDM6B	JMJD3

New Name	Old Name
KMT1	
KMT1A	SUV39H1
KMT1B	SUV39H2
KMT1C	G9a
KMT1D	EuHMTase/GLP
KMT1E	ESET/SETDB1
KMT1F	CLL8
KMT2	
KMT2A	MLL1
KMT2B	MLL2
KMT2C	MLL3
KMT2D	MLL4
KMT2E	MLL5
KMT2F	hSET1A
KMT2G	hSET1B
KMT2H	ASH1
KMT3	
KMT3A	SET2
KMT3B	NSD1
KMT3C	SYMD2
KMT4	DOT1L
KMT5	
KMT5A	Pr-SET7/8
KMT5B	SUV4-20H1
KMT5C	SUV4-20H2
KMT6	EZH2
KMT7	SET7/9
KMT8	RIZ1

New Name	Old Name
KAT1	HAT1
KAT2	
KAT2A	hGCN5
KAT2B	PCAF
KAT3	
KAT3A	CBP
KAT3B	P300
KAT4	TAF1
KAT5	TIP60/PLIP
KAT6	
KAT6A	MOZ/MYST3
KAT6B	MORF/MYST4
KAT7	HBO1/MYST2
KAT8	HMOF/MYST1
KAT9	ELP3
KAT10	
KAT11	
KAT12	TFIIIC90
KAT13A	SRC1
KAT13B	ACTR
KAT13C	P160
KAT13D	CLOCK

KDM: Lysine (K) demethylase,; KMT: lysine (K) methyltransferase; KAT: lysine (K) acetyltransferase

Table 1.2 Histone methylation, methyltransferases, demethylases and their functions in mice (Allis et al., 2007, Lall, 2007, Li et al., 2007, Bhaumik et al., 2007, The-UniProt-Consortium, 2008, Kouzarides, 2007, Hemberger, 2007, Vakoc et al., 2006, Wagschal et al., 2008, Schotta et al., 2008)

Modification	HMTase	Demethylase	Functions
H3K9me1	Ehmt1	LSD1, JHDM2a, JHDM2b	Transcriptional repression Heterochromatin formation
H3K9me2	Ehmt1, Ehmt2, ESET	LSD1, JHDM2a, JHDM2b, JMJD2D, JMJD2A, JMJD2C	Transcriptional repression X inactivation
H3K9me3	Suv39h1, Suv39h2, ESET, Ehmt2	JMJD2B, JMJD2D, JMJD2A, JMJD2C	Transcriptional repression Heterochromatin formation Imprinted gene
H3K4me1	MLL3, MLL2, MLL4, MLL5, Set1A, Set1B, ASH1	LSD1, JARID1B	Transcriptional activation
H3K4me2	MLL3, MLL2, MLL4, MLL5, Set1A, Set1B, ASH1	LSD1, JARID1B, JARID1C, JARID1D	Transcriptional activation
H3K4me3	MLL3, MLL2, MLL4, MLL5, Set1A, Set1B, ASH1	JARID1B, JARID1C, JARID1D	Transcriptional activation
H3K27me1	Ezh2		Heterochromatin formation
H3K27me2	Ezh2	UTX	Transcriptional repression
H3K27me3	Ezh2	UTX, JMJD3	Transcriptional repression X-inactivation Imprinted gene
H4K20me1	SetD8		Transcriptional repression, elongation
H4K20me2	Suv420h1, Suv420h2		Transcriptional repression
H4K20me3	Suv420h1, Suv420h2		Heterochromatin formation Imprinted gene
H3K36me1	Smyd2	JHDM1a, JHDM1b	Transcriptional elongation
H3K36me2	Smyd2, NSD1	JHDM1a, JHDM1b, JMJD2a, JMJD2c	Transcriptional elongation
H3K36me3	Setd2, NSD1	JMJD2a, JMJD2c	Transcriptional activation
H3K79me1	Dot1L		Transcriptional activation
H3K79me2	Dot1L		Transcriptional activation
H3K79me3	Dot1L		Transcriptional repression, heterochromatin

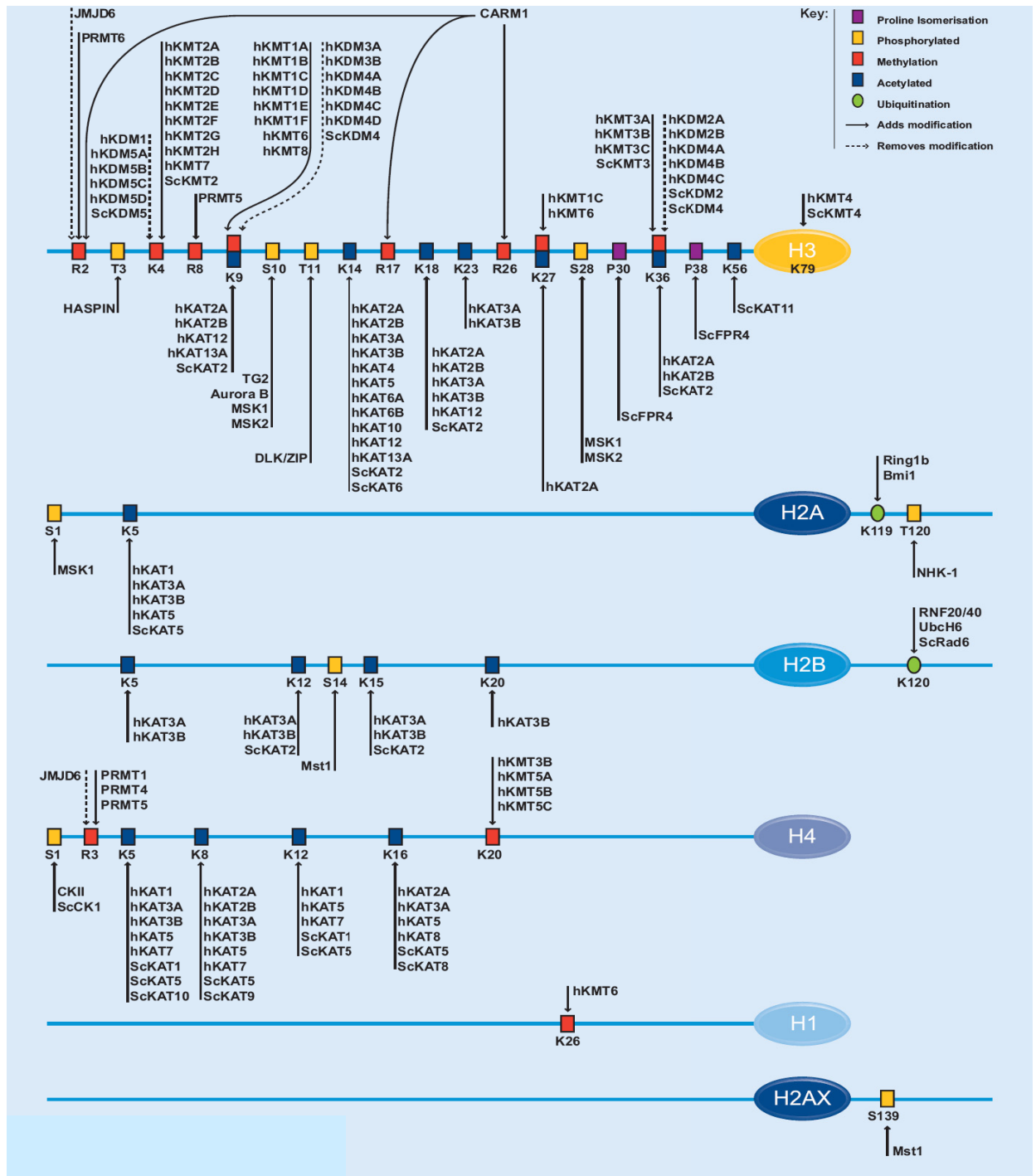
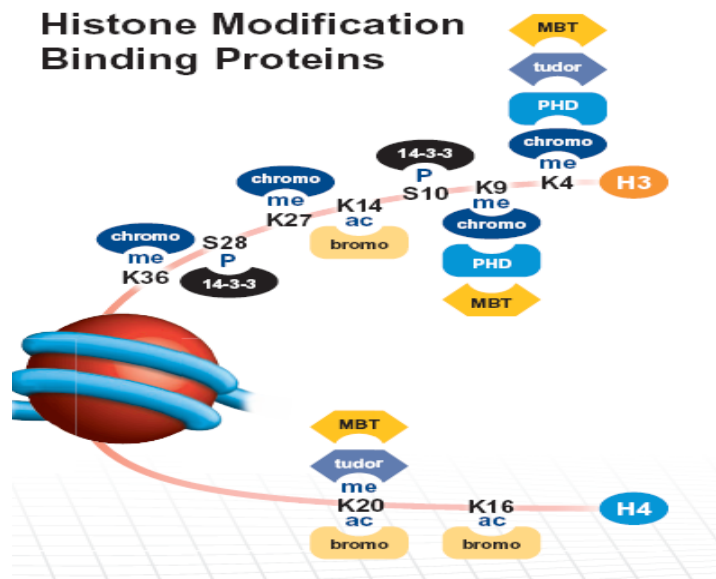


Figure 1.5 Summary of histone modifying enzymes. KAT, KMT and KDM are new nomenclature (see former name in Table 1.5). “h” represents human, “sc” represents *S. cerevisiae* (Image is modified from www.abcam.com).

1.3.7 Reading the Histone Code

It has been proven that the post-translational modified histones serve as selective binding platforms for specific regulatory proteins that drive distinct nuclear processes. It is interesting that a simple and very small chemical modification on a comparatively huge histone can make such a dramatic difference in specific nuclear functions (Turner, 2000, Jenuwein and Allis, 2001). Recent findings indicate that certain evolutionarily conserved domains found within specific regulatory proteins possess the ability to selectively bind a certain histone modification with very high affinity, meaning that specific regulatory proteins can read the histone code to initiate DNA-template programs (Turner, 2000, Jenuwein and Allis, 2001). Many domains and proteins have been found to specifically recognise histone modifications (Lall, 2007) (summarised in Figure 1.6), for example, the bromodomain, a conserved motif found within many transcription factors, binds to acetylated lysine residues on histone H3 and/or H4 (Loyola and Almouzni, 2004). This specific binding may result in the initiation, stabilisation and enhancing of the transcriptional machinery at the target regions (Loyola and Almouzni, 2004). By contrast, regions containing hypoacetylated histones are unable to effectively bind these transcription factors resulting in transcriptional inactivation of the target region (Loyola and Almouzni, 2004, Grewal and Jia, 2007). Notably, inactive regions also contain modified histones which are likely serve to maintain this inactivated state. For instance, the methylation of histone H3 on lysine residue 9 (H3K9) and H4 on lysine 20 (H4K20) are shown to occur specifically in inactivated chromatin (also known as heterochromatin) (Grewal and Jia, 2007). It was shown that methylated H3 K9 can recruit and bind a transcriptionally repressor protein, heterochromatin protein 1 (HP1), via its evolutionarily conserved chromodomain (LeRoy et al., 2009, Grewal and Jia, 2007). This interaction could lead to the structural formation of compacted chromatin that physically inhibits the access of the transcriptional machinery to the underlying DNA (Grewal and Jia, 2007).



Domain	Binding protein	Histone modification	
Chromodomain Proteins	chromo	CHD1	H3K4me2/3
		HP1/spSwi6	H3K9me2/3
		spChp1	H3K9me2/3?
		CDY1	H3K9me2/3
		PC1/PC2/PC/LHP1	H3K27me3, H3K9me3
		MSL3	H3K36me3
		hMRG15	H3K36me, H3K4me
		scEaf3	H3K36me, H3K4me
MBT Proteins	MBT	PHF20L1	H3K4me1, H4K20me1
		SFMBT	H3K9me1/2, H4K20me1/2
		L(3)MBTL1	H4K20me1/2, H1bK26me1/2
PHD Proteins	PHD	scYng1	H3K4me2/3
		ING1,2,3,4,5	H3K4me2/3
		BPTF/dmNURF301	H3K4me2/3
		scSpp1	H3K4me2/3
		scSet3	H3K4me2/3
		scJhd1	H3K4me3
		RAG2	H3K4me3
		TAF3	H3K4me3
		ICBP90 (Np95)	H3K9me2/3
		JARID1C	H3K9me3
14-3-3 Proteins	14-3-3	H3S10ph, H3S28p	
Tudor Proteins	tudor	JMJD2A	H3K4me3, H4K20me3
		53BP1	H4K20me1/2
		spCrb2	H4K20me2
		PHF20	H4K20me2
Bromodomain Proteins	bromo	Polybromo/BAF180	H3ac
		scSnf2	H3ac, H4ac
		Brd2,3,4,7	H3ac, H4ac
		TAF1	H3ac, H4ac
		P/CAF	H3ac, H4ac, H4K16ac
		CBP/p300	H3ac, H4ac
		scBdf1	H4ac
		hBRG1	H3K14ac
		scRsc1,2,4	H3K14ac (Rsc4)
		scGcn5	H4ac, H4K16ac

Figure 1.6 Summary of histone modification binding domains/proteins (modified from www.abcam.com).

1.3.8 DNA Methylation

DNA methylation is associated with chromatin structure and transcriptional repression and occurs mainly at the fifth position of cytosine (5mC) in the dinucleotide CpG. In humans CpGs occupy about 1% of the genome and more than 70% of CpGs are methylated (Ehrlich et al., 1982). Some localised areas of the genome contain a high frequency of CG dinucleotides called CpG islands which are typically 300-3000 base pairs in length (Fatemi et al., 2005). They correspond to approximately 40% of promoters in mammals (Fatemi et al., 2005) and about 70% in humans (Saxonov et al., 2006). CpG islands are usually non-methylated in genes except imprinted genes, the female X chromosome, germ line specific genes and tissue specific genes (Bird, 1986). In mouse, approximately 5% of CpG islands are tissue-specific differentially methylated regions (TDMs). The expression of genes associated with these regions is correlated with methylation status (Song et al., 2005). The majority of the TDMs appear to be associated with 5' promoter CpG islands and may have important roles in establishing or maintaining gene silencing during or after tissue differentiation (Song et al., 2005). Notably, the majority of DNA methylation occurs in repetitive sequences which make up approximately 40% of the mammalian genome (Goodier and Kazazian, 2008, Yoder et al., 1997). The role of DNA methylation may be to inactivate repetitive sequences such as transposable element to maintain genomic stability (Goodier and Kazazian, 2008).

Several hypotheses have been proposed to explain the mechanism of gene regulation by DNA methylation. The first possibility is that DNA methylation prohibits the binding of CpG specific transcription factors to promoters (Tate and Bird, 1993). Second, the DNA methylation may alter nucleosome structure so that gene silencers may bind more effectively to the promoter than the transcription machinery (Kass et al., 1997). The third possibility is that DNA methylation may recruit specific factors that prevent the binding of transcription factors (Lewis et al., 1992). In mammalian cells, DNA methylation is maintained and established by three DNA methyltransferases (Dnmt). Dnmt1 has a high affinity for hemi-methylated DNA rather than unmethylated DNA; this enzyme is critical for the maintenance of DNA methylation after DNA replication

(Bird, 2002). Dnmt1 is expressed in ES cells and all somatic cells, except in oocyte where Dnmt1o, oocyte specific form is active and essential for maintenance imprints during early development (Latham et al., 2008). Dnmt2 is not essential for DNA methylation and mice development; however, it has been reported that Dnmt2 is an RNA methyltransferase with specificity for aspartic acid tRNA (Goll et al., 2006). Dnmt3 has several splice variants, Dnmt3a and Dnmt3b, which are particularly important for *de novo* methylation, are highly expressed in ES cells and are down regulated upon differentiation (Okano et al., 1999). Dnmt3L shares homology with Dnmt3a and 3b but it lacks the catalytic domain activity necessary for DNA methyltransferase (Latham et al., 2008). Dnmt3L can interact with Dnmt3a and 3b to enhance the efficiency of methylation activity. Dnmt3L is not necessary for zygotic development but is important for establishment of paternal and maternal imprints through mouse development (Latham et al., 2008, Arnaud et al., 2006, Bourc'h et al., 2001, Hata et al., 2006) and Dnmt3L-deficient males have impaired spermatogenesis (Webster et al., 2005, Hata et al., 2006). It is believed that Dnmt3L together with Dnmt3a2 isoform is responsible for the establishment of imprints in the germ line (Sakai et al., 2004).

1.3.9 Polycomb and Trithorax proteins

Polycomb proteins (PcG) are usually associated with gene silencing and they are essential to epigenetic maintenance and normal development in multicellular organisms (Ringrose and Paro, 2004, Schwartz and Pirrotta, 2007). PcG can be biochemically and functionally separated into two major multiprotein complexes, polycomb repressive complex 1 (PRC1) and PRC2 which is also known as initiation complex (Lund and van Lohuizen, 2004). The well known complex of PRC2 comprises Eed, Ezh1, Ezh2 and Suz12 while PRC1 complexes contain Cbx2, Cbx4, Eed1, Eed2, Ring1, Rnf2, Rnf110, Bmi-1 and Znf134 (Lund and van Lohuizen, 2004). Bmi-1 and Ring1B of the PRC1 are associated with histone ubiquitination, Ring1B is an E3 ligase that participates in the ubiquitination of lysine 119 of histone H2A, and the binding of Bmi-1 stimulates the E3 ligase activity (Li et al., 2006).

Unlike PcG, trithorax proteins (TrxG) are commonly linked to the general transcription process and gene activation, and they work as antagonists to PcG (Ringrose and Paro, 2004, Schwartz and Pirrotta, 2007). PcG and TrxG regulate many developmentally important genes (Hanson et al., 1999, Boyer et al., 2006) and also associate with X chromosome inactivation (Schwartz and Pirrotta, 2007). Moreover, most of the PcG-occupied genes contain nucleosomes modified for H3K27methylation, suggesting that the role of PcG and TrxG might be related to H3K27 methylation both in methyltransferase function and histone-protein complexes (Boyer et al., 2006).

1.3.10 Heterochromatin Protein 1 (HP1)

In mammals, three isoforms of HP1 have been identified, HP1 α , HP1 β and HP1 γ . These are associated with constitutive heterochromatin as well as some forms of facultative heterochromatin (Jones et al., 2000, Li et al., 2002). In somatic mammalian cells, HP1 α and HP1 β are predominantly localised at the pericentric heterochromatin, whereas HP1 γ uniformly distributes at both heterochromatin and euchromatin (Schmiedeberg et al., 2004, Dialynas et al., 2007, Vakoc et al., 2005, Fischle et al., 2005). Notably, HP1 α , but not HP1 β and HP1 γ , remain associated with heterochromatin during cell division (Fischle et al., 2005, Schmiedeberg et al., 2004). Studies of their chromatin binding proteins showed that HP1 α has a higher affinity for either chromatin or DNA than HP1 β and HP1 γ (Gilbert et al., 2003, Remboutsika et al., 1999, Meehan et al., 2003). Although all HP1 isoforms may be associated with gene silencing (Smallwood et al., 2007), HP1 γ seems to be connected to transcription activation (Vakoc et al., 2005). Collectively this makes HP1 α a more specific marker for constitutive heterochromatin and a true epigenetic mark.

1.4 Chromatin Modification in Mouse Development

In the mammalian reproductive system, the male and female gametes have very different chromatin organisations. This chapter summarises the chromatin modifications during gamete development and post-fertilisation to post-implantation development. The diagram of mouse development is shown in Figure 1.7 and 1.8.

1.4.1 Chromatin Modification in Early Germ Cell Development

Germ cells are the progenitors of gametes. Mouse primordial germ cells (PGC) begin to form at embryo day 7.5 (E7.5) in the posterior primitive streak and then migrate to genital ridge by day E11.5 (McLaren, 2003). There they undergo global epigenetic and chromatin reprogramming. Most of the DNA methylation at imprinted genes and repeat sequences is removed in this process (Hajkova et al., 2002, Hajkova et al., 2008, Morgan et al., 2005). Some DNA demethylation occurs specifically at paternally inherited imprinted genes (Hajkova et al., 2002). Although the DNA methylation at repeat sequences is mostly removed, some retrotransposons such as LINE 1 and IAPs are resistant (Lane et al., 2003, Hajkova et al., 2002). The differential erasure of DNA methylation persists until new imprints are established (Hajkova et al., 2002, Lee et al., 2002).

Recently Hajkova and colleagues (2008) reported dynamic changes in chromatin modifications during mouse early germ line development. Initially at E8.5 loss of heterochromatin marker H3K9me3 is observed despite the presence of histone methyltransferase G9a. Conversely, repressive histone mark H3K27me3, histone methyltransferase Ezh2 and active histone modifications such as H3K4me2, H3K4me3 and H3K9ac seem to increase as well as the symmetrical methylation of arginine 3 on H4 (H4R3me2s) and H2A (H2AR3me2s) (Hajkova et al., 2008). A massive remodelling of heterochromatin and chromatin modifications is found at E11.5, when linker histone H1 and heterochromatin markers such as H3K9me3, H3K27me3, HP1 α , HP1 β , HP1 γ , ATRX and M33 become undetectable or redistribute simultaneously with the loss of chromocentres (stained with DAPI) as well as nuclear enlargement (Hajkova et al., 2008). In addition, the active mark H3K9ac is also removed (Hajkova et al., 2008)

and new arrangements or reappearance of chromatin modifications as well as heterochromatin are observed around E12.5 (Hajkova et al., 2008). The loss of many repressive marks and heterochromatin markers may suggest that the chromatin of germ cells may be in the loose and open state to allow resetting of the epigenetic and chromatin profile.

Although male germ cells enter a mitotic arrest at E13.5 that lasts until after birth, epigenetic and chromatin remodelling does not stop and seems to be dynamic (Yoshioka et al., 2009). A temporal and spatial reorganisation of chromocentres and heterochromatin markers H3K9me3 and HP1 α has been found (Yoshioka et al., 2009). A diagram of epigenetic change in germ cell development is shown in Figure 1.8.

1.4.2 Chromatin Modification in Oogenesis

The main function of the female gamete formed by oogenesis is to produce and store cytoplasmic enzymes, mRNA, organelles and metabolic substrates needed to initiate and maintain metabolism and development (Gilbert, 2000). After arriving at urogenital ridge PGCs differentiate to self-renewing stem cells called oogonia, they undergo mitotic proliferation but most of oogonia die during this period, then at E12.5 the remaining oogonia enter meiosis these cells are named primary oocytes (Edson et al., 2009, Gilbert, 2000). Thereafter primary oocytes progress through the first meiotic prophase, at this stage genetic reassortment occurs by homologous chromosome exchange, a phenomenon called chromosome crossing over (Alberts et al., 2002, Gilbert, 2000). The oocyte arrest at diplotene stage of meiosis I and do not resume meiosis until sexual maturity progressing to metaphase of meiosis II (usually called metaphase II). Only after fertilisation is meiosis completed (Edson et al., 2009) (see Figure 1.7 and 1.8).

Oogenic meiosis conserves the volume of oocyte cytoplasm in a single cell rather than splitting it equally among four progeny. When the primary oocyte divides, its nucleus, called the germinal vesicle, breaks down, and then metaphase spindle migrates to the periphery of the cell (Gilbert, 2000). At telophase, the oocyte divides to produce a two daughter cells, one cell contains very little cytoplasm (known as the first polar body),

whereas the other cell has nearly the entire volume of cellular constituents referred to as the secondary oocyte (Gilbert, 2000). During the second division of meiosis (meiosis II), sister chromatids are separated and the genome becomes haploid (Cooper, 2000). An unequal cytokinesis also takes place, most of the cytoplasm is retained by the mature oocyte (metaphase II oocyte) (Gilbert, 2000).

During oocyte development, chromatin structure is globally altered, and most genes are transcriptionally silenced (Kageyama et al., 2007, Kim et al., 2003). Many marks for histone methylation, acetylation and DNA methylation significantly increase from primary oocytes to germinal vesicle stage (GV) (Kageyama et al., 2007). This global change in chromatin modification coincides with increased gene expression of both methyltransferases and acetyltransferases (Kageyama et al., 2007). Gene repressive as well as active histone marks seem to co-localise with heterochromatin regions (Kageyama et al., 2007). Moreover, somatic linker histone H1 is replaced by an oocyte specific H1 variant named H1Foo, which is abundant in lysines and has many potential sites for methylation and acetylation (Kimmins and Sassone-Corsi, 2005). Taken together, it seems likely that epigenetic and chromatin modifications take place during oogenesis in association with chromatin architecture but this does not seem to be related to global gene expression.

After meiosis, acetylation of all histones is reduced to an undetectable level by HDAC (Kim et al., 2003). A study of deacetylation in meiotic oocytes showed that p34^{cdc2} kinase activity and/or ATRX (A member of SWI/SNF family, a chromatin remodelling protein) are responsible for the activation of HDACs (Akiyama et al., 2004, De La Fuente et al., 2004).

1.4.3 Chromatin Modification in Spermatogenesis

The development of male germ cells (spermatogenesis) differs from female (oogenesis) in many ways (see Table 1.6 for comparison). The main function of sperm is to transfer paternal genomic information to the oocyte during fertilisation, to form the zygote. During spermatogenesis, sperm chromatin undergoes dramatic changes, during which the histones are replaced by special proteins named protamines. This results in an

extremely compact chromatin compared to somatic cell nuclei (Ward and Coffey, 1991). However the histone replacement by protamines is incomplete; it has been shown that the proteins associated with DNA are approximately 85% protamine and 15% histone and other proteins (van der Heijden et al., 2006, Oliva, 2006, Gatewood et al., 1990). Most histone modifications are significantly decreased or absent after spermatogenesis while the remaining modifications such as acetylation of H4K8 and K12 are transmitted to the zygote after fertilisation (van der Heijden et al., 2006). The DNA methylation status at specific sites in three spermatogenesis-specific genes, Pgc-2, ApoA1 and Oct-3/4 is unmethylated in adult spermatogenic cells in the testis, but remethylated in mature spermatozoa in the vas deferens (Ariel et al., 1994). A sperm chromatin architecture study showed that the centromere are organised in a chromocentre, well positioned inside the nucleus while the telomeres forming dimers are positioned at the nuclear periphery (Oliva, 2006). Additionally, several epigenetic modifiers are important for spermatogenesis, for example, disruption of Suv39h2 or G9a or Jmjd1a (H3K9-HMT) which are preferentially expressed in the testis, results in infertility due to spermatogenetic failure (Tachibana et al., 2007, Peters et al., 2001, Okada et al., 2007)

Table 1.3 Differences between male and female gametogenesis (Handel and Eppig, 1998).

Female oogenesis	Male spermatogenesis
Meiosis initiated once in a finite population of cells	Meiosis initiated continuously in a mitotically dividing stem cell population
One gamete produced per meiosis	Four gametes produced per meiosis
Completion of meiosis delayed for months or years	Meiosis completed in days or weeks
Meiosis arrested at first meiotic prophase and reinitiated in a smaller population of cells	Meiosis and differentiation proceed continuously without cell cycle arrest
Differentiation of gamete occurs while diploid, in first meiotic prophase	Differentiation of gamete occurs while haploid, after meiosis ends
All chromosomes exhibit equivalent transcription and recombination during meiotic prophase	Sex chromosomes excluded from recombination and transcription during first meiotic prophase

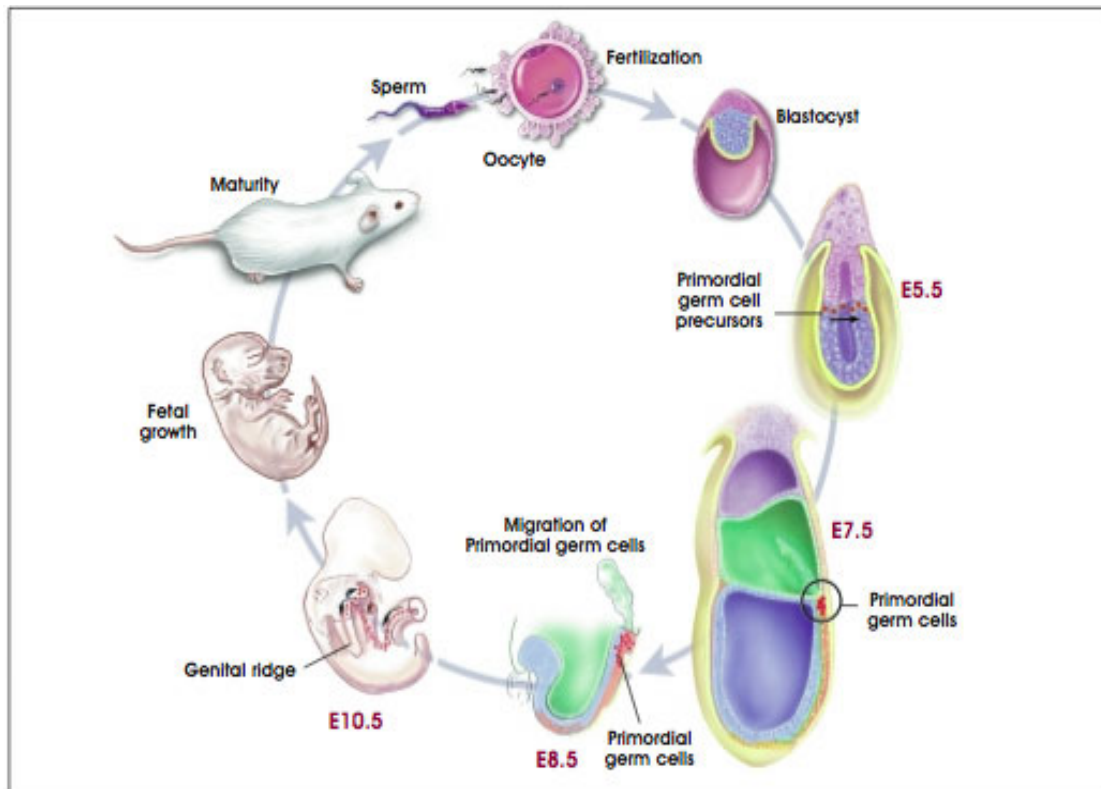


Figure 1.7 Diagram of mouse development. (Image from NIH stem cell information website <http://stemcells.nih.gov/>)

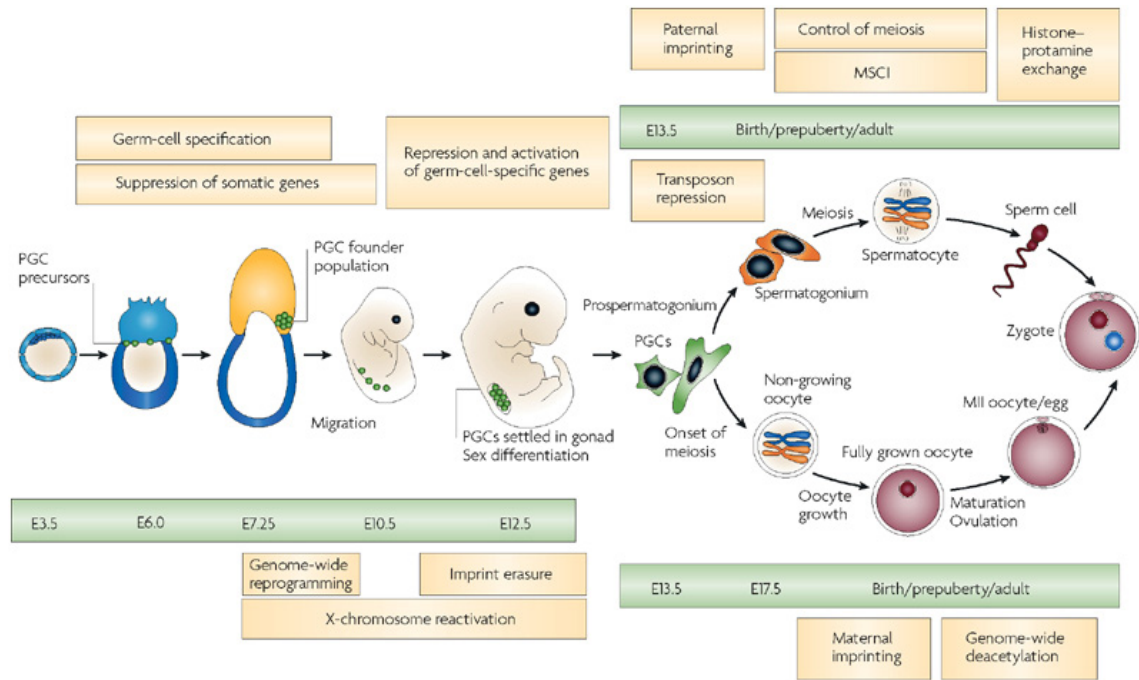


Figure 1.8 Epigenetic changes in germ cell development. Dashed lines indicate that the level of epigenetic modification is lower during these periods than that shown by the solid lines. FG, fully grown; MSCI, meiotic sex-chromosome inactivation; MI, metaphase I; MII, metaphase II; NG, non-growing; PGC, primordial germ cell (Image from Sasaki and Matsui, 2008).

1.4.4 Chromatin after Fertilisation

At fertilisation, the parental genomes are in different stages of the cell cycle and have different chromatin organisations. The paternal chromatin has been delivered by sperm, and is mostly densely packaged with protamines rather than histones (Oliva, 2006). By contrast, the maternal chromatin is arrested at metaphase II and is packaged with histones (van der Heijden et al., 2005). Upon fertilization, sperm nucleoprotamine is decondensed and rapidly replaced with new histones, after which modification may occur (van der Heijden et al., 2005). Maternal chromatin, however; already contains histones and has abundant DNA methylation and chromatin modifications at fertilisation (van der Heijden et al., 2006, Santos et al., 2002). Previous studies under fluorescence microscopy reveal that most types of histone methylation such as H3K4, H3K9, H3K27, H4K20 are already present in female chromatin whereas only H3K9me1, H3K4me1, H4K20me1 are found in male chromatin (Erhardt et al., 2003, Torres-Padilla et al., 2006, van der Heijden et al., 2005). After chromatin decondensation, active DNA demethylation occurs in male chromatin by an unknown mechanism (Santos et al., 2002, Mayer et al., 2000, Yamazaki et al., 2007); at the same time new histones are modified (van der Heijden et al., 2005, Santos et al., 2005) including H3K4me1, H4K20me1, and acetylation of H3 and H4 (van der Heijden et al., 2005, Santos et al., 2005, Adenot et al., 1997, Kim et al., 2003). Both the mechanism and the function of paternal genome demethylation are still unclear. It is hypothesised that the oocyte cytoplasm contains demethylation factors that are specifically targeted to sperm chromatin (Beaujean et al., 2004b, Yoshida et al., 2006). Histone variant H3.3 is already present in the oocyte as a maternal factor, and then incorporates preferentially into the male pronucleus before gamete activation (Torres-Padilla et al., 2006). That paternal chromatin has DNA demethylation and fewer histone modifications may associated with the fact that zygotic gene transcription of paternal chromatin is higher and occurs earlier than in maternal chromatin (Aoki et al., 1997, Schultz, 2002). Furthermore, both H3K79me2 and H3K79me3 are decreased soon after fertilisation. The level of H3K79me2 is maintained until morula stage, whereas H3K79me3 is not detected throughout preimplantation (Ooga et al., 2008). However recent studies using chromatin immunoprecipitation and western blot from sperm extract found that most histone

modifications are found in spermatozoa (Dindot et al., 2009, Hammoud et al., 2009, Brykczynska et al., 2010). This may explain that the amounts of many histone modifications in sperm are very low and could not be detected by fluorescence microscope in single sperm sample.

Zygotic gene activation (ZGA), the critical event that direct the transition from maternal to embryonic control of development, occurs during the 2-cell stage in mice (Schultz, 2002). The demethylation of H3K9 methylation (Liu et al., 2004) and significant chromatin reorganisation also happens simultaneously at the 2-cell embryo stage (Martin et al., 2006, Probst et al., 2007, Schultz, 2002). For these reasons, it may be suggested that a highly permissive chromatin in 2-cell embryo allows access of the transcriptional machinery so as to activate zygotic gene expression. The dynamic changes of histone methylation are shown in Figure 1.9.

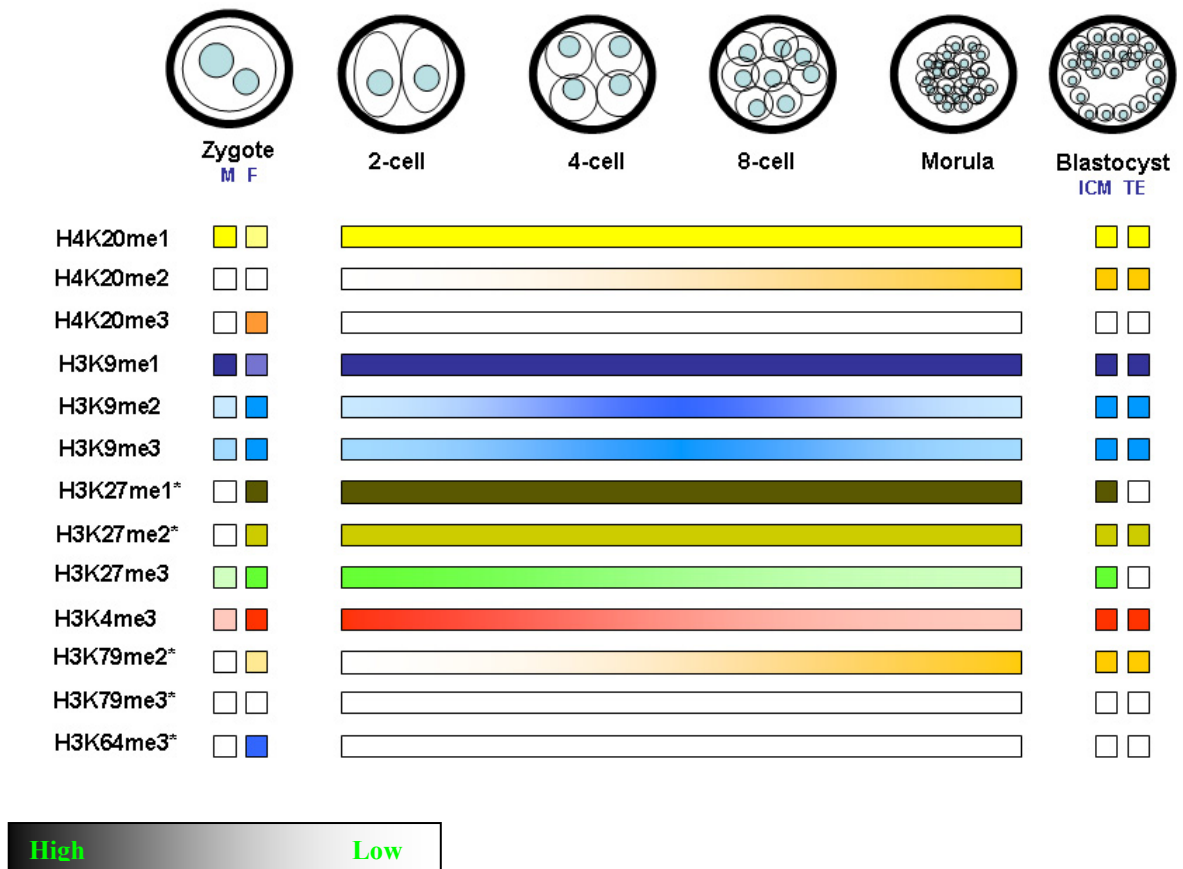


Figure 1.9 Dynamics of histone methylation in preimplantation development. M=male pronuclei, F=Female pronuclei, ICM=Inner cell mass and TE=trophectoderm. Most of data come from the present studies except data mark with* is mean data from previously published report and so the quantification of histone methylation cannot be performed. H3K27me1 (Erhardt et al., 2003, van der Heijden et al., 2005); H3K27me2 (Erhardt et al., 2003, van der Heijden et al., 2005); H3K79me2 (Ooga et al., 2008); H3K79me3 (Ooga et al., 2008), and H3K64me3 (Daujat et al., 2009). H3K9me1, H3K9me2, H3K9me3, H4K20me1, H4K20me2, H4K20me3, H3K4me3, H3K27me3 was from the present study (Tuempong result in Chapter 3). The gradient of each colour represents the level of histone methylation.

1.4.5 Chromatin at Periimplantation

Blastocyst is the stage during which the embryo prepares to implant in the uterine epithelium. There are two populations of cells at this stage; inner cell mass (ICM) which develops into the future postimplantation embryo, and trophoctoderm (TE) which forms the placenta. It has been shown that DNA methylation and H3K27me3 are significantly higher in ICM than in TE, suggesting that they are essential to establish and maintain pluripotency and separate cell population (Erhardt et al., 2003, Santos et al., 2002).

Histone arginine methylation can also regulate pluripotency in the early mouse embryo. One study showed that higher levels of H3 arginine methylation predisposes blastomeres to contribute to the pluripotent cells of the ICM (Torres-Padilla et al., 2007). Increase of H3K9me3 and H4K20me3 was found in the neural tube of postimplantation embryos (E12.5) indicating that they may be associated with neural differentiation (Biron et al., 2004).

Many studies using gene knock-out mice models have demonstrated that epigenetic mechanisms are essential for development. A summary of the phenotypes of gene knock-out mice is shown in Table 1.2.

Table 1.4 Epigenetic modifiers that are critical for mouse development (knock-out mice model).

Name	Function	Embryonic lethality	Extraembryonic tissue	References
Ehmt1	H3K9me	E9.5	Chorioallantoic fusion defect	(Tachibana et al., 2005)
Ehmt2/G9a	H3K9me	E8.5	Chorioallantoic fusion defect, small placenta, reduction of giant cell	(Tachibana et al., 2002, Wagschal et al., 2008)
ESET	H3K9me	E3.5-E5.5	Abnormal blastocyst	(Dodge et al., 2004)
Suv3-9h	H3K9me	E14.5	No data	(Peters et al., 2001)
Ezh2	H3K27me	E7	Amnion and chorion defect	(O'Carroll et al., 2001)
PRMT1	Arginine methylation	E6.5	Lack of amnion and ectoplacental cavity	(Pawlak et al., 2000)
Dnmt1	DNAme	E9.5	Chorioallantoic fusion defect	(Li et al., 1992)
Suv4-20h	H4K20me	E17-E19	Labyrinth trophoblast defect, small size of placenta	(Schotta et al., 2008) and (Schotta personal communication)
Dicer	RNAi	E9.5	No data	(Bernstein et al., 2003)
Dnmt3L	DNAme	E9.5	No labyrinth formation, less spongiotrophoblast, more trophoblast giant cell	(Arima et al., 2006)
Dnmt3b	DNAme	E15.5	No data	(Okano et al., 1999)
Eed	H3K27me	E9.5	Secondary giant trophoblast defect	(Wang et al., 2002, Faust et al., 1995)
Nsd1	H3K36me	E10.5	Lack of allantoids	(Rayasam et al., 2003)
HDAC1	deacetylase	E9.5	Lack of allantois formation	(Lagger et al., 2002)
Suz12	H3K27me	E10	Lack of chorion, amnion and ectoplacental cavity	(Pasini et al., 2004)
Dot1L	H3K79	E9.5-10.5	abnormal vascular morphology in yolk sac, severe cardiac dilation	(Jones et al., 2008)

1.5 Genomic Imprinting Controlled by Epigenetics

Genomic imprinting is a genetic phenomenon in which certain genes called imprinted genes are expressed in a parent-of-origin-specific manner. It is an inheritance process independent of classical Mendelian genetics, which describes the inheritance of traits as due to either dominant or recessive gene. Unlike Mendelian laws, in which both parental copies are equally likely to contribute to the phenotype or gene expression, imprinted gene expression depends only on which parent it was inherited from (Swales and Spears, 2005, Ideraabdullah et al., 2008). For example, gene H19 is expressed only from the allele inherited from the mother whereas IGF-II is expressed from the allele inherited from the father (Ideraabdullah et al., 2008). Studies in many species indicate that imprinting is broadly conserved among placental mammals, including primates, rodents, ruminants and marsupials (Monk et al., 2006, Lucifero et al., 2006, Umlauf et al., 2004, Vu et al., 2006).

Many imprinted genes are found in clusters throughout the genome. Their gene expression is regulated by imprinting control regions (ICRs), which are marked by DNA methylation, histone modification, polycomb protein and non-coding RNA on one of two parental alleles (Delaval and Feil, 2004, Ideraabdullah et al., 2008). These allelic epigenetic marks are established in either the female or male germ line, following the epigenetic reprogramming in the primordial germ cells. The parental imprinted genes escape from the global epigenetic reprogramming after fertilisation, so that their epigenetic marks are maintained during preimplantation development (Ideraabdullah et al., 2008).

DNA methylation is arguably the most studied mechanism in epigenetically controlled imprinted genes. The expressed imprinted genes are not marked with DNA methylation, while repressed imprinted genes are enriched for DNA methylation (Fowden et al., 2006). Histone modifications also play a vital role in the regulation of imprinted genes. DNA methylated ICRs are associated with H4K20me3 and H3K9me3, whereas the unmethylated allele has H3K4me2 and H3ac (Delaval et al., 2007).

Another type of genomic imprinting is the imprinted X chromosome inactivation (XCI) which is found in female cells that have two X chromosomes (XX). XCI is a developmentally regulated process that causes one of the two X chromosomes to become transcriptionally silenced, thus equalising the expression of X-linked genes between male and female (Sado and Ferguson-Smith, 2005, Thorvaldsen et al., 2006). In postimplantation embryos, either the parental or maternal X-chromosome has to be inactivated, which is referred to as random XCI, whereas in the preimplantation embryo and extra-embryonic lineages, XCI is paternally imprinted (Thorvaldsen et al., 2006). The imprinted XCI is marked by repressive epigenetic markers such as H3K27me3 (Erhardt et al., 2003), H4K20me1, H3K9me2 (Erhardt et al., 2003) and DNA methylation (Sado and Ferguson-Smith, 2005) whereas the active X chromosome is marked by active markers such as histone acetylation and H3K4me3 (Ideraabdullah et al., 2008).

It has been demonstrated that imprinted genes play vital roles in fetal and placental growth and development (Arnaud and Feil, 2005, Fowden et al., 2006, Nafee et al., 2008). They affect the growth, morphology and nutrition by controlling nutrient demand and supply between fetus and placenta (Fowden et al., 2006). Abnormal development in fetus and placenta has been found in mice in which imprinted genes were genetically deleted or altered (see table 1.3). Epigenetic dysregulation could also affect the fetal and placenta growth through malfunction of imprinted genes (see Table 1.2).

Table 1.5 Imprinted genes that affect embryo and placental development (Modified from Fowden et al., 2006)

Allele	Gene	Gene product	Knockout proportion of normal weight, %		Placenta localisation
			Fetus	Placenta	
Paternally expressed	Igf2	IGF II growth factor	50	60	All
	Igf2P0	IGF II growth factor	75	70	Labyrinth trophoblast
	Peg1	α/β hydrolase	87	86	Labyrinth blood vessel
	Peg3	Zinc finger transcription	80	72	Labyrinth and spongiotrophoblast
	Ins ½	Insulin	80	No result	Yolk sac
	Slc 38a4	System A amino acid transporter	80	No result	Labyrinth trophoblast
Maternally expressed	H19	Non-coding RNA	130	140	All
	Igf2r	IGF II receptor	140	140	All
	Ip1	Cytoplasmic protein	100	140	Labyrinth trophoblast
	Grb10	Adaptor protein	146	130	Labyrinth trophoblast
	p57 ^{kip2}	Cyclin-dependent kinase inhibitor	100	140	All

1.6 Epigenetic Alterations by Reproductive Biotechnology

In the last decade, the use of reproductive biotechnologies such as hormonally induced ovulation, artificial insemination (AI), *in vitro* maturation of oocyte (IVM), *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) have been proven to resolve some infertility problems in humans (Horsthemke and Ludwig, 2005, De Rycke et al., 2002), to increase production of farm animals and to save endangered species (Andrabi and Maxwell, 2007, Paterson et al., 2003). However, the recent realisation of the critical importance of epigenetic information and its impact on health has focused attention on assisted reproductive technique (ART) births (Horsthemke and Ludwig, 2005, De Rycke et al., 2002).

There is increasing evidence that *in vitro* culture of preimplantation embryos may be associated with epigenetic alterations resulting in abnormal growth, phenotypic abnormalities, and developmental failure. One possible cause is an inappropriate use of culture medium. Khosla *et al.* (2001) showed that the culture mouse embryos in M16 containing fetal calf serum (FCS) induces aberrant DNA methylation of imprinted genes causing repression of the H19 gene. Furthermore, the culture of preimplantation ovine and bovine embryos in medium containing serum frequently causes overgrowth and diverse developmental abnormalities during fetal and postnatal development and is related to abnormal pattern of DNA methylation at ICR controlling the imprinted IGF2R gene (Young et al., 1998). These aberrant phenotypes are named ‘large offspring syndrome’ (Young et al., 1998, Young et al., 2001), which is the model for *Beckwith-Wiedemann syndrome* (BWS) in human. Recent studies in mouse also showed that *in vitro* mouse embryo culture can affect global DNA methylation patterns (Zaitseva et al., 2007) but not histone modifications such as acetylation of H4, methylation of H3K9, and phosphorylation of H3 serine 10 (Huang et al., 2007).

Moreover, aberrations of DNA methylation by unknown mechanisms during ART may cause abnormalities at imprinted genes. There are few clinical investigations of epigenetic errors that may arise during early development caused by ART (De Rycke et

al., 2002, Kelly and Trasler, 2004, Dean et al., 2005), but they are thought to mimic certain genetic disorders. Firstly, the *Beckwith-Wiedemann syndrome* (BWS) is a pre/postnatal overgrowth syndrome associated with errors at the imprinting cluster. BWS is thought to be a result of the inappropriate epigenetic imprinting at specific loci on the maternal allele, such as inactivation of the cyclin-dependent kinase CDKN1C or defects of the H19 DMR. Secondly, *Angelman Syndrome* (AS) is caused by epigenetic alteration resulting in loss of function in the brain of the maternal copy of Ube3a.e. Thirdly, retinoblastoma sometime is caused by hypermethylation of the promoter region of RB gene (tumour suppressor gene) through interactions with DNMT1. Finally, *ATR-X syndrome* is an X-linked syndrome characterized by severe mental retardation, reduced or absent speech, delayed developmental milestones, and also facial dysmorphism, α -thalassemia, and sexual dysgenesis, and is associated with DNA methylation defects in specific regions of the genome.

Advances in biotechnology such as nuclear transfer (NT) techniques have allowed the propagation of multiple genomic copies of an animal utilizing both embryonic and somatic cells as donors of genetic materials (Wilmut et al., 1997, Wilmut et al., 2002). The employment of NT has also provided a way to control the genetic content and accurate genetic modification of animal species, thus providing a route for the study of gene function, production of biopharmaceutical proteins (Schnieke et al., 1997), modification of production traits or disease susceptibility (Wilmut et al., 2000), preservation of endangered species (Gomez et al., 2004) and creation of pluripotent stem cells to serve in patient-specific cell transplantation for treatment of degenerative disease (Yang et al., 2007b). Notwithstanding, there are many advantages of NT, the success rate of reconstructive embryo to ES cell and full term offspring is extremely low (Wilmut et al., 2002). The majority of cloned animals die during pre-implantation and gestation due to developmental abnormalities (Wilmut et al., 2002). Epigenetic malfunction has been observed in several species of cloned embryos (Santos et al., 2003, Yang et al., 2007a, Beaujean et al., 2004a); this may produce inaccurate gene expression and lead to developmental failure (Eilertsen et al., 2007, Vignon et al., 2002, Blelloch et al., 2006, Inoue et al., 2006). For these reasons, successful cloning is likely to require the reorganisation of donor chromatin to a state compatible with embryonic development. The transferred genome must properly activate genes important for early

embryonic development and also adequately suppress differentiation-associated genes that are transcribed in the original donor cell (Suzuki et al., 2006).

In order to these resolve epigenetic abnormalities, several studies have attempted to use epigenetic reagents to improve the developmental success rate of NT and round spermatid injection (ROSI). For example, Trichostatin A (TSA), a deacetylase inhibitor, can induce global histone acetylation in many cells. Treating mouse cloned embryos with TSA after nuclear implantation of specific cell types can significantly improve the development to blastocyst (Rybouchkin et al., 2006) and full term development (Kishigami et al., 2006a). Another study in ROSI shows that unusual remethylation of the paternal genome occurs after normal active demethylation, but using TSA can reduce the DNA remethylation of paternal chromatin (Kishigami et al., 2006b). Furthermore, studies in bovine nuclear transfer show that partial removal of DNA methylation by using either TSA or 5-aza-2'-deoxycytidine (a DNA methyltransferase inhibitor) can increase blastocyst development of bovine cloned embryos (Enright et al., 2003).

1.7 Epigenetic Control of Pluripotency and Differentiation

The definition of pluripotency refers to cells such as embryonic stem (ES) cells that have the capacity to differentiate into three germ layers: endoderm, mesoderm, or ectoderm, and have an ability for infinite propagation (Smith, 2001). There are many applications for ES cells: first, the unique property of ES cells is that they can be easily subjected to gene introduction, after which genetically modified ES cells can be injected into blastocysts to produce transgenic animals (Wobus and Boheler, 2005). Second, the ability to differentiate into germ layers is comparable to postimplantation development; so this characteristic can be used as a model for developmental biology and pathology (Wobus and Boheler, 2005, Keller, 2005). Furthermore, *in vitro* differentiated ES-derived progenitor cells might be of use as a source for cell transplantation for intractable diseases such as cardiovascular diseases, neurodegenerative diseases,

diabetes, and reproductive disease (Nagano, 2007, Laflamme and Murry, 2005, Schroeder et al., 2006).

There is recent evidence that DNA and histone modifications might play an important role in the regulation of gene expression patterns in ES cells. It has been shown that the promoter region of active genes in ES cells, such as *Oct4* and *Nanog*, are marked by acetylation of H3 and H4, and H3K4me3 which is an important mark for active gene transcription; then after differentiation these modifications are replaced with repressive markers such as DNA methylation, H3K9me3, H3K27me3 (Hattori et al., 2004, Kimura et al., 2004, Feldman et al., 2006, Hattori et al., 2007). Also remarkable and important is the finding of bivalent chromatin structures in ES cells. This unusual chromatin modification profile is highly specific to ES cells (Azura et al., 2006, Bernstein et al., 2006) and has been demonstrated for histone modification patterns at multiple lineage-control gene loci in mouse ES cells. The critical transcription factor genes for cell lineage determination such as *Sox1*, *Nkx2-2*, *Msx1*, *Irx3*, and *Pax3*, that are not expressed in ES cells, are associated with both active (H3K4me3) and repressive (H3K9me3 and H3K27me3) histone modifications within their promoter loci. The bivalent histone modification patterns disappear after the differentiation of ES cells into the neuronal lineage. Only H3K4me3 remains at the promoters of neural genes whereas H3K27me3 is removed; conversely, the promoters of other genes that were silent in neurons lose H3K4me3, and are enriched for H3K27me3. This is consistent with other studies showing the important role of polycomb proteins associated with H3K27me3 in maintaining ES cell pluripotency and plasticity (Boyer et al., 2006). This evidence suggests that H3K27me3 might be functionally important for preventing expression of lineage-control genes in ES cells

Furthermore, evidence from various sources indicates that chromatin might generally be less compact and more transcriptionally permissive in undifferentiated ES cells compared to differentiated cells. For example, differentiation of mouse ES cells results in the progressive increase in the cluster number of pericentric heterochromatin (Kobayakawa et al., 2007). Moreover, from a different perspective, the global chromatin status of ES cells can be assessed by measuring the exchange rate of chromatin-associated proteins using fluorescent recovery after photobleaching (FRAP)

(Meshorer et al., 2006). Histones H2B and H3 and the heterochromatin-associated protein HP1, which bind H3K9me2 and H3K9me3, were found to have a markedly increased exchange rate in ES cells compared with differentiated cells. The higher recovery rate presumably reflects a loose binding of these proteins to the chromatin, so that chromatin is more accessible to transcription factors and chromatin modifiers. At the same time, the exchange rates of the histone variant H3.3, which marks actively transcribed regions, was generally unaltered upon differentiation (Meshorer et al., 2006).

There are many publications that report that ES cells can survive without certain heterochromatin associated proteins but subsequently fail to undergo proper differentiation. These results suggest that these proteins are not necessary for ES cells and that chromatin in ES cells is flexible and/or does not form mature or real heterochromatin (see table 1.4).

Table 1.6 *Pluripotency and differentiation defects are caused by epigenetic alteration*

Name	ES cell viability	Differentiation abnormality	References
Dicer	No	Yes (mesoderm)	(Bernstein et al., 2003)
Dicer	Yes	Yes (mesoderm, endoderm)	(Kanellopoulou et al., 2005)
Suv39h	Yes	No data	(Peters et al., 2001)
Ehmt2	Yes	No data	(Tachibana et al., 2002)
Suv420h	Yes	No data	(Benetti et al., 2007)
Ezh2	No	No data	(O'Carroll et al., 2001)
Ehmt1	Yes	No data	(Tachibana et al., 2005)
Dnmt1,3a,3b	Yes	Yes (cardiocyte, erythoid, myeloid)	(Jackson et al., 2004)
Dot1L	Yes	No data	(Jones et al., 2008)
ESET	No	No data	(Dodge et al., 2004)

1.8 Manipulation of Epigenetic Reprogramming

By nature, cells and organisms possess mechanisms that can epigenetically reprogram gene expression. One notable example is the epigenetic reprogramming that occurs during development; a phenomenon which is critical for normal development and relies on a variety of endogenous chemical and enzymatic mechanisms (Reik, 2007, Morgan et al., 2005). Not only mechanisms of endogenous origin, but also exogenous factors can be used to manipulate epigenetic profiles and in this way can alter cell fate. There are currently several techniques used to manipulate epigenetic profiles such as nuclear transfer, cell fusion, treating cell with ES cell extraction or small molecule, and over expression of defined transcription factors (Jaenisch et al., 2005, Collas and Gammelsaeter, 2007, Takahashi et al., 2007). Cell reprogramming strategies may be useful for future therapies for example: the treatment of cancers which have epigenetic abnormalities, or cell replacement therapies for degenerative tissue, and also as a model for regenerative and developmental biology.

Studies from different species using the nuclear transfer technique have shown that eggs or oocytes have the ability to erase old epigenetic pattern (“epigenetic memory”) of the donor nucleus and replace these with new marks (Ng and Gurdon, 2005, Martin et al., 2006, Lee et al., 2002, Shi et al., 2003, Yang et al., 2007a). After a somatic cell is transferred into an oocyte, its nuclear epigenetic marks are removed and replaced with the embryonic epigenetic pattern (Eggan and Jaenisch, 2003, Rideout et al., 2001). However reprogramming by nuclear transfer is incomplete as most nuclear transfer embryos fail to achieve full term development (Mann et al., 2003, Kang et al., 2001, Young et al., 2001).

The idea that pluripotent cells such as embryonic germ (EG) cells, ES cells and embryonal carcinoma (EC) cells have an ability to reprogram a differentiated cell to a pluripotent or multipotent cell was exhibited by cell fusion experiments (Tada et al., 1997, Ambrosi and Rasmussen, 2005). After the ES cell is fused with a somatic cell, epigenetic reprogramming is promoted by the pluripotent cell’s proteins, which in turn switches on pluripotent gene expression (*Oct4*, *Nanog*, *Sox2*) in somatic cells (Tada et al., 1997, Pells et al., 2002). However the use of such hybrid cells is considered to be

impractical due to the presence of double set of chromosomes and the method cannot produce vast cell numbers. An alternative technique involves the use of nuclear extracts from pluripotent cells. This may be more appropriate, as the previous studies have shown that pluripotent cell extracts can retain the factors capable of epigenetic reprogram *Oct4* promoter by removing DNA methylation (Taranger et al., 2005) and repressive histone methylation (Collas and Taranger, 2006).

Recently, it has been demonstrated that a cocktail of ectopic expression or recombinant proteins of the pluripotency transcription factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28) is sufficient to reprogram somatic cell into ES-like cell in mouse (Takahashi and Yamanaka, 2006, Zhou et al., 2009) and human cells (Park et al., 2008b, Takahashi et al., 2007, Kim et al., 2009, Yu et al., 2009), these cells have been named as induced pluripotent stem cells (iPS cells) (Takahashi and Yamanaka, 2006). The study of their global epigenome shows that these techniques can induce global epigenetic reprogramming from fibroblast to ES-like cell epigenome (Maherali et al., 2007, Takahashi et al., 2007). iPS cells are a promising tool for deriving either patient-specific or disease-specific pluripotent cells. These cells might be used for cell therapy, drug screening, and as a model for the pathogenesis of degenerative diseases such as Alzheimer's, Parkinson's or multiple sclerosis (Park et al., 2008a). The study of iPS cells that are corrected for a gene mutation to rescue sickle cell anaemia in mouse model has been a 'proof of principle' for the use of iPS cells combined with gene therapy for disease treatment (Hanna et al., 2007). Although the mechanisms of epigenetic reprogramming by transcription factors are still poorly understood, the general hypothesis is that epigenetic reprogramming in iPS cells may be the result of two steps: firstly, exogenous chromatin remodelers, Klf4 and Myc (Jiang et al., 2008, Knoepfler et al., 2006) might govern global chromatin to be more permissive to allow the transcription machinery access to the chromatin domain. Secondly, the exogenous pluripotent transcriptional factors, Oct4 and Sox2 access the open chromatin and initiate global gene expression, and subsequently expression of endogenous pluripotent genes such as *Oct4*, *Nanog*, *Sox2*. This is consistent with recent evidence showing that Oct4 protein is at the heart of epigenetic reprogramming in ES cells through histone demethylases *Jmjd1a* and *Jmjd2c* to help maintain pluripotency (Loh et al., 2007).

In the last decade, a variety of small molecules have been created and discovered. Some small molecules have the potential to alter epigenetic marks whilst others can reprogram terminally differentiated cells to progenitor cells. One example includes demethylating agents such as 5-azacytidine. 5-azacytidine (5-AzaC) is a cytosine analogue that can cause extensive global DNA demethylation and reduce DNA methyltransferase activity in the cells (Taylor, 1993). It was originally developed as an antitumor agent, and has been useful in the treatment of both childhood and adult leukaemia (Taylor, 1993). 5-AzaC can reverse the differentiation state of ES cells through its DNA demethylating activity at differentiation-related genes (Tsuji-Takayama et al., 2004). However the effect of 5-azacytidine is unpredictable it can cause either reverse differentiation or induce differentiation (Taylor, 1993). Another type of modifiers includes histone deacetylase (HDAC) inhibitors such as valproic acid (VPA) and trichostatin (TSA). VPA has been utilised for decades as a treatment for epilepsy, as a mood stabiliser and in migraine therapy, while TSA is currently applied as an anticancer medicine (Butler and Bates, 2006, Marks and Jiang, 2005). It has been shown that both VPA and TSA can inhibit HDACs and then trigger active global demethylation of the mammalian epigenome, causing reprogramming of gene expression (Milutinovic et al., 2006). VPA combined with two factors, Oct4 and Sox2 enables reprogramming of primary human fibroblasts, without the need for the oncogenes c-Myc or Klf4 to become iPS cells that resemble human ES cells in pluripotency, global gene expression profiles and epigenetic states (Huangfu et al., 2008). In addition, histone methylation inhibitors such as BIX-01294. (a diazepin-quinazolin-amine derivative) have been demonstrated to selectively impair G9a (Ehmt2) HMTs and H3K9me2 (Kubicek et al., 2007). A combination of BIX-01294 with defined factors with or without c-Myc (a chromatin remodeller) could increase the cellular and epigenetic reprogramming rate of iPS methodology (Shi et al., 2008).

1.9 Objectives of the Project

Cell reprogramming post-fertilisation is fundamental and has major implications for the application of modern technologies such as nuclear transfer, and iPS cell generation. However the success rate of reprogramming for both technologies is extremely low and the mechanisms involved are not well understood. One aspect believed to be very important for reprogramming is the resetting of chromatin and epigenetic signature. To improve the technology we need to understand the principle of natural reprogramming using mouse preimplantation mouse development as a model. In the present study, chromatin and epigenetic signatures and the mechanisms involved were explored during normal mouse development.

The overall objective of the PhD project was to study chromatin organisation in normal mouse development. These studies will provide crucial information on the developmental transitions in nuclear chromatin organisation, and increase our understanding of how heterochromatin is established after fertilisation. In addition, they could serve as a model for improvement of reproductive biotechnologies such as somatic cell reprogramming, diagnosis of genetic abnormalities and medical applications of stem cell technology for other species such as human and livestock. More specifically, the aims of the research project were:

1. To investigate epigenetic heterochromatin formation in developing mouse embryos derived from natural fertilisation.
2. To investigate the epigenetic heterochromatin transition from the *in vivo* inner cell mass of blastocysts to *in vitro* embryonic stem cells.
3. To investigate global changes of epigenetic heterochromatin markers during *in vitro* early differentiation of ES cells as a model for early differentiation in mouse development.
4. To investigate heterochromatin maturation in mouse development from preimplantation to postimplantation.
5. To investigate the mechanism of epigenetic and chromatin reprogramming in the preimplantation embryo.
6. To investigate the function of epigenetic modifiers such as histone

methyltransferases and demethyltransferase in mouse preimplantation embryos.

The critical hypothesis tested in these studies was whether heterochromatin in preimplantation embryos is more immature than that of postimplantation embryos and pluripotent embryonic stem cells.

Finally, the results from this project are expected to provide benefits by assisting in the creation of novel techniques for epigenetic manipulation. It may improve epigenetic reprogramming protocols for either nuclear transfer or transcription factor reprogramming, and for directing ES cell differentiation into specific lineages. Moreover, further understanding of epigenetic and chromatin modification in mouse development will provide useful models for human and other species to study the mechanisms at the basis of developmentally epigenetic abnormalities and cancer development from progenitor or stem cells.

CHAPTER 2

General Materials and Methods

Chapter 2

Materials and Methods

In this Chapter the manipulations of animals and embryos, cell culture techniques, methods to detect epigenetic and chromatin modifications, gene expression assays, and gene knock-down technology are described. The full details of reagents and chemicals are shown in Appendix 1. Media preparation is described in Appendix 2.

2.1 Animal and embryo manipulation

2.1.1 Animals

The mouse colony (B6CBAF1) was maintained in phase I of the animal unit at the Chancellor's Building of the University of Edinburgh, under 12 hour dark/light cycle. Six to eight week old B6CBAF1 (C57BL/6 x CBA) female mice were used to collect oocytes and embryos. Eight to ten week old male mice were used for natural mating. The experiments and scientific procedures on living animals were performed under project licences and personal licences that were approved by the Home Office.

2.1.2 Immature oocyte recovery and *in vitro* maturation

Follicle development was induced by intraperitoneal injections (i.p.) of 5 IU pregnant mare serum gonadotrophin (PMSG) (Intervet) into female mice. After 47 hours, mice were euthanised by cervical dislocation and ovaries were transfer to M2 medium (Sigma). Immature oocytes were collected using small needle (26 Gauge) to puncture follicles. Cumulus oocyte complexes (COCs) were washed three times in M2 medium before transferring to a drop of 40 μ l of α MEM (Invitrogen) supplemented with 0.5 IU/ml sheep FSH (Sigma), 1 mg/ml Fetuin (Sigma) and 1ng/ml EGF (Sigma) in 60 mm cell culture dishes (Falcon) Ten to fifteen COCs were cultured in the same drop of

culture medium and were incubated at 37°C in 5% CO₂ 95% air atmosphere in a humidified incubator for 17 hours to mature.

The maturation rate was approximately 90%, observed by the presence of the first polar body and metaphase plate using Hoechst staining (Sigma). Oocytes were incubated in Hoechst 33342 (5 µg/ml) diluted in M2 medium for 5 minutes in a 37°C warm box, then washed in M2 three times before being transferred into PBS for observation of the metaphase plate using a fluorescence microscope.

2.1.3 Zygote collection and culture

Female mice were superovulated by intraperitoneal injections of 5 IU pregnant mare serum gonadotrophin (PMSG) (Intervet) and 5 IU of human chorionic gonadotrophin (hCG) (Intervet) given 47 hours apart. After hCG injection, mice were allowed to mate with 8 to 10 week-old male mice. Female mice were euthanised by cervical dislocation 21 hours post hCG. Ovaries and oviducts were removed, and zygotes were recovered from the ampulla of the oviducts using tweezers to puncture the oviduct membrane. Thereafter, cumulus cells were removed from zygotes by short incubation (30 seconds) in 1% hyaluronidase (Sigma) in M2 medium at 37 °C. Denuded zygotes were then cultured in 40 µl drops of Potassium Simplex Optimised Medium (KSOM) (Millipore) under mineral oil (Fluka) at 37°C in 5% CO₂ 95% air atmosphere humidified incubator. Each drop contained 10-15 zygotes. Embryos were cultured in 60 mm cell culture dishes (Falcon) for 4-5 days to the blastocyst stage. A diagram of embryonic development is shown in Figure 2.1.

2.1.4 Peri/Postimplantation embryo collection

To understand epigenetic changes that take place during implantation, different postimplantation stages of embryos were collected. Mice were naturally mated at night and the presence of vaginal plugs the next day was considered day 0.5 post-mating (E0.5). Preimplantation embryos (E3.5) and periimplantation embryos (E4.5) were collected by flushing the uterus with 1 ml of M2 medium with blunt needle (26 Gauge). Postimplantation embryos (E5.5, E6.5, E7.5, E8.5, E14.5 and E17.5) were dissected using a needle and forceps, the embryo was released by making a small puncture at the

implantation side then using forceps to press around puncture site to release embryo. The stages of post-implantation development are shown in Figure 2.1.

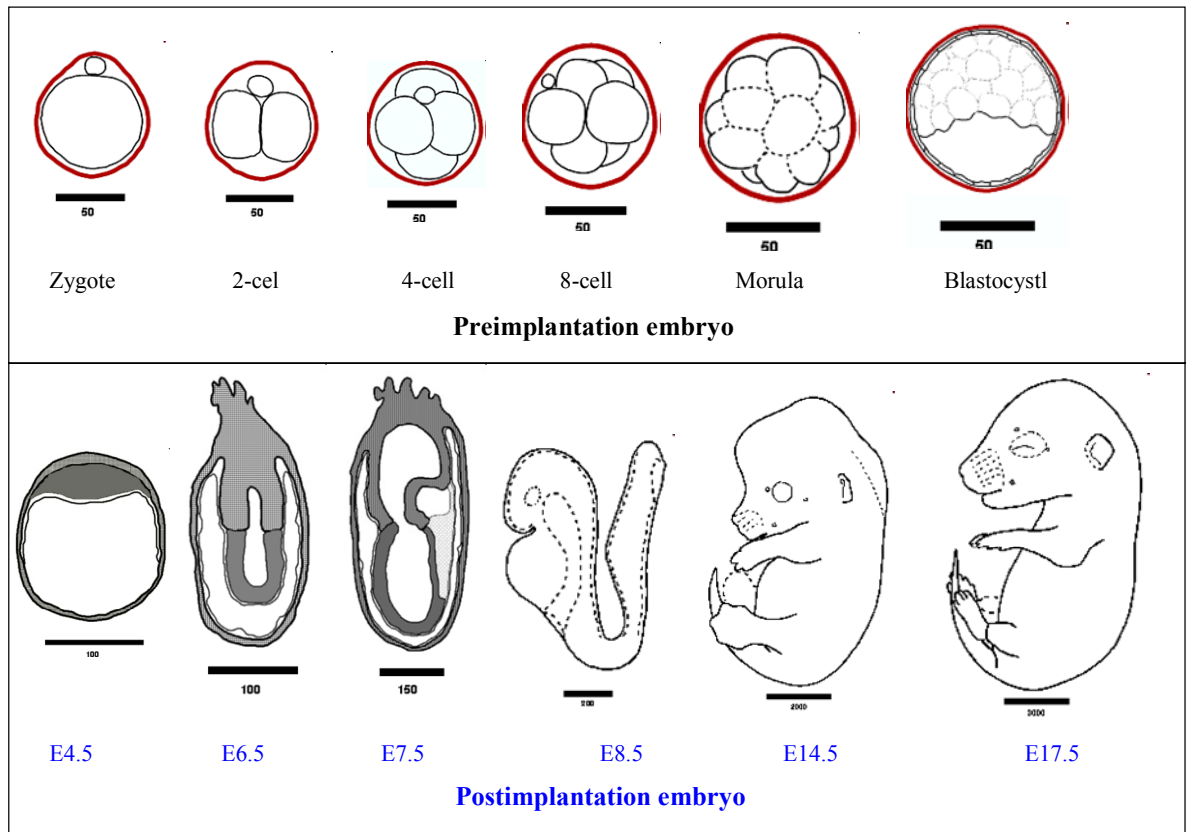


Figure 2.1 Diagram of embryonic stages collected in this experiment. The scale bar represents 50 μm for preimplantation embryos, 100 μm for E4.5 and E6.5 embryos, 200 μm for E7.5 and E8.5 embryos, 2,000 μm for E14.5 embryos and 3,000 μm for E17.5 embryos. (Images were modified from Edinburgh mouse atlas project; <http://genex.hgu.mrc.ac.uk/>).

2.2 Cell culture

In the present studies, fibroblast cells from adult or foetal mice were compared to ES cells and embryos. Fibroblast cells represent finally differentiated cells whereas ES cells served as an *in vitro* model for embryo development.

2.2.1 Primary mouse adult fibroblast culture

Primary adult fibroblast cells were obtained from skin of B6CBAF mice and cultured in fibroblast medium containing DMEM (Invitrogen) supplemented with 10% Foetal bovine serum (FBS) (Invitrogen) and 100 IU/ml penicillin/100 ug/ml streptomycin (Sigma). Pieces of mouse ear skin were cut and sliced using a surgical blade, and left in a small volume of culture medium allowing the skin tissue to attach to the culture dish (10cm dish). Skin tissue was incubated at 37°C in 5% CO₂ 95% air atmosphere humidified incubator for 1-2 days. After fibroblast-like cells were visible, medium was added to cover the tissues and incubated until the cells were 80-90% confluent. Thereafter cells were passaged (1:3) using 0.25% trypsin (see Appendix 2) and moved into new culture dishes. After 3-4 passages, only adult fibroblast cell remained.

2.2.2 Primary mouse embryonic fibroblast (MEF) culture

Primary foetal fibroblast cells were obtained from 14.5 days foetuses (E14.5, see Figure 2.1). Foetuses were collected from the uterus and then left in 37 °C in PBS (Lonza). Head and visceral organs were removed and the body skin was cut into small pieces and incubated in 0.25% trypsin for 10-30 minutes. Foetal cells were centrifuged 700 xg for 2.5 minutes, and then PBS was removed. The wash and centrifuge steps were repeated three times. The supernatants were removed and pellets were resuspended in fibroblast medium (see 2.2.1), before culture at 5% CO₂ and 95% air atmosphere humidified in a 37°C incubator. After 3 to 4 passages (Split 1:3), only fibroblast cells remained, and the MEF passage number was considered to be passage one.

2.2.3 Embryonic stem cell culture

Established ES cells and new ES-like cells were used. ES cells (E14tg2a) were

originated from 129/J mice (Hooper et al., 1987) and these cells were provided by Institute of Stem cell Research, the University of Edinburgh. ES-like cells (noo01) were derived during this project and early passages of these cells were used. ES cells were cultured on gelatinised culture plate (0.1% gelatine) (Sigma) using ES cell medium. ES cell medium contained knockout-DMEM (Invitrogen), 15% FBS (Invitrogen), 100 IU/ml leukaemia inhibition factor (LIF) (Chemicon), 100 μ M 2-mercaptoethanol (Invitrogen), 100 μ M non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen) and 100 IU/ml penicillin/100 ug/ml streptomycin (Sigma). ES cells were cultured at 37°C in 5% CO₂ 95% air atmosphere humidified incubator. Ratio for ES cell splitting is 1:3.

2.2.4 Isolation of ES-like cell from blastocysts

ES cells were isolated from late blastocysts. MEF cells were used as a feeder layer and they were prepared one day before ES cell isolation. MEF cells were inactivated by 10 μ g/ml mitomycin C in fibroblast medium and incubated at 37°C in 5% CO₂ 95% air atmosphere humidified incubator for 3 to 4 hours. Thereafter feeder cells were washed with PBS and cultured with ES cell medium at 37°C in 5% CO₂ incubator for 24 hours. Expanded blastocysts were used for isolation of ES cells. The zona pellucida was removed using acid Tyrode's solution (Sigma). Zona free blastocysts were then placed in a 4-well culture plate containing feeder layer and ES cell medium at 37°C in 5% CO₂ incubator. After 4-6 days, inner cell mass (ICM) outgrowths were disaggregated by placing into 0.025% trypsin (see Appendix 2) drop for 2-3 minutes. Mechanical aspiration was done using a pipette until the ICM broke into small clumps. These cells were transferred to new feeders and ES medium (see 2.2.3). After 1-3 days ES-like colonies were visible, cells were disaggregated and placed into 30 cm culture dishes; this step was considered first passage.

2.2.5 *In vitro* differentiation of ES cells

ES cell differentiation into embryoid body (EB) is commonly used as a model for early differentiation of mouse development (Wobus and Boheler, 2005). ES cells (4×10^6 cell) were induced to form EB by placing ES cell into 10 cm Petri dish (Sterilin) and cultured

with differentiation medium (similar to ES cell medium but without LIF) at 37°C in 5% CO₂ 95% air atmosphere humidified incubator. EBs were cultured for 4 days with a daily change of medium. Thereafter they were collected for protein or RNA extraction. For immunofluorescence EBs were transferred to a chamber slide to allow to attachment to the slide for 24 to 48 hours at 37°C in 5% CO₂ incubator before staining.

For cardiomyocyte spontaneous differentiation, EBs were cultured for 2-4 weeks in cell culture dish (10 cm) to allow EBs to attach to the dish surface. Differentiation medium was changed daily. Beating cardiomyocytes appeared approximately 2 weeks after differentiation was initiated.

2.2.6 *In vitro* implantation and blastocyst outgrowth

In order to observe very early implantation embryos, an *in vitro* implantation technique was used. Expanded blastocysts were incubated in ES cell medium on gelatinised chamber slides. After 24 hours, trophectoderm (TE) attached the plate and differentiated to trophoblast while ICM would grow and form colony-like epiblast cells. Embryos were collected approximately 24 hours post-culture were described as *in vitro* periimplantation embryos while embryos collected later than 24 hours were categorised as postimplantation embryos.

2.2.7 Cell cryopreservation

Confluent cells from 10cm culture dish were trypsinised and centrifuged 700 xg for 5 minutes. Supernatants were removed and cells were resuspended with culture medium. Cells were counted and diluted to 2×10^6 cell/ml and 2x-freezing medium (see Appendix 2) with equal volume of culture medium (500 µl) was slowly added into cell suspension. One ml of cell suspension (1×10^6 cells) was pipetted into 1.8 ml cryopreservation tube. Tubes were placed into a slow freezing foam box and then a box was placed into -80 °C freezer for overnight. The next day, the tubes were transferred into liquid nitrogen for long term storage. For thawing, frozen cells in the cryotube were thawed in 37 °C water bath for 1-2 minutes. Cell suspensions were transfer to a 15 ml centrifuge tube (Corning) and diluted with 5 ml culture medium. Cell suspensions were centrifuged 700 xg for 5 minutes, and then supernatants were discarded. Cell pellets were resuspended

with culture medium and transfer to appropriate culture dish (a 10cm dish is suitable for 1×10^6 cells).

2.3 Detection of global epigenetic and chromatin modifications.

Several techniques were used to detect epigenetic and chromatin modifications. DNA methylation, histone modification, transcription factor and related proteins were detected by using highly specific antibodies. Western blot and indirect immunofluorescence, with and without flow cytometry, were used to observe epigenetic changes in cultured cells. Whole-mount immunofluorescence was used for preimplantation embryos and early postimplantation embryos. Western blot and immuno/histofluorescence were used for postimplantation embryos (E8.5-E17.5). Dilution of antibodies and their source is shown in Table 2.1, and details of the antibodies used are shown in Appendix 1.

2.3.1 Immunofluorescence

2.3.1.1 Immunofluorescence for cell culture and whole mount embryo.

Specimens were fixed in 4% paraformaldehyde (PFA, Sigma) in PBS (Invitrogen) (see Appendix 2) for 15 minutes at room temperature. For the detection of DNA methylation, specimens were treated with 4M Hydrochloric Acid (HCl) at room temperature (10 minutes for embryos, 5 minutes for cells) before permeabilisation to denature the DNA to allow antibody binding to methyl-C groups. After washing twice with PBS, specimens were permeabilised in 0.2% Triton X-100 (Sigma) for 15 minutes. After that, specimens were washed twice with 0.01% Tween (Sigma) in PBS and incubated in blocking solution 2% bovine serum albumin (BSA) (Sigma) or 5% donkey serum (DS) (Sigma) for either 2 hours or overnight. Specimens were then incubated with primary antibodies either at room temperature for an hour or at 4 °C for overnight. After washing three times, specimens were incubated with secondary antibodies for an

hour. Then specimens were washed three times and mounted with 1.5 µg/ml DAPI diluted in glycerol (Vectashield)

2.3.1.2 Immunohistofluorescence for postimplantation embryo

Postimplantation embryos (E8.5 to E17.5, see Figure 2.1) were prepared in paraffin section or cryosection. For paraffin section, samples were fixed in 4% PFA for an hour, and then held in 70% ethanol overnight at room temperature. Embryos were embedded in paraffin by Centre for Reproductive Biology (CRB) histology service (University of Edinburgh). Sections were cut by microtome (Leica) with a thickness of 3 to 5 µm. After section, slides were dried in 60 °C incubator for overnight and then the antigen retrievals were performed (see 2.3.1.3). Thereafter, sections were deparaffinised and rehydrated in sequence: xylene two changes for 5 minutes, 100%, 95%, 80%, 50% ethanol for 20 seconds each. Slides were rinsed in PBS and they were ready for immunofluorescence.

For cryosection, samples were placed in OTC Embedding Medium (Raymond A Lamb) and immediately frozen on dry ice for 30 minutes. Samples were stored in -80 °C until ready for cryosection. The cryosection was performed using cryostat machine (Leica) in CRB histology unit. After cryosection, slides were dried 5 minutes, and then fixed in 4% PFA 5 minutes before immunofluorescence.

For immunofluorescence, samples from paraffin section or cryosection were permeabilised with 0.1% Triton X100 in PBS, washed in PBS for 5 minutes and blocked with 2% BSA+5% donkey serum for an hour. Samples were washed in PBS for 5 minutes and then incubated with primary antibodies either at 4°C overnight or at room temperature for 2 hours. Thereafter, samples were washed in 0.05% Tween in PBS three times for 15 minutes each and one time in PBS for 5 minutes. Samples were incubated with secondary antibodies at room temperature for 2 hours. Next, the samples were washed in 0.05% Tween in PBS three times for 15 minutes each, and one time in PBS for 5 minutes. Excess liquid was absorbed by using paper and then mounted with 1-2 drop of Prolong Gold antifade reagent with DAPI (Invitrogen). Mounting reagent was left for curing overnight at room temperature in the dark. Finally slides were sealed with

nail varnish and stored at 4 °C until observation with a fluorescence microscope (see section 2.3.1.4).

2.3.1.3 Antigen retrieval

After the paraffin embedding, some antigens may lose sensitivity and it may be necessary to ‘unmask’ or retrieve the antigen through pre-treatment of the specimens before staining. This step is known as antigen retrieval or recovery. Two methods, either pressure cooker (heating method) or trypsin digestion (enzymatic method) were used in these experiments.

For the heating method, two litres of antigen retrieval buffer (see Appendix 1 and 2) were added into Tefal Clypso pressure cooker and then boiled. Dewaxed and rehydrated slides were placed on cooker and boiled for 5 minutes. The slides were removed and washed in cold water.

For the enzymatic method, trypsin (Sigma) was used. The trypsin tablet was dissolved in deionized water (1mg/ml) and then dropped on specimen slides. Slides were incubated at room temperature for 10 minutes. Afterwards each slide was washed with PBS (Invitrogen) for 5 minutes.

2.3.1.4 Image Analysis and Software

Images were captured by both fluorescence microscopy and laser confocal microscopy. The microscopes and software used in these studies were Zeiss fluorescence microscope (Zeiss Axioscope 2) with OpenLab Software (Improvision), Nikon fluorescence microscope (Nikon) with Velocity (Improvision), Zeiss laser confocal microscope (Zeiss LSM) with LSM Meta software and Leica laser confocal microscope (Leica SP5) with Leica image software. The details of software, manuals and relevant websites are shown in Appendix 1.

Z stack series were mainly obtained on the Zeiss LSM using LSM-meta software. 3D, 2D and merged images were created by LSM-image browser. Image adjustment and measurements of fluorescence intensity were performed using the programme ImageJ (Rasband, 1997-2004).

The volume of each stage of embryo is varies considerably with development, so when the embryo decreases in nuclear volume (through division), the chromatin density increases (Sawicki et al., 1974). Thus, the nuclear volume has an effect on nuclear intensity and also fluorescence intensity (Beaujean et al., 2004). To resolve the effect of nuclear volume on chromatin intensity, the nuclear total fluorescence intensity (NTFI) method was used for semi-quantitative analysis of fluorescence intensity. The NTFI was calculated using an average of 2D nuclear intensities multiplied by nuclear volume. This method was modified from Fuoka Aoki's Lab, University of Tokyo (Akiyama et al., 2006, Kageyama et al., 2007, Liu et al., 2004). The nuclear volume was calculated using ellipsoid volume equation $\frac{4}{3}\pi abc$. (abc = radii). Radii were measure by LSM-image browser. The intensity was measured over all nuclei of each embryo, except in morula where at least eight nuclei were randomly measured, and in blastocysts where at least eight nuclei from inner cell mass and eight nuclei from trophoectoderm were measured.

2.3.1.5 Flow cytometry analysis

Flow cytometry was used to analyse the relationship of histone modification and early differentiation in ES cells, and also applied to analyse transfection efficiency and apoptosis rate after siRNA transfection. Flow cytometry allows simultaneous multi-parameter analysis of single cells, it can analyse more than ten thousand cells in less than 5 minutes, and it is more efficient and accurate than manual visual cell counting. Not only can it detect fluorescence signal, it also can detect cell size and complexity (sometimes called granularity). The signals of fluorescence intensity, cell size and complexity together can be used to distinguish cell populations.

Cultured cells were trypsinised and then centrifuged at 700 xg for 5 minutes. Suspension was removed and washed with PBS-azide (Sigma), and centrifuged three times. Then cells were fixed in 0.4% PFA in 1.5 ml centrifuge tubes at room temperature for 15 minutes. After centrifugation, the fixative was removed and washed with PBS. Cells were permeabilised with 0.2% TritonX-100 for 15 minutes. Thereafter, cells were blocked with 2%BSA+5%Donkey serum for 30 minutes, and primary antibodies were added later (see Table 2.1). Cells were washed in PBS and rotated for 5 minutes and then centrifuged at 700 xg for 5 minutes. This washing step was repeated

three times. After that the cells were incubated with secondary antibodies in light protected tubes. Cells were washed in PBS and rotated for 5 minutes and centrifuged at 700 xg for 5 minutes. This wash step was repeated three times. Flow cytometry analysis was performed using FACSCalibur® flow cytometer (BD Bioscience). Data were analysed using the programme Cell Quest (BD Bioscience). Samples incubated with blocking solution alone or secondary antibodies alone were used as negative controls for autofluorescence and non-specific fluorescence.

2.3.1.6 Positive and Negative control for immunofluorescence

Mouse embryonic fibroblasts were used as positive control for histone antibodies and ES cells for pluripotency marker as it show in many publications. Samples were treated with either no antibody or only secondary antibodies and then observed under fluorescence microscopy and flow cytometry to evaluate autofluorescence and non-specific binding of secondary antibodies.

2.3.2 Protein electrophoresis

Western blot analysis was used to detect global histone modification and heterochromatin proteins in fibroblast and ES cells in order to find the association between chromatin modification and cell differentiation.

2.3.2.1 Histone extraction (Acid extraction)

Histone extracts were prepared from either cultured cells or embryos. Cultured cells were trypsinised and centrifuged 700 xg for 5 minutes. Supernatants were removed and lysis buffer was added to the pellets and mixed using a pipette. For embryos, lysis buffer was added to a metal bead then homogenised for 1 minute by homogeniser (Ball Mill Model MM301, Retsch). The lysis buffer contained 10 mM HEPES pH 7.8 (Sigma), 1.5 mM MgCl₂ (Sigma), 10 mM KCL (Sigma), 0.5 mM DTT (Sigma), 1.5 mM PMSF (Sigma). Hydrochloric acid (HCl) was added into a final concentration of 200 mM. The samples were incubated at 4°C for 30 minutes then taken through a brief

sonication (1 Watt for 2 seconds) (XL2000, Microson). Samples were centrifuged at 13,000 xg, 4 °C for 10 minutes. Supernatants containing the acid-soluble proteins were collected and stored at -20 °C before western blot analysis. In order to concentrate the histone protein, eight volumes of acetone were added to the samples which were then incubated at -20°C overnight. Thereafter, samples were centrifuged 2,000 xg at 4 °C for 10 minutes and supernatants were removed. Samples were washed with acetone/HCL (100 mM stock) (10:1), and centrifuged 2,000 xg at 25 °C for 10 minutes. This was repeated twice. Supernatants were removed and pellets were washed with acetone (10 volumes), then centrifuged 2,000 xg at 25 °C for 10 minutes. This was repeated three times. Supernatants were removed and then pellets were dried at room temperature. The pellets were either stored at -80 °C until or dissolved in 0.2 M hydrochloric acid before being stored. The full details regarding preparation of cell extraction buffer is in Appendix 2.

2.3.2.2 Whole cell extraction

In order to analyse a house keeping protein (Beta-actin) and pluripotency marker (Oct4 and Nanog), whole cell extract solution (RIPA buffer) was used to extract whole cell protein. RIPA buffer contained 50 mM Tris HCl pH 8 (Sigma), 150 mM NaCl (Sigma), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA pH 7.4, 1.5 mM PMSF. Samples were incubated in lysis buffer at 4 °C for 30 minutes, and then briefly sonicated. Samples were centrifuged at 13,000 xg 4 °C for 10 minutes, and supernatants were stored at -20 °C. The full details regarding preparation of cell extraction buffer is in Appendix 2.

2.3.2.3 Protein concentration measurement

Protein concentration was measured using the DC protein assay (Bio-Rad) which is a colorimetric assay developed from the well-documented the Lowry assay (Lowry et al., 1951). Firstly, working reagent A' was prepared by adding 5 µl of reagent S into 250 µl of reagent A' (working reagent A was stable for 1 week). BSA solution 0.2 to 1.5 mg was used to prepare a standard curve. One µl of standards and samples were added into the cuvette. Thereafter, 5 µl of working reagent A' was added into each cuvette. Finally 200 µl of reagent B was added, and then vortexed to mix. Samples were incubated at

room temperature for 15 minutes before measuring absorbance at 750 nm using spectrophotometer (Thermo).

2.3.2.4 Western blot analysis

The western blot is a method of detecting specific proteins in a sample from tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide.

Western blot was performed using the NuPAGE[®] system (Invitrogen) which is a pH discontinuous SDS-PAGE, precast polyacrylamide mini-gel system. Samples (10 to 20µg protein) were mixed with NuPAGE[®] LDS sample buffer and NuPAGE[®] Reducing agent (500mM dithiothreitol), then heated to denature proteins at 70°C for 10 minutes. Protein electrophoresis was performed using precast 4-12% gradient Bis-Tris-Gels (Invitrogen), Xcell Surelock[™] Mini-Cell and NuPAGE[®] MES SDS running buffer plus NuPAGE[®] antioxidant. The running conditions were 200 V constant voltages, starting at 110-125 mA/gel, ending with 70-80 mA/gel for 35 minutes. Thereafter, proteins were transferred onto a PVDF membrane (Amersham) with a XCell II[™] Blot module using NuPAGE[®] transfer buffer plus NuPAGE[®] antioxidant. Conditions were 30 V constant voltage, starting at 170 mA, ending with 100 mA, for 1 hour.

After the protein transfer step, membranes were removed from the block. They were blocked with 5% skim milk, and then incubated with primary antibodies for either 1-3 h at room temperature or at 4°C for overnight (see Table 2.1). Membranes were washed five times with 0.1 % Tween 20 in PBS for 10 minutes. Thereafter membranes were incubated with secondary antibodies conjugated with HRP (see Table 2.2). Chemofluorescence was produced by reaction with ECL solution (Amersham) and was detected with X-ray film (Fujifilm) and Kodak imaging system (Kodak). All membranes were stained with anti-histone H3 or anti-histone H4 or β-actin primary antibodies to confirm equal protein loading.

Table 2.1 Primary antibodies used in the experiments

Antibodies	Dilution						Temp (°C)	Block	Supplier
	WE-F	EF-IF	EC-IF	C-IF	FC	WB			
H3k9me1	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H3K9me2	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H3k9me3	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H4K20me1	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H4K20me2	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H4k20me3	1:200	1:100	1:200	1:500	1:500	1:1000	4	BSA	Upstate
H3K27me3	1:200	Not used	Not used	1:500	1:500	1:1000	4	BSA	Upstate
H3K4me3	1:200	Not used	Not used	1:500	1:500	1:1000	4	BSA	Upstate
HP1 β	1:300	Not used	Not used	1:600	1:600	1:5000	4	DS	Euromedex
HP1 α	1:100	1:50	1:100	1:300	1:300	1:5,000	RT	DS	Chemicon
Oct4	1:100	Not used	Not used	1:300	1:300	1:3000	4	DS	Santa Cruz
Nanog	1:500	Not used	Not used	1:1000	1:1000	1:5000	RT	DS	Chemicon
β -actin	Not used	Not used	Not used	Not used	Not used	1:20000	4	Milk	Abcam
H3	Not used	Not used	Not used	Not used	Not used	1:20000	4	Milk	Upstate
H4	Not used	Not used	Not used	Not used	Not used	1:1000	4	Milk	Upstate
5MeC	1:200	Not used	Not used	1:500	Not used	Not used	RT	BSA	Eurogentec

WE-IF = Whole embryo immunofluorescence. EF-IF = Embryo paraffin section immunofluorescence
 C-IF = Cell immunofluorescence. FC = Flow cytometry. WB = Western blotting. DS=Donkey serum.
 BSA= Bovine serum albumin. RT = Room temperature. Temp = Incubation temperature

Table 2.2 Secondary antibodies used in the experiments.

Ab/companies	Dilution					Supplier
	WE-IF	histo	FC	C-IF	WB	
Anti-mouse FITC	1:200	1:200	1:200	1:200	Not used	Jackson
Anti-rabbit FITC	1:200	1:200	1:200	1:200	Not used	Jackson
Anti-rabbit TRITC	1:200	1:200	1:200	1:200	Not used	Jackson
Anti-mouse Alexa 488	1:500	1:200	Not used	Not used	Not used	Molecular probe
Anti-rabbit Alexa 488	1:500	1:200	Not used	Not used	Not used	Molecular probe
Anti-rabbit Alexa 543	1:500	1:200	Not used	Not used	Not used	Molecular probe
Anti-mouse HRP	Not used	Not used	Not used	Not used	1:5000	Santa Cruz
Anti-rabbit HRP	Not used	Not used	Not used	Not used	1:5000	Amersham
Anti-rabbit PE	Not used	Not used	Not used	1:200	Not used	Santa cruz

Histo = histofluorescence. WE-IF = Whole embryo immunofluorescence.

C-IF = Cell immunofluorescence. FC = Flow cytometry. WB = Western blotting.

2.4 Gene expression analysis

The Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique was used to qualify and quantify the mRNA expression level in cells and embryos. These steps involved total RNA extraction, RNA concentration measurement, cDNA synthesis, semi-quantitative PCR and Quantitative real-time PCR.

2.4.1 Sample preparation for total RNA extraction

Cells were trypsinised into a 1.5 ml tube, and cell number was counted. RNA was extracted from up to 1×10^7 cells using the RNeasy kit (Qiagen). Cells were centrifuged at 700 xg for 4 minutes and supernatants were discarded. The pellets were stored at -80 °C.

Embryos were washed in M2 medium and then placed into 200 µl PCR tubes (ABgene) containing 10 µl of RNALater buffer (Ambion). Samples were incubated at 4°C overnight and then transferred to -80 °C for long term storage.

2.4.2 Total RNA extraction by RNeasy kit (Qiagen)

Lysis buffer was freshly prepared by mixing 990 µl of RLT with 10 µl of betamercaptoethanol. 350 µl of lysis buffer were used for less than 5×10^6 cells, if more than that 600 µl was used. Lysis buffer was added into the cell extract (this step was performed on ice) and then mixed. Thereafter the samples were homogenised by pipette the lysate onto QIAshredder spin columns (Qiagen), centrifuged at 9,000 x g for 2 minutes. One volume of 70% ethanol was added to the lysate and mixed using a pipette. Samples were applied to RNeasy minicolumns in 2-ml collection tubes and centrifuged at 8,000 xg for 15 seconds, and the flow-through was discarded. Before the next step DNase I was prepared by gently mixing 70 µl RDD buffer with 10 µl DNase I. Then 80 µl of DNase I was gently pipetted into sample columns, and incubated at room temperature for 15 minutes to remove DNA. 350 µl of buffer RW1 was added into columns and then centrifuged at 8,000 xg for 15 seconds. Flow-through and used collection tubes were discarded. Columns were transferred to new 2-ml collection tubes and 500 µl of buffer RPE was added to columns then centrifuged at 8,000 xg for 15

seconds. Flow-through and collection tubes were discarded before 500 μ l of buffer RPE was added to columns, centrifuged at 8,000xg for 2 minutes. Next, columns were transferred to 1.5 ml collection tubes for elution step. RNase-free water was added to columns (30 μ l for cells, 50 μ l for embryos) and centrifuge at 8,000 xg for a minute. The RNA was stored at -80 °C.

2.4.3 Precipitation of embryo RNA using Pellet Paint® Co-Precipitant

As the quantity of RNA yield from embryo was small. Pellet Paint® Co-Precipitant (Novagen) was used to concentrate embryonic RNA. Pellet Paint is a visible dye-labelled carrier formulated specifically for use in alcohol precipitation of nucleic acids. To each tube of embryo RNA 2 μ l of Pellet Paint was added with 0.1 volumes (i.e. 5 μ l) of 3M sodium acetate and mixed briefly. Thereafter, two volumes (i.e. 100 μ l) of 100% ice-cold ethanol was added and then mixed briefly. Samples were incubated at room temperature for 2 minutes. Afterward samples were centrifuged at 15,000 xg for 30min at 4°C, and supernatants were carefully removed. Pellets were washed with 500 μ l of ice-cold 70% ethanol in DEPC-treated water, mixed briefly and centrifuged at 15,000 xg 4 °C for 25 min. The supernatants were removed, centrifuged again briefly and as much ethanol as possible was removed. Pellets were dried on ice with tubes lids opened for approximately 10-15 minutes. Pellets were resuspended in 9 μ l RNase-free water and transferred RNA to pre-labelled 0.5 ml PCR tubes. Tubes were stored on ice, before proceeding to cDNA synthesis, or stored in -80 °C for storage.

2.4.4 cDNA synthesis

cDNA synthesis was performed by using either Cloned AMV first strand synthesis kit (Invitrogen) or Verso™ cDNA Kit (ABgene).

For Cloned AMV first strand synthesis kit (Invitrogen), all steps were performed in 0.5 ml PCR tubes (ABgene), on ice. The RNA in 9 μ l RNase-free water (for cells we used 1 μ g), 1 μ l of random hexamer and 2 μ l of 10mM dNTP were mixed, then heated at 65 °C for 5 minutes. The master mix was prepared on ice as follows: 4 μ l of 5x cDNA synthesis buffer (vortex prior to use), 1 μ l of 0.1M DDT, 1 μ l of RNaseOUT, 1 μ l of DEPC treated water, 1 μ l of cloned AMV RT. Control (RT-ve) sample consisted of

RNA without reverse transcriptase. Eight μl of mastermix reaction was added to each tube of denatured RNA on ice. RNA was transferred to Hybaid PCR machine (Hybaid) to perform cDNA synthesis. The reverse transcription cycling program was as follows: (1) 25°C 10 minutes, (2) cDNA synthesis 50°C for 50 minutes, (3) inactivation 85°C for 5 minutes. The product can be stored at -20 °C before performing PCR.

For Verso™ cDNA synthesis, Verso™ reverse transcriptase was used as RNA dependent DNA polymerase with a significant attenuate RNase H activity can synthesise long cDNA up to 11 kb between the temperatures 42 and 57°C. The recommended amount of starting RNA is between 1 pg to 1 μg . RT enhancer was used to eliminate contaminating DNA during RT step. RNA was heated at 70 °C for 5 minutes to reduce secondary structure, and then immediately placed on ice. 11 μl diluted RNA was mixed with 4 μl of 5XcDNA synthesis buffer, 2 μl dNTP mix, 1 μl of random hexamer and 1 μl of Verso enzyme mix. Thereafter, tubes were transferred to Hybaid PCR machine (Hybaid) to perform cDNA synthesis. The reverse transcription cycling program was as follows: (1) cDNA synthesis 50°C for 30 minutes, (2) inactivation 95°C for 2 minutes.

RT-negative samples were prepared similar to normal sample except adding reverse transcriptase enzymes. RT-negative samples were used to ensure the RNA used was free of DNA contaminants.

2.4.5 Polymerase Chain Reaction (PCR)

The ThermoStart PCR mix (2x) (ABgene) containing dNTP's 1.5mM MgCl_2 and hot-start taq polymerase was used for PCR. PCR mastermix was prepared containing: 12.5 μl PCR buffer, 1.25 μl forward primers, 1.25 μl reverse primer and 8 μl distilled water. The 23 μl of mix were added to each tube in PCR hood. On lab bench 2 μl of cDNA was added, mixed well, centrifuged tubes briefly, kept tubes on ice. Tubes were transferred to PCR machine (Hybaid). PCR programme was, 1 cycle of 95 °C for 15 min, 35 to 40 cycles of 95°C for 30 sec, 55 to 65°C for 30 sec, followed by 72°C for 45 sec, 1 cycle of 72°C for 5 to 7 min, cool to 4°C and hold at 4°C. A list of the primers and relevant references is shown in Table 2.3.

2.4.6 Agarose Gels for DNA Electrophoresis

The PCR products were run on an agarose gel. Agarose (Fisher Scientific) was added into 1X Tris Borate EDTA (TBE) buffer (see Appendix 2) to make 1.5% agarose gel. SYBR gold (Molecular probes, Invitrogen) (1 μ l in 100 ml of TBE) was added into mixture. The mixture was heated using a microwave until the agarose had dissolved. The gel was cooled before pouring into the gel cassette and allowed to polymerise. 15 μ l of PCR product were loaded into each lane on 1.5% agarose gel. Electrophoresis was run in TBE buffer with 90 Volts for 30 to 60 minutes. PCR products were observed by UV light and images were captured using Gel-DOC programme (Bio-Rad). The 10 μ l of 100 bp DNA ladder (New England Biolab) was used to determine size of PCR products.

2.4.7 Real time Quantitative Polymerase Chain Reaction (QPCR)

QPCR is becoming important for gene expression analysis. One method for real-time quantification uses SYBR Green I, a dye that fluoresces when bound non-specifically to double stranded DNA. SYBR Green I is more economical and sensitive than using probes; however, it is less specific. The QPCR using SYBR Green requires optimisation of the primer concentration and PCR conditions before doing the experiment. Basically, the fluorescent dye SYBR Green I binds to the minor groove of the DNA double helix. The unbound dye molecule weakly fluoresces, producing a minimal background fluorescence signal which is subtracted during computer analysis. After the annealing step, a few dye molecules can bind to the double strand and then more dye molecules bind to the newly synthesised DNA during the elongation step, resulting in a dramatic increase of the SYBR Green I molecules emitting light on excitation. Upon denaturation of the DNA before the next heating cycle, the dye molecules are released and the fluorescence signal significantly decreases. Fluorescence measurement was performed at the end of the elongation step of every PCR cycle to monitor the real-time continuously increasing amount of amplified DNA. A melt curve analysis was usually performed subsequently to the PCR to identify primer dimer, and DNA contamination. So using PCR with the SYBR Green I together with melt curve analysis provides an excellent tool for specific product identification and quantification.

A 2-Step QPCR technique was used in the present studies. cDNA synthesis was performed by using Verso™ cDNA synthesis kit. For PCR step, we use ABsolute™ Blue QPCR SYBR green with low ROX Mix (ABgene) contained Therm-Start™ DNA polymerase for doing PCR and ROX dye which is an internal passive reference used to normalise the fluorescent reporter signal generated in qPCR. The final concentration of ROX dye was 25 nM in 1x reaction. The concentration of MgCl₂ was 3mM in the final 1x reaction. The DNA polymerase was activated at 95°C for 15 minute before PCR. 3 step QPCR program was used as follow: (1) denature 95°C for 15 sec, (2) annealing temperature 15 seconds, (3) Extension 15 seconds for product size less than 200 kb, 30 seconds for product size between 200 to 500 kb, 60 seconds for product size more than 500 bp. The PCR was repeated for 40 cycles and then melt curve analysis was performed. In this study we used a PCR platform called the Rotor-Gene™ 6000 (Corbett life science). The total reaction volume was 25 µl in 200 µl PCR tubes (Corbett Life Science). QPCR primer sequences were obtained from publications (see Table 2.4) and primer bank (Wang and Seed, 2003). The names of genes, primers sequence and reference are shown in Table 2.4.

2.4.8 Quantification strategy in QRT-PCR

Generally, three major strategies are performed in real-time RT-PCR to measure the levels of expressed genes: (1) absolute quantification, (2) relative quantification and (3) comparative quantification.

Absolute quantification is used to determine the amount of sample mRNA (copy number or ng, etc). Basically, it translates the PCR signal to a defined unit of interest by calculation with a calibration curve using known DNA standard molecules. The reliability of an absolute real-time RT-PCR assay depends on the condition of 'identical' amplification efficiencies for both the native target and the calibration curve in RT reaction and in the following kinetic PCR. Absolute quantification is commonly used for diagnosis purposes such as viral or bacterial load determination, amount of microorganism in food and the percentage of genetic modified organisms in food (Pfaffl, 2004).

Relative quantification measures the relative change in mRNA expression levels between multiple samples and is represented as a ratio or fold change. Unlike absolute quantification, a calibration curve is not necessary for relative quantification. However it may require a serial dilution of calibrator to give a standard curve which can be used to calculate reaction efficiency. The reaction efficiency should represent the repeatability and reliability of the experiments and is also inserted into the mathematical model for calculating relative gene expression. Genes of interest have to be normalised with reference genes which are stable expression genes, for example, house keeping genes Gapdh and beta-actin. The reliability of the result is dependant on the stability of the gene used for normalisation, so it makes the selection of reference genes critical for valid results. The relative quantification method is widely used to study the pattern of gene expression in many experiments such as a comparison between control and treatment or between different tissues.

Comparative quantification is quite similar to relative method, but reference genes are not required and sometimes reaction efficiency may be unnecessary. It can be used to study a high throughput of different gene expression in various samples.

Table 2.3 Primer sequences for conventional RT-PCR (The concentration for all primers is 10 μ M)

Gene	F/R	Sequence (5'-3')	Size (bp)	Temp (C°)*	Source
Suv3-9h1	F	CGCATCGCATTCTTTGCC	184	60	(Lehnertz et al., 2003)
	R	AAGCCGTTGTCCCACATTTG			
Suv3-9h2	F	AAATCCAACCAGGCACTCCC	278	60	(Lehnertz et al., 2003)
	R	CTCGTAGTCCAGGTCAAAGAGGTA			
Suv4-20h1	F	CACGACAGTGACAGCAACCT	655	58	Wongtawan, T
	R	GTCTGAAGGCCCTATGTGGA			
Suv4-20h2	F	TGCGTGAAGAGGATGAAGAC	800	58	Wongtawan, T
	R	TAGGCGGGTAAGTTCCACAC			
Oct4	F	GGCGTTCTCTTTGGAAAGGTGTTC	312	55	(Chambeyron and Bickmore, 2004)
	R	CTCGAACCACATCCTTCTCT			
Gapdh	F	AACAACCCCTTCATTGACCTC	458	60	(Lehnertz et al., 2003)
	R	TTCTGAGTGGCAGTGATGGC			
Ehmt1	F	CCTGGAGGAGCCAGTAAATG	350	55	Wongtawan, T
	R	GCTGTCTGGGACTTTGTGGT			
Ehmt2	F	ATCCTTAAGCGGGAGACCAT	221	58	Wongtawan, T
	R	CAGTGGGGACAGAAGACCAT			
HP1a	F	AGGGCAACCTACCTGTTGTG	547	55	Wongtawan, T
	R	GGCTCCTCTCCAAAAATTCC			
Jmjd1A	F	GCCTAGCATTCTTGGCTTTG	597	58	Wongtawan, T
	R	ATGACATTGGCTGCATCAGA			
Jmjd2a	F	GCTGCAGCACAGACATCAAT	504	58	Wongtawan, T
	R	GGAAGCACGTGATGAAGACA			
Jmjd2c	F	GATGACTGGCCTTACGTGGT	702	58	Wongtawan, T
	R	GTCCCAATGTGTACCCCAAG			
Ezh2	F	AAGCACAATGCAACACCAAA	170	58	Taylor, J
	R	AGACGGTGCCAGCAGTAAGT			

*Annealing temperature. F=Forward primer. R=Reverse primer

Table 2.4 Primer sequences for QPCR

Gene	F/R	Sequence (5'-3')	Size (bp)	Temp (C°)	Conc (nM)	Source
Oct4	F	ACATCGCCAATCAGCTTGG	50	60	900	(Brambrink et al., 2008)
	R	AGAACCATACTCGAACCATCC			900	
Sox2	F	ACAGATGCAACCGATGCACC	52	60	50	(Brambrink et al., 2008)
	R	TGGAGTTGTACTGCAGGGCG			300	
Gapdh	F	TTCACCACCATGGAGAAGGC	51	60	300	(Brambrink et al., 2008)
	R	CCCTTTTGGCTCCACCCT			300	
Ehmt2	F	ATCCTTAAGCGGGAGACCAT	221	60	900	Wongtawan, T
	R	CAGTGGGGACAGAAGACCAT			50	
Suv3-9h1	F	CGCATCGCATTCTTTGCC	184	60	900	(Lehnertz et al., 2003)
	R	AAGCCGTTGTCCCACATTTG			900	
Suv3-9h2	F	AAATCCAACCAGGCACTCCC	278	60	300	(Lehnertz et al., 2003)
	R	CTCGTAGTCCAGGTCAAAGAGGTA			900	
Jmjd2a	F	TAATGTCTGAAAGCGCTTCTG	112	60	900	(Wang and Seed, 2003)
	R	TTCTGTTTGAAACTGACCAGG			900	
Jmjd1a	F	CAGCAACTCCATCTAGCAAGG	161	60	900	(Wang and Seed, 2003)
	R	TGTTCTCGGTAATTCAGGTTTTG			300	
Jmjd2c	F	TGGAGAGTCCCCTAAATCCA	129	60	300	(Wang and Seed, 2003)
	R	CCTTGCAAGACCTGCTCG			900	
Nanog	F	CACCCACCCATGCTAGTCTT	130	60	900	(Masui et al., 2007)
	R	ACCCTCAAATCCTGGTCCT			900	
Eset	F	CCTGGGTGCATGAGTTTGG	118	60	300	(Wang and Seed, 2003)
	R	TGTAAGTACGAAGTTCCTCCATA			300	
MuERV	F	TTCTCAAGGCCACCAATAGT	301	58	300	(Sonehara et al., 2008)
	R	GACACCTTTTTTAATATGCGAGCT			900	
Elf1a	F	AAAAACAGGCGCAGAGGTA	142	58	300	(Wang and Seed, 2003)
	R	TCCTCACACCGTCAAAGCAC			900	
Zcan4D	F	TGTAGCCGTCGCTTTCGCCA	196	58	900	Wongtawan, T
	R	GTGGCAATCCACAAGCATGTTA			900	
Rpl13a	F	CCTGCTGCTCTCAAGGTTGTT	146	60	300	(Wang and Seed, 2003)
	R	CGATAGTGCATCTTGGCCTTT			900	
β -actin	F	CAACGAGCGGTTCCGATG	67	60	300	(Lefever et al., 2009)
	R	GCCACAGGATTCCATACCCA			300	
Cdx2	F	AGGCTGAGCCATGAGGAGTA	124	60	300	(Masui et al., 2007)
	R	CGAGGTCCATAATCCACTCA			300	

*Annealing temperature. F=Forward primer. R=Reverse primer

2.5 Gene knock-down by siRNA

The short interfering RNA (siRNA) or silencing RNA, is a class of 20-25 nucleotide-long double-stranded RNA molecules that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of a specific gene. The use of siRNA for gene silencing is a rapidly evolving tool in molecular biology to study gene expression and function. In these experiments, siRNA was used to study the function of HMTase and DHTMase.

2.5.1 Transfection siRNA into ES cells

ES cells (2×10^5 cells) were plated into each well of 6-well plate, and cultured with 2 ml of ES cell medium (without antibiotics) for 1 hour, at 37°C in 5% CO₂ incubator. After 45 minutes, siRNA solution was prepared as follows: 125 pmole siRNA (6.25 µl) was added to 250 µl Opti-MEM, mixed gently. Diluted transfection reagent was prepared by adding 5 µl Lipofectamine™ 2000 into Opti-MEM®, gently mixed and incubated at room temperature for 15 minutes. The 250 µl of siRNA was gently mixed with 250 µl Lipofectamine™ 2000, and incubated at room temperature for 15 minutes to form a complex. After that, 500 µl of complexes were added to each well containing cells and medium, gently mixed by rocking the plate back and forth. Finally cells were incubated at 37°C in 5% CO₂ incubator for 16-24 h.

2.5.2 siRNA injection into zygote

For embryo knock-down experiments, intracytoplasmic injection technique was used to deliver siRNA into embryonic cytoplasm. Pipettes were purchased from Biomedical Instruments Ltd (Germany, cat no.PI1.2). The diameter of the injection needle was approximately 1.2 µm. Injection was performed using Narishige micromanipulation system connected to a Nikon Microscope by Dr. Jane Taylor (University of Edinburgh). The volume of liquid injection was controlled using a PV820 PicoPump (World Precision Instruments Ltd.). One to two microlitres of siRNA (50 to 100 µM) was loaded into injection pipette and approximately 1 pl of liquid was injected into zygote cytoplasm. Injection of either RNAi-negative control (Negative Control #1 siRNA,

Ambion) or buffer was used as negative controls while Oct4 siRNA was used as a positive control. The siRNA sequences and suppliers are shown in Table 2.5.

Table 2.5 *siRNA sequences*

Gene	Sequence	OligoID	Suppliers
Jmjd2c sense	UGAACAGGAGUACAAUGCAACUGUU	MSS233760	Invitrogen
Jmjd2c antisense	AACAGUUGCAUUGUACUCCUGUUCA	MSS233760	Invitrogen
Oct4 sense	GGAUGUGGUUCGAGUAUGGtt	151960	Ambion
Oct4 antisense	CCAUACUCGAACCACAUCct	151960	Ambion
Jmjd2a sense	GCCACACAGACAGGUUGCUUGGAAA	MSS200432	Invitrogen
Jmjd2a antisense	UUUCCAAGCAACCUGUCUGUGUGGC	MSS200432	Invitrogen
Jmjd1a sense	CAGGAGAGCCGAGUGUGAAGAAGAA	MSS214043	Invitrogen
Jmjd1a antisense	UUCUUCUUCACACUCGGCUCUCCUG	MSS214043	Invitrogen
Negative control*	Stealth™ RNAi negative control duplex	12935-300	Invitrogen
Transfection control*	Block-it™ alexa Fluor® Red Fluorescent Oligo	14750-100	Invitrogen

* No sequence information provide from companies.

CHAPTER 3

Dynamics of histone lysine methylations, modifiers and satellite sequences in mouse preimplantation embryos

Chapter 3

Dynamics of histone lysine methylations, modifiers and satellite sequences in mouse preimplantation embryos.

3.1 Introduction

3.1.1 Genetic reprogramming in the preimplantation embryo

One of the most critical events for the beginning of new life is the transition of maternal oocyte to embryonic zygote upon fertilisation. Oocytes synthesise and store transcripts and proteins that are important for genetic and epigenetic reprogramming postfertilisation (Ma et al., 2008, Bachvarova, 1985, Zeng et al., 2004, Vitale et al., 2007). The transition of the maternal oocyte to embryonic zygote is comprised of three steps: first, degradation of maternally inherited transcripts and proteins occurs immediately after fertilisation (Bachvarova, 1985, Piko and Clegg, 1982, Clegg and Piko, 1982, Flach et al., 1982). This degradation is specific for mRNA used during oogenesis, but not mRNA required for embryo development (Alizadeh et al., 2005). Second, stored maternal transcripts and proteins are replaced with new zygotic transcripts and proteins such as housekeeping genes at the one to two-cell stage (Schultz, 2002, Minami et al., 2007, Flach et al., 1982). Third, expression of new embryonic-specific transcripts starts at the time point which is called zygotic gene activation (ZGA) or embryonic gene activation (EGA) (Schultz, 2002, Minami et al., 2007).

ZGA in mice mainly occurs at the 2-cell stage, and requires changes in gene regulation and chromatin organisation (Bell et al., 2008, Schultz, 2002). Gene enhancers become more active after the ZGA event in order to relieve the transcriptional repressive state (Henery et al., 1995, Wiekowski et al., 1993). Approximately 70 % of transcripts in the oocytes are derived from TATA-containing promoters; this fundamentally changes after fertilisation to utilise TATA-less promoters in preimplantation embryos. It is likely that TATA-less promoters are more efficient and can accelerate the expression of vital

totipotent genes (Davis and Schultz, 2000, Schultz, 2002). Two-cell embryos contain both maternal mRNA and new transcripts of which approximately 50 % are found in oocytes (Evsikov et al., 2004, Evsikov et al., 2006) and 15 % of transcripts correspond to repetitive sequence such as transposons (Ma et al., 2001, Evsikov et al., 2004). Many transcripts identified in oocytes are *cis*-encoded RNAs that are transcripts from the same gene but in the opposite orientation. For example, Suv39h1 mRNA is not detected in oocytes but Suv39h1 antisense appears in the oocyte; this antisense RNA may have a role in regulating mRNA (Evsikov et al., 2006).

Another wave of gene expression in mouse preimplantation development is found between the 4 to 8 cell stages, named mid-preimplantation gene activation (MGA). This gene expression contributes to cell lineage specification and is particularly important for the formation of the inner cell mass and trophectoderm of the blastocyst (review by Bell et al., 2008).

3.1.2 Protein synthesis in preimplantation embryos

After fertilisation, oocyte proteins remain in the zygote but very few persist to the two-cell stage (Howe and Solter, 1979, Levinson et al., 1978). Many new proteins appear in two-cell embryos and some of these are two-cell specific proteins which disappear at the four-cell stage (Howe and Solter, 1979, Levinson et al., 1978). Stage-specific proteins are also found in four to eight-cell stage and blastocyst embryos (Howe and Solter, 1979, Levinson et al., 1978). Protein synthesis significantly changes (mainly increases) in two-cell embryos, but is stable in four-cell embryos (Latham et al., 1991).

3.1.3 Histone lysine methylation in preimplantation embryos

Asymmetry of parental histone modifications has been found in zygote to two-cell stage embryos. Typically, paternal chromatin lacks many types of histone methylation while maternal chromatin is fully modified. Asymmetry of histone methylation disappears at the four-cell stage, suggesting that the parental genomes are homogeneously mixed after this stage. (Santos et al., 2005, Arney et al., 2002, Lepikhov and Walter, 2004, Erhardt et al., 2003, Torres-Padilla et al., 2006, Sarmiento et al., 2004, van der Heijden et al., 2005). The asymmetry of parental epigenetic and chromatin modifications may play an

important role in cellular reprogramming and ZGA. For example, embryos reconstructed by nuclear transfer mainly fail at the two-cell stage and show aberrant expression of ZGA (Inoue et al., 2006, Suzuki et al., 2006, Sebastiano et al., 2005). The epigenetic asymmetry of parental genomes does not occur in cloned two cell embryos (Merico et al., 2007, Yoshida et al., 2007). The somatic patterns of chromocentres and histone methylation persist in cloned embryos (Martin et al., 2006a, Martin et al., 2006b, Merico et al., 2007) and the resulting aberrant methylation pattern at the two-cell stage causes early development failure (Shi and Haaf, 2002).

Histone methylations are regulated by histone methyltransferases (HMTs) and demethylases. Eset, Ehmt1, Ehmt2, Suv3-9h1 and Suv39h2 are histone methyltransferases that catalyse H3K9 methylation in mammalian cells. Deletion of each gene causes developmental failure at a different stage (see Table 1.2 in Chapter 1). Histone methylations were widely believed to be irreversible (Bannister et al., 2002) until the first histone demethylase was recently discovered (Shi et al., 2004). Little is known about the function of histone demethylases in mouse development. H3K27 demethylases Jmjd3 and UTX are involved in HOX gene regulation and development in *C. elegans* (Agger et al., 2007) and zebrafish (Lan et al., 2007). Jmjd3 is also involved in cell differentiation in mouse epidermal stem cells (Sen et al., 2008) and is required for neural commitment in mouse ES cells (Burgold et al., 2008). H3K9me1/2 demethylase Jmjd1a has an important role in spermatogenesis and the disruption of this gene may cause male infertility (Okada et al., 2007). Jmjd1a and Jmjd2c also play important roles for maintaining pluripotency in mouse ES cells by prevention of methylation at promoter regions of pluripotency genes (Loh et al., 2007).

3.1.4 Non-coding transcripts from repetitive element in preimplantation embryos

Repeat sequences are usually epigenetically silenced in differentiated cells compared to early developmental cells or pluripotent cells. This is associated with DNA methylation and histone methylation marks such as H3K9me2, H3K9me3, H4K20me2 and H4K20me3 (Martens et al., 2005). Epigenetic reprogramming at repetitive sequences can occur at early development (Santos et al., 2002, Santos et al., 2005, Reik, 2007) and during germ cell formation (Hajkova et al., 2008, Surani et al., 2008, Reik, 2007) and it

may activate the transcription process at repeat sequences. After fertilisation, DNA methylation is significantly decreased, compared to parental germ cells and somatic cells, at least at early transposon (Etn) and centromeric satellites but not intracisternal-A particle (IAP) (Kim et al., 2004). A comprehensive analysis of transposable element expression in mouse unfertilised oocytes revealed that long terminal repeat (LTR) class III retrotransposons make a large contribution to the maternal mRNA pool (12 %), which persist in cleavage stage embryos, and expression of transposable elements (TES) is developmentally regulated (Peaston et al., 2004). Furthermore, TES can act as alternative promoters and first exons for a subset of host genes, regulating their expression in oocyte and cleavage embryos (Peaston et al., 2004). Other reports also show that many endogenous retrovirus (ERV) particles and non-LTR transposons are also highly expressed in mature oocytes (Puschendorf et al., 2006).

Transcriptome analysis of two-cell embryos revealed that 8 % of transcripts are repetitive elements in which 80 % of them are retrotransposons including ERV (Evsikov et al., 2004). This is consistent with other meta-analyses showing that many transposons are highly expressed in two-cell embryos (Stanton and Green, 2001). Murine endogenous retrovirus (MuERV-L) is the one of the earliest transcribed genes in mouse zygotes and is highly expressed in two cell embryos and is suppressed in four-cell embryos (Peaston et al., 2004, Kigami et al., 2003, Svoboda et al., 2004) while IAP expresses highly at eight-cell and blastocyst stages (Svoboda et al., 2004). Inhibiting the RNAi pathway by knocking down Dicer causes an increase in expression of these two repetitive elements (Svoboda et al., 2004). These ERVs were found to act as alternative promoter and first exon to diverse genes in two-cell embryos, synchronising their expression (Peaston et al., 2007). Inhibition ERVs activity using antisense RNA causes embryos to arrest at the four-cell stage (Kigami et al., 2003).

3.15 Objectives.

Currently there is a lack of reports profiling the level of histone methylation and their modifiers in mouse preimplantation embryos. The aim of this study was to investigate dynamic changes in global histone methylation and their modifiers during preimplantation development.

3.2 Materials and methods

3.3 Experimental design

Zygotes were collected from B6CBAF1 mice and cultured in KSOM medium. Embryos from all preimplantation stage were collected for immunofluorescence and RT-PCR. A total of approximately 30 embryos per stage for each antibody were collected to do immunofluorescence. Each time, ten embryos per stage were stained and observed under the fluorescence microscope. The experiment was repeated three times with different sample groups.. For QPCR, thirty embryos in total were collected to quantify gene expression by qPCR. Fifteen embryos per stage were pooled for RNA extraction and each pooled sample was performed in triplicate. The PCR was repeated twice with a different pooled sample. These details of the techniques used are described in Chapter 2 Materials and Methods.

3.4 Comparative and Relative gene expression Analysis

In this experiment, comparative and relative quantification was used to study the trend of gene expression in preimplantation embryos (see Chapter 2, Materials and Methods). Relative gene expression requires the use of an endogenous control such as a housekeeping gene to monitor RNA integrity, concentration and PCR efficiency (Fleige et al., 2006, Pfaffl, 2006). Many previous reports show that gene expression of housekeeping genes in embryos is highly dynamic during preimplantation development, (Mamo et al., 2007, Goossens et al., 2005, Kuijk et al., 2007), and comparative expression (non-normalisation with house keeping gene) may be suitable and not bias the data (Pfaffl, 2006). My studies reveal that expression of the housekeeping gene *Gapdh* either per embryo or per individual cells is dynamic especially during oocyte to two-cell stage where mRNA degradation occurs (Figure 3.1), however; the overall result suggested that normalisation with a housekeeping gene could be used to compare only some stage of embryos (Figure 3.1). Alternatively, some studies suggest the use of an external control such as synthetic RNA to monitor sample RNA concentration (McGraw et al., 2007) but this cannot replace the use of an endogenous gene as it cannot measure RNA integrity which may cause changes to the relative concentration

(Fleige et al., 2006). Taking together, I have shown both gene expression ratios per embryo and also relative to house keeping gene (Gapdh) and believe this may be the best way to demonstrate the results without bias. Gapdh can be used as endogenous control for RNA concentration, RNA quality and PCR efficiency.

In the present studies the cell number of the embryos and the use ROX dye helped to adjust or 'normalise' the amount of input material and volume variation in each tube. Quantification of individual transcripts was performed using the comparative quantification analysis included in the Rotor-gene 600 series software (Corbett life science, UK). The feature "Comparative Quantification" was used to obtain threshold cycle (Ct) value and amplification efficiency, which were comparable to reaction efficiency. Gene expression was mathematically compared between calibrator and sample (Warton et al., 2004). The software calculates the Ct value using a take-off point defined as the cycle at which 20 % of the maximum level of fluorescence is reached in the reaction, and also indicates the end of the noise and the transition into the exponential phase (Figure 3.2). The reaction efficiency (sample amplification) was calculated at exponential phase, by detection of the average increase in raw data four cycles following the take-off (Figure 3.3). A 100 % efficient reaction would result in an amplification value of 2 for every sample, which means that a doubling of amplicons takes place in every cycle. The mean efficiency of a group was used to calculate a fold change according to the formula 1 for comparative quantification. For relative quantification the gene of interest (GOI) was normalised with a reference gene (REF) as shown in formula 2. This is one of the best models for relative gene expression due to the fact it can minimise the interference of variation from PCR efficiency, manual error, and RNA stability (Pfaffl, 2004, Pfaffl et al., 2002, Pfaffl, 2001, Fleige et al., 2006, Pfaffl, 2006).

$$R = E_{(GOI)}^{Ct1-Ct2} \quad (1)$$

$$R = \frac{E_{(GOI)}^{Ct1-Ct2}}{E_{(REF)}^{Ct1-Ct2}} \quad (2)$$

R = gene expression ratio, E = amplication efficiency, $Ct1$ = take off value of calibrator, $Ct2$ = take off value of sample, GOI = gene of interest and REF = reference gene (Pfaffl, 2001).

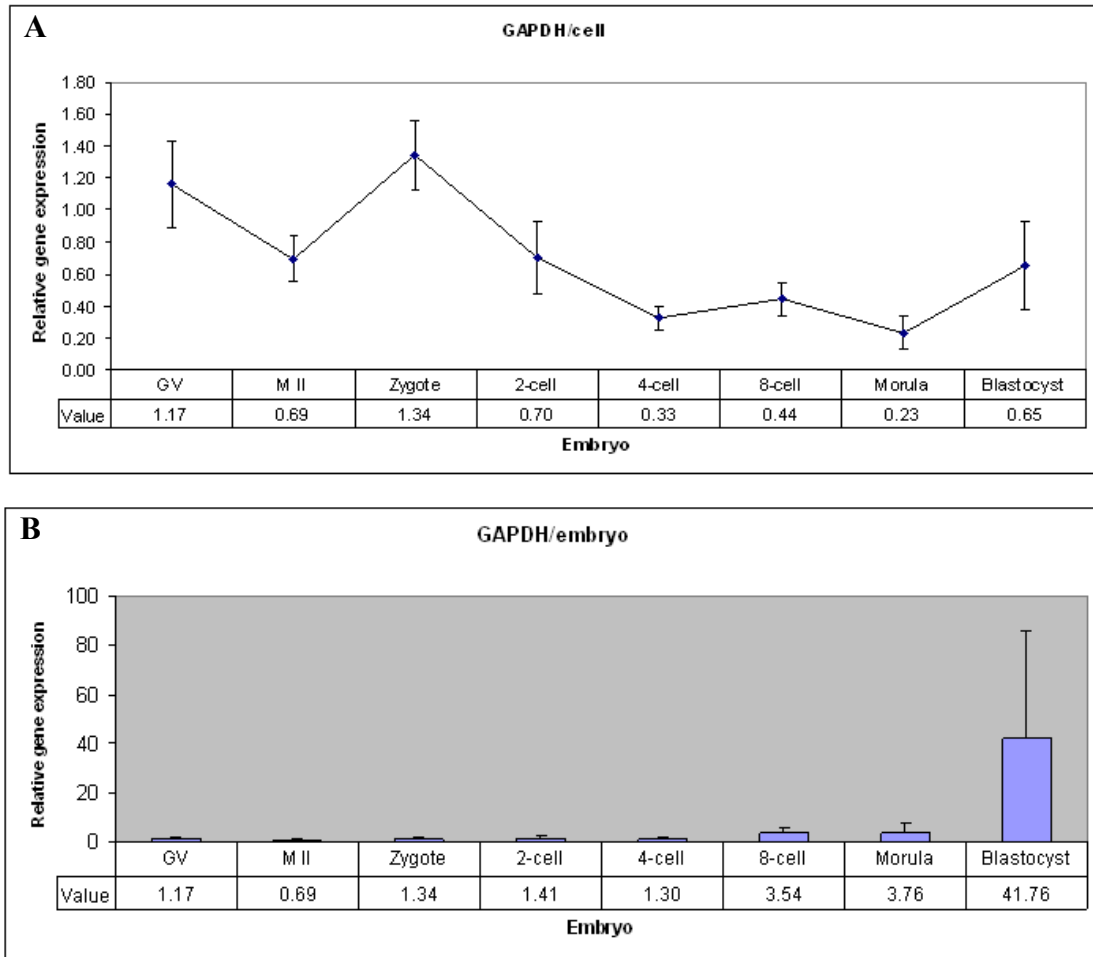


Figure 3.1 Comparative expression of reference gene Gapdh in oocytes and preimplantation embryos. (A) Expression of Gapdh per cell. (B) Expression of Gapdh per embryo. The value shown is gene expression ratio (GV embryo was used as a calibrator). Error bar represented standard deviation.

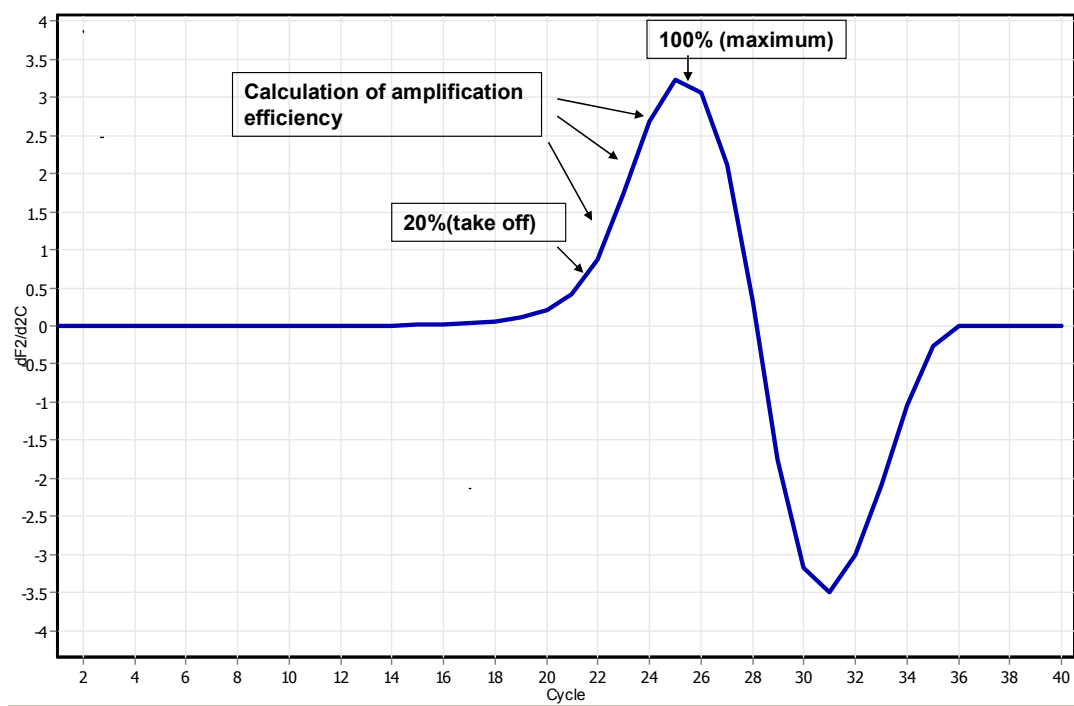


Figure 3.2 Model for calculation of Ct value. Ct value was calculated using take-off point and reaction efficiency (sample amplification). This image was modified from Rotor Gene software manual.

3.5 Statistics

Statistical software SPSS14 was used to analyse statistical changes in fluorescence intensity. Data was evaluated for normal distribution. Total nuclear fluorescence intensity is shown in mean \pm standard error. Statistic difference was analysed using ANOVA and Student's t test. For comparison between embryos and ES cells, the REST software was used, this software use the Pair Wise Fixed Allocation Randomisation Test to calculate statistical differences (Pfaffl et al., 2002, Horgan and Rouault, 2000).

3.3 Results

3.3.1 Asymmetry of histone lysine methylation in zygotes

My immunofluorescence results showed that H3K9me₂, H3K9me₃, H3K27me₃, H3K4me₃ and H4K20me₃ were strongly detected after fertilisation in female chromatin, whereas the signal from paternal chromatin was very weak or undetectable (Figures 3.4, 3.5, 3.8, and 3.10). Unlike other histone methylations, H4K20me₁ and H3K9me₁ were clearly found in both male and female chromatin (Figure 3.3 and 3.6), but were preferentially enriched in male rather than female chromatin based on quantification of fluorescence intensity (Figure 3.11 and 3.12). This may suggest that these markers have an important role in the heterochromatin of the paternal genome. Only H4K20me₂ was not detected in either parental chromatin (Figure 3.7 and 3.12). This asymmetry of histone methylation between parental genomes found in the present study was mostly similar to previous reports (van der Heijden et al., 2005, Santos et al., 2005) with the exception of H3K27me₃, reported to be present in male and female pronuclei (Puschendorf et al., 2008) and H3K9me reported to be detectable only in female chromatin (van der Heijden et al., 2005). These differences between previous and the present studies may reflect the specificity and sensitivity of each antibody to the antigen epitope. Moreover, some diversity of pattern of histone methylations may also occur between mouse strains (See Table 1) (Peaston and Whitelaw, 2006).

Table 3.1 Mouse strains that are commonly used to study embryo epigenetics during development

Mouse strain	References
B6CBAF1 (C57BL/6 x CBA)	Wongtawan T, (van der Heijden et al., 2005, Martin et al., 2006b, Kim et al., 2004)
C57BL6	(Puschendorf et al., 2008, Houlard et al., 2006, Lewis et al., 2006)
B6D2F1 (C57BL/6 x DBA/2)	(Chung et al., 2003, Kageyama et al., 2007a, Moreira et al., 2003)
CF1	(Puschendorf et al., 2006)
CD1 (ICR)	(Puschendorf et al., 2006, Godmann et al., 2007, Kigami et al., 2003)
B6CDF1 (SLC)	(Kim et al., 2003)

3.3.2 Similar dynamic patterns of H3K9me2 and H3K9me3 but not H3K9me1

Many studies show that methylations of H3K9 are strongly associated with gene repression (Lachner et al., 2003, Mikkelsen et al., 2007, Huebert et al., 2006). The quantification in the present study showed that highly dynamic changes in methylation of H3K9 were found in H3K9me2 and H3K9me3 but not H3K9me1 which was more stable. The patterns of H3K9me2 and H3K9me3 are very similar: in two-cell embryos, H3K9me3, H3K9me2 fluorescence signal was dramatically reduced (approximately seven times reduction), but then the signal became stronger in four-cell embryos (Figures 3.4, 3.5 and 3.11). This result suggests that active demethylation of H3K9me2, H3K9me3 occurs in two-cell embryos while *de novo* methylation occurs in four-cell embryos. Moreover, quantitative analysis showed that at eight-cell embryo stage, the total intensity of H3K9me2, H3K9me3 was highest (Figure 3.11). Interestingly, at the morula stage, H3K9me2 and H3K9me3 seem to have undergone a passive demethylation (two times reduction) and maintenance demethylation is instated in the blastocyst. The demethylation and remethylation of H3K9me2 at two to four cell stage has been observed in a previous study (Liu et al., 2004), showing that it occurs in both male (androgenetic embryo) and female (parthenogenetic embryo) chromatin.

3.3.3 Distinct pattern of H4K20me1, H4K20me2 and H4K20me3

H4K20me1 was the only type of H4K20 methylation found in all preimplantation stages. The highest signal was observed in the paternal pronucleus of the zygote. The signal was stable from zygote (maternal pronuclei) to eight-cell stage, and then the signal decreased at morula and blastocyst (Figure 3.12). H4K20me2 was not found at zygote and two-cell, but it became visible but very weak in four-cell embryos and increased at eight-cell embryos to blastocysts (Figure 3.2 and 3.7). There was no difference between TE and ICM for H4K20me1 and H4K20me2 (Figure 3.6, 3.7, 3.12).

H4K20me3 was found in female chromatin and was specifically localised at perinucleolar ring (Figure 3.8) where pericentric heterochromatin is located (Probst et al., 2007). In my studies, H4K20me3 was completely undetectable from two-cell embryos until blastocysts. Demethylation of H4K20me3 was also noted in previous study with a hardly detectable level of H4K20me3 (Kourmouli et al., 2004).

3.3.4 H3K4me3 and H3K27me3

Generally, H3K4me3 marks transcriptionally active promoters and permissive chromatin whereas H3K27me3 is related to gene repression in euchromatin (Lopez-Bigas et al., 2008, Schubeler et al., 2004, Mikkelsen et al., 2007). A bivalent modification was reported in ES cells, in which H3K4me3 and H3K27me3 bind to the same promoter of many developmental genes (Bernstein et al., 2006, Azuara et al., 2006). The bivalent modification is believed to play an important role in maintaining pluripotency and prepares the cell for differentiation (Bernstein et al., 2006, Azuara et al., 2006). In the present study, the global level of H3K4me3 was highest in zygotic female chromatin (Figure 3.09, 3.13) and then demethylation occurred at two- and four-cell embryos. Fluorescence intensity was low and steady until early blastocyst, thereafter remethylation occurred in late blastocyst, but only in ICM, not TE.

The level of H3K27me3 was highly detected in male pronuclei; the signal was steady from two-cell to eight-cell stage (Figure 3.13). Thereafter the fluorescence signal decreased (around two times) in morula and then remethylation occurred only in ICM of blastocysts (Figure 3.13). It seems likely that remethylation of both H3K4me3 and

H3K27me3 concurrently occur at ICM and may be related to the bivalent modification of pluripotent ICM.

3.3.5 Most histone demethylases but not methyltransferases were detected throughout preimplantation development

In order to investigate the mechanism of dynamic histone methylations in preimplantation embryos, quantitative gene expression analysis of HMTs and demethylases was used. Relative gene expression was normalised to either reference gene *Gapdh* or embryo number.

The present studies showed that most histone demethylases (Figure 3.16 and 3.17) but not methyltransferase were detected throughout preimplantation (Figure 3.14 and 3.15). *Suv3-9h1* and *Suv3-9h2* work together to catalyse H3K9me3 at constitutive heterochromatin in somatic cells (Peters et al., 2001). *Suv3-9h1* and 2 however, behave differently; *Suv3-9h2* was predominantly found in unfertilised oocytes and early preimplantation stages whereas *Suv3-9h1* was clearly detected in late preimplantation embryos (Figure 3.15 and 3.16). *Eset* is responsible for H3K9me3 in euchromatin, it was found throughout preimplantation development but the expression was dynamic. *Ehmt2* preferably catalyses for both H3K9me2 and H3K9me3 (Wang et al., 2003), and was detected only in the blastocyst stage (Figure 15). The expression pattern of *Ehmt2*, *Suv3-9h1* and *Suv3-9h2*, but not *Eset*, were similar to a previous report (Kageyama et al., 2007b).

Histone demethylase *Jmjd2c* was found in all stages but at a low level in oocytes compared to preimplantation embryos. *Jmjd2c* mRNA is synthesised in unfertilised oocytes and decreases rapidly after fertilisation. The expression increased slightly at the two-cell stage and then increased after four-cell stage. *Jmjd1a* was lowly expressed in immature oocytes and was undetectable in mature oocytes and zygotes. The expression of *Jmjd1a* was detected again after the two-cell stage but there was a substantial reduction at the blastocyst. *Jmjd2a* was found mostly in all stages except in four-cell stage.

3.3.6 Histone demethylation of H3K9 at the two-cell stage

In my immunofluorescence experiments, the active demethylation of H3K9me3 and H3K9me2 occurred at two-cell embryos and remethylation happened in the four-cell embryos (Figure 3.4 and 3.5), so I questioned whether demethylase enzymes were responsible for this event. Comparative gene expression RT-PCR analysis from unfertilised oocytes, zygote, two-cell and four-cell embryos showed that methylases Eset and Suv39h2 were highly expressed in unfertilised oocytes and then substantially decreased after fertilisation, whereas Suv39h1 and Ehmt2 were not detected in these stages (Figure 3.14, and 3.15). Demethylases Jmjd2c and Jmjd2a but not Jmjd1a were found in unfertilised metaphase II oocytes (Figure 3.16 and 3.17). However, only Jmjd2a and Jmjd1a transcripts increased postfertilisation specifically at zygote and two-cell stages.

It seems that most histone methyltransferase transcripts were not detected or very low between zygote to two-cell stage where most demethylase transcripts were present. These results could also suggest that the presence and relative increase of histone demethylases in zygotes and two-cell stage embryos and lack of some H3K9 histone methylases may create an unbalanced status of epigenetic modifiers and lead to histone demethylation of H3K9me2 and H3K9me3 at the two-cell stage.

3.3.7 Suv4-20h detection in the late preimplantation embryos

Suv4-20h1 and Suv4-20h2 are responsible for H4K20me2 and H4K20me3 (Schotta et al., 2008), neither of these gene transcripts were found in oocytes and early preimplantation embryos (Zygote to Morula). Transcripts of both genes were clearly detectable in the blastocyst, which may be related to the presence of H4K20me2 at the blastocyst stage and of H4K20me3 in the postimplantation embryos (Figure 4.3, Chapter 4). These observations are comparable to a previous report that revealed neither Suv4-20h is found in mature oocytes and early preimplantation embryos (Kageyama et al., 2007b).

3.3.8 Differential expression of histone modifiers in blastocysts and ES cells

This experiment extends the previous studies and compares preimplantation blastocysts, postimplantation blastocysts and ES cells. There were statistical differences in the expression of demethylases (Figure 3.21) but not histone methyltransferases (Figure 3.20). The results show that most histone methyltransferases (Eset, Suv39h1, Suv39h2) tend to increase their expression level towards postimplantation whereas demethylases Jmjd2c, Jmjd2a decrease their expression. One exception was the expression of Jmjd1a which tended to increase in expression during postimplantation.

In comparison to ES cells, expression of Eset, Jmjd2c, Jmjd2a and Suv39h1 in preimplantation blastocysts was higher whereas expression Jmjd1a and Suv39h2 was lower.

3.3.9 The expression profile of major satellite and minor satellite DNA sequences

The expression profiles of satellite sequences are shown in Figure 3.18 and 3.19. Major satellite DNA is located at pericentric heterochromatin while minor satellite DNA is found at centromeric heterochromatin (Schotta et al., 2008). Notably, the majority of both satellites were observed at unfertilised mature oocytes (MII), eight-cell and two-cell stage embryos. Moreover, two-cell embryos had a higher transcript level than zygote and four-cell embryos correlating with the histone demethylation at the two-cell embryos. It could be suggested that increased expression of repeat sequences at the two-cell stage might result from demethylation of the repressive histone mark (H3K9me2, H3K9me3 and H4K20me3).

3.4 Discussion

In the present study I found that demethylation of H3K9me2, H3K9me3 and H4K20me3 occurs in the two-cell embryo. Repressive histone methylation peaked at the eight-cell stage. An imbalance of H3K9 modifiers was found in preimplantation embryos and may be responsible for histone demethylation at the two-cell stage.

Demethylase *Jmjd2a* was specifically expressed at blastocyst while *Jmjd1a* was specific in ES cells. Demethylation at the two-cell stage may cause up regulation of satellite transcripts from pericentric and centromeric heterochromatin. Satellite transcripts were also highly expressed in mature oocytes.

3.4.1 Reprogramming of histone lysine methylation

In somatic cells, H3K9me₃, H3K27me₁, H4K20me₃ are involved in formation of pericentric heterochromatin. This process is sequential: the first step requires the activities for H3K27me₁ and H3K9me₁, then Suv3-9h1 and Suv3-9h2 enzymes catalyze H3K9me₃. Thereafter stabilized binding of HP1 α and HP1 β to H3K9me₃ in nucleosomes occurs, followed by recruitment of the Suv4-20 enzyme to catalyze H4K20me₃ (Schotta et al., 2004).

In the present study heterochromatin marker H3K9me₃ (Figure 3.4), H3K9me₂ (Figure 3.5) and H4K20me₃ (Figure 3.8) were demethylated at the two-cell stage, then remethylation occurred in four-cell embryos, except for H4K20me₃ where remethylation was not observed until postimplantation (see Chapter 4). Notably, demethylation of heterochromatin at the two-cell stage occurs at the same time as zygotic gene activation starts (Kanka, 2003). The majority of transcription during ZGA is from repetitive elements; especially LTR retrotransposons, which are abundant in two-cell embryos (Evsikov et al., 2004). These active repetitive sequences such as retrotransposons might have a possible role in introducing genomic variation, creating alternative promoters or a first exon to synchronise their expression, and trigger zygotic gene expression in the early embryos (Evsikov et al., 2004, Peaston et al., 2007, Peaston et al., 2004). Moreover, the present studies showed that satellite transcripts from centromeric and pericentric heterochromatin were up regulated at the two-cell stage. Taking the data together, the advantage of erasing heterochromatin markers in preimplantation embryos may lie in the reactivation of repetitive sequences which are commonly silenced in somatic differentiated cells (Martens et al., 2005, Peaston et al., 2007).

H4K20me₃ removal was shown in this study, which is consistent with previously published reports (Kourmouli et al., 2004, van der Heijden et al., 2005), however the

mechanism is still unknown. In Chapter 4 I propose that the possible mechanism of H4K20me3 demethylation could be associated with the lack of HP1 α in the preimplantation embryo (see Chapter 4). However, absence of H4K20me3 in this work may result from several other reasons such as a lack of prerequisite HMTs specific for H4K20me3 and HP1 α or the presence of unknown demethylases in two-cell embryos. Histone modifications in somatic cells are removed by the activity of recently identified histone demethylases. The enzyme named Jmjd1a is responsible for demethylation of H3K9me2, while Jmjd2 is specific for both H3K9me2 and H3K9me3 (Klose and Zhang, 2007). However, the demethylation enzymes for H4K20me3 have not been discovered yet.

3.4.2 Repressive histone lysine methylations peak at the eight cell embryo stage

Quantitative analysis showed that the total intensities of repressive heterochromatin markers H3K9me1, H3K9me2, H3K9me3, H4K20me1, H4K20me2 and H3K27me3 were highest at the eight-cell embryo stage (Figure 3.11-3.13). The function of this temporary enrichment in these histone markers in eight-cell embryos is still unclear. However, it could be explained by previous reports in nuclear transfer in which blastomeres from eight-cell stage embryos are less effective as donor cells in achieving development compared with two- and four-cell blastomeres (Egli et al., 2007, Hiiragi and Solter, 2005, Wilmut et al., 2002). Taking together, this may suggest that the lower developmental success rate of nuclear transfer may be associated with enrichment of epigenetic heterochromatin markers.

3.4.3 Function of satellite transcripts in oocytes

In this study, an abundance of minor and major satellite transcripts were found in mature oocytes. An earlier study in ES cells showed that most satellite transcripts are double stranded RNA (dsRNA) (Martens et al., 2005) that can be a precursor of small RNA used in RNAi pathway (Watanabe et al., 2008). The presence of many small RNAs in mouse oocytes and the fact that they are mainly derived from repeat sequences suggests that they may act as master controllers of mobile elements and regulated protein encoding genes. This data is consistent with other published reports (Tam et al., 2008, Watanabe et al., 2008). Disruption of Dicer and Argonaute 2 which are essential

for the RNAi pathway, results in lethality early in development (Bernstein et al., 2003, Lykke-Andersen et al., 2008). This could suggest that satellite transcripts may act as small RNA precursors that can be used to silence maternal genes before fertilisation and degrade maternal RNA after fertilisation.

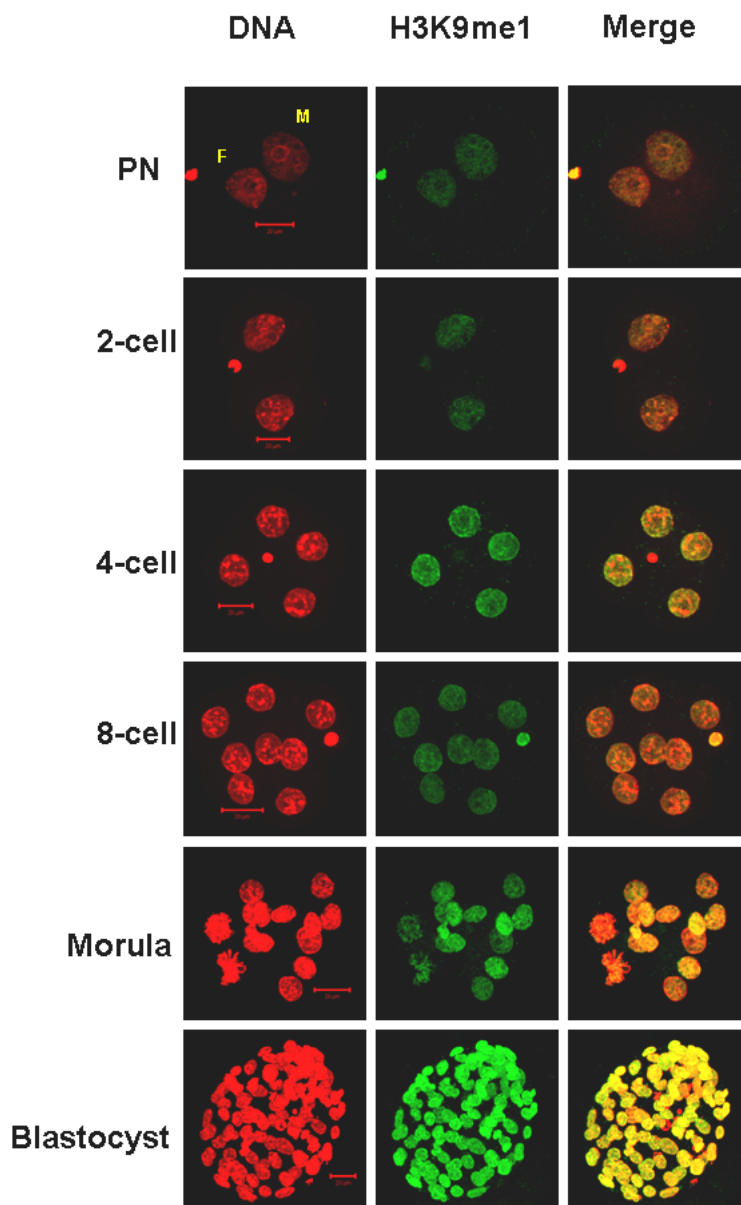


Figure 3.3 H3K9me1 in preimplantation embryos. Embryos were immunostained with anti-H3K9me1 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocolour red). In the merged panels, the yellow colour designates colocalisation between the two markers. The scale bar represents 20 μm . Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

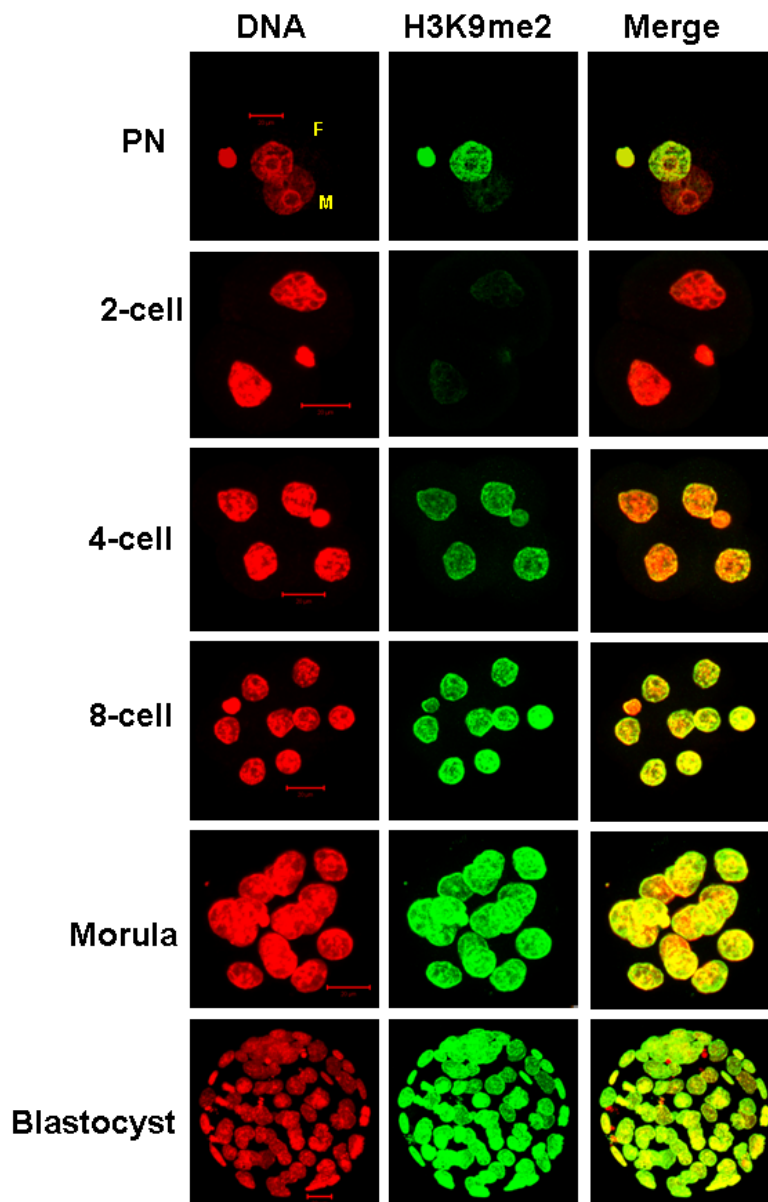


Figure 3.4 H3K9me2 in preimplantation embryos. Embryos were immunostained with anti-H3K9me2 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocoloured red). In the merged panels, the yellow colour designates colocalisation between the two markers. In zygotes, female chromatin was strongly detected for H3K9me2 while male chromatin was very weak or undetectable. Demethylation occurred at the two-cell stage and *de novo* methylation at the four-cell stage. Scale bar represents 20 μm . Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

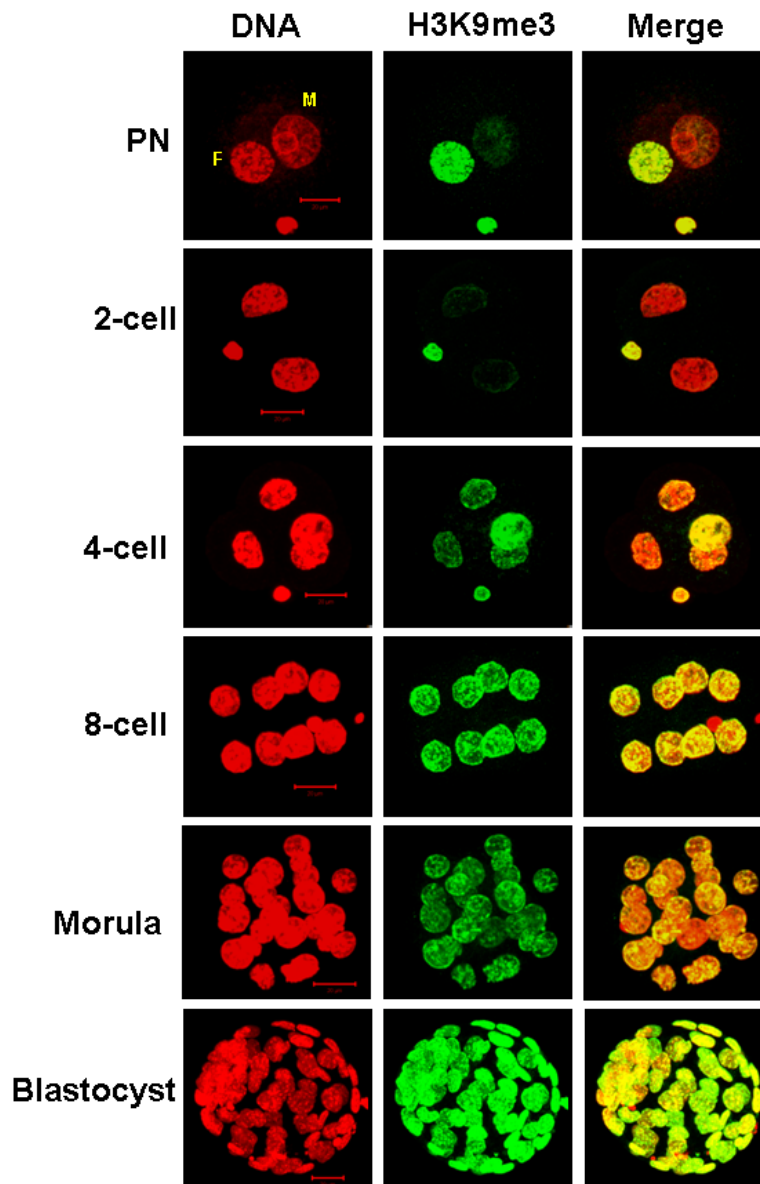


Figure 3.5 H3K9me3 in preimplantation embryos. Embryos were immunostained with anti-H3K9me3 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocoloured red). In the merged panels, the yellow colour designates colocalisation between the two markers. In zygotes, female chromatin was strongly detected for H3K9me3 while male chromatin was very weak or undetectable. Demethylation occurred at the two-cell stage and *de novo* methylation at the four-cell stage. Scale bar represents 20 μm . Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

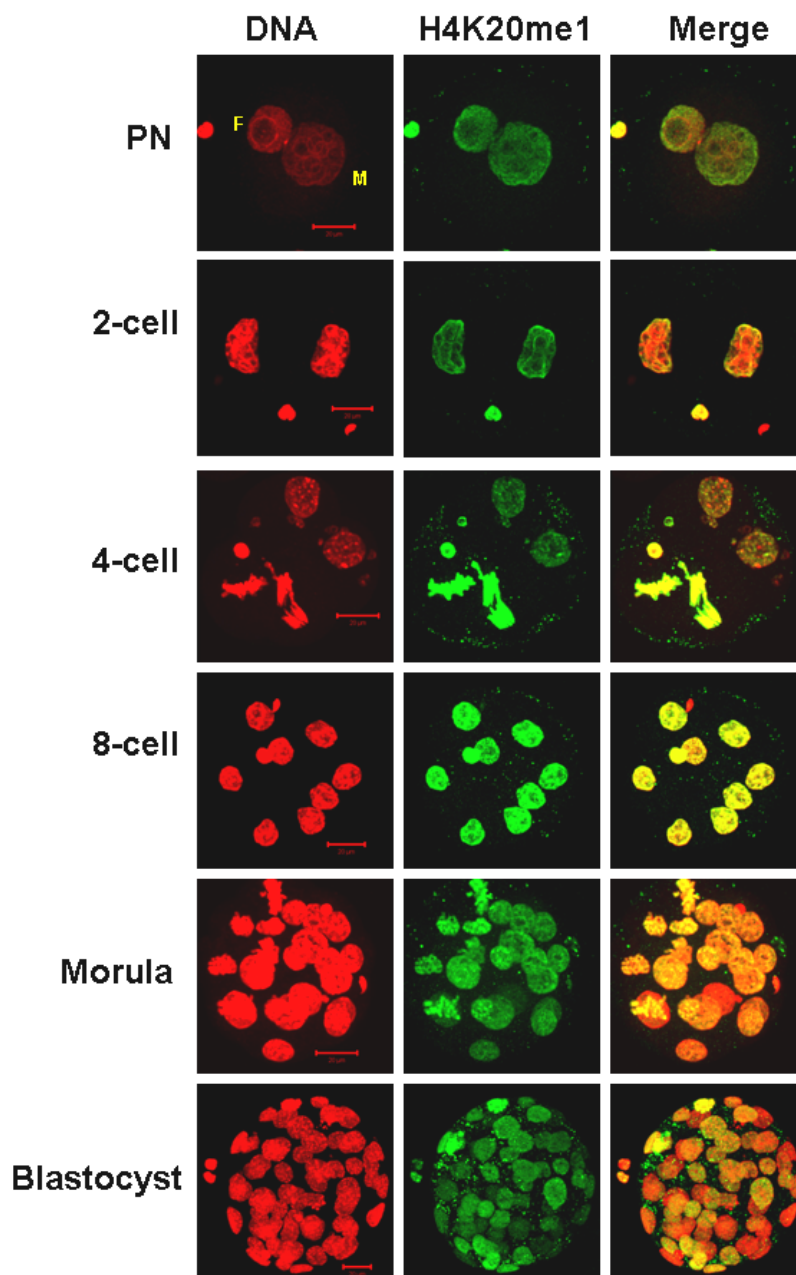


Figure 3.6 H4K20me1 in preimplantation embryos. Embryos were immunostained with anti-H4K20me1 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocolour red). In the merged panels, the yellow colour designates colocalisation between the two markers. Scale bar is 20 μ m. Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

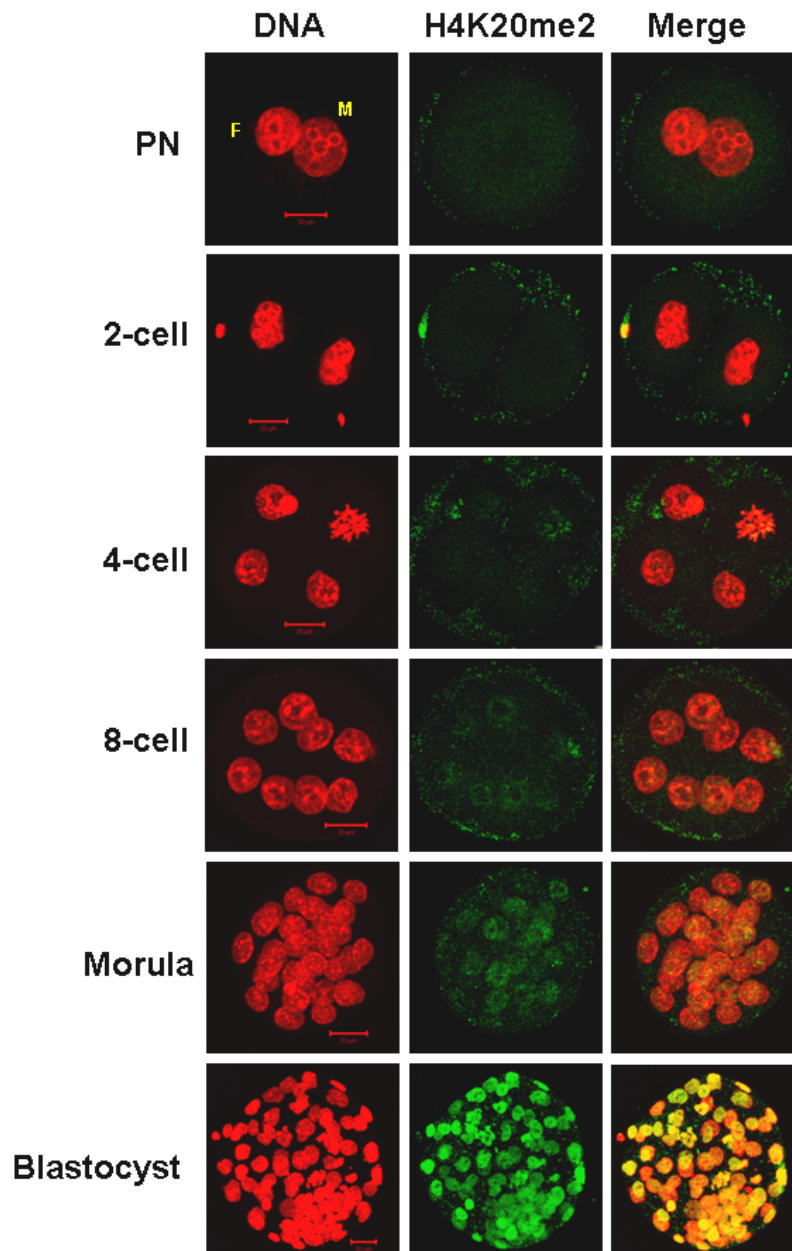


Figure 3.7 H4K20me2 in preimplantation embryos. Embryos were immunostained with anti-H4K20me2 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocolour red). In the merged panels, the yellow colour designates colocalisation between the two markers. H4K20me2 was not found in early preimplantation embryos but was detected from eight-cell stage. Scale bar is 20 μ m. Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

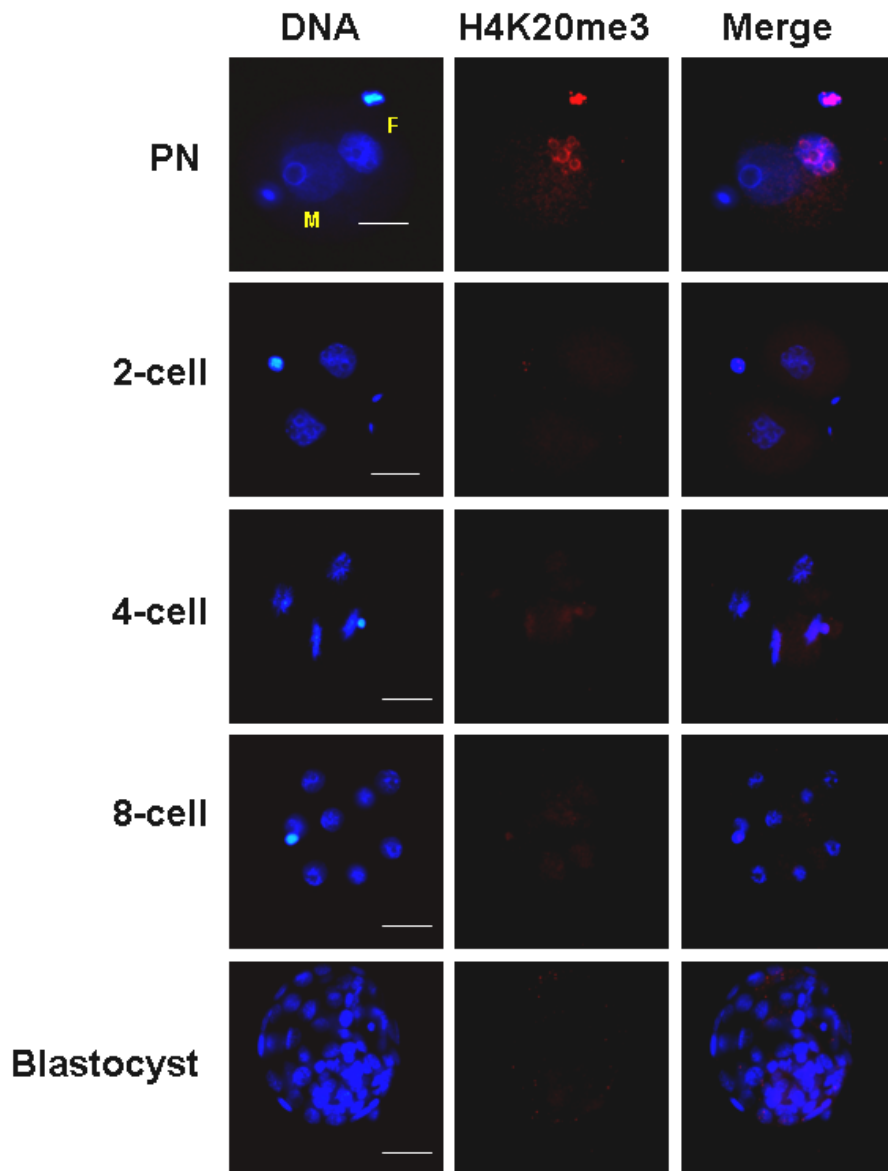


Figure 3.8 H4K20me3 in preimplantation embryos. Embryos were immunostained with anti-H4K20me3 and TRITC conjugated secondary antibody (red), and DNA dye DAPI (blue). In the merged panels, the pink colour designates co-localisation between the two markers. H4K20me3 was found only in female chromatin of zygotes and specifically stained pericentric heterochromatin and co-localised with dense DAPI staining. Demethylation occurred at the two-cell stage. The scale bar represents 20 μm . Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

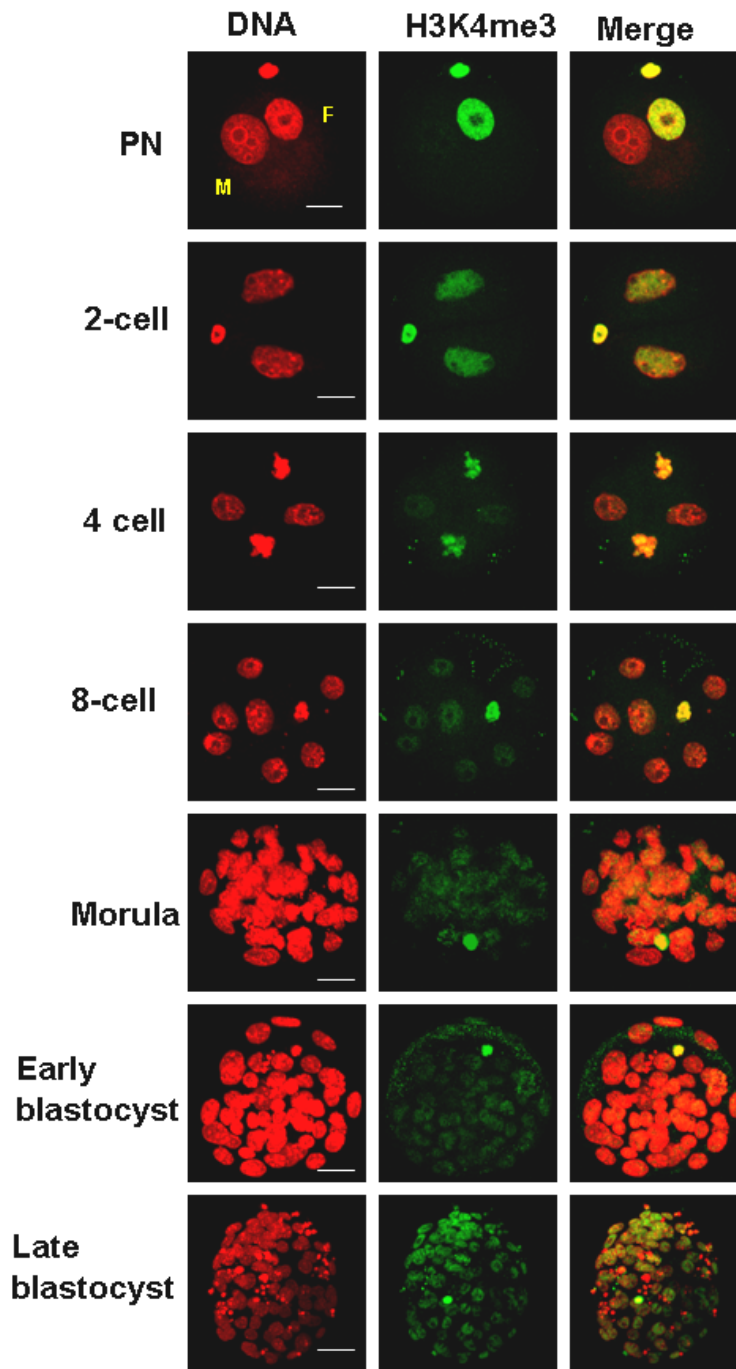


Figure 3.9 H3K4me3 in preimplantation embryos. Embryos were immunostained with anti-H3K4me3 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocoloured red). In the merged panels, the yellow colour designates co-localisation between the two markers. The scale bar represents 20 μm . Thirty embryos per stage were immunostained and all embryos showed a similar pattern of staining. In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

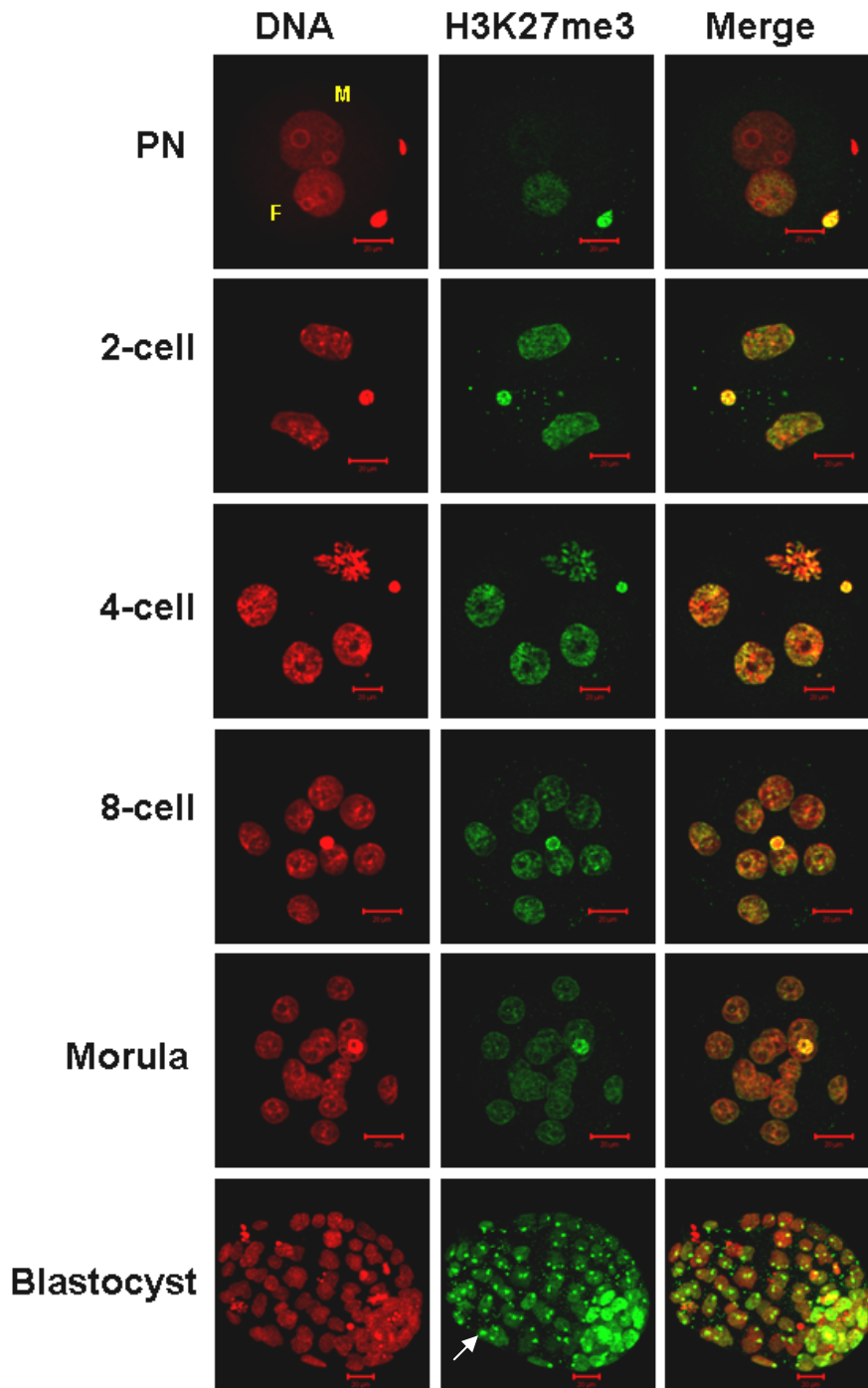


Figure 3.10 H3K27me3 in preimplantation embryos. Embryos were immunostained with anti-H3K27me3 and FITC conjugated secondary antibody (green), and DNA dye DAPI (pseudocoloured red). In the merged panels, the yellow colour designates co-localisation between the two markers. The scale bar represents 20 μm . Thirsty embryos per stage were immunostained and all embryos showed similar pattern of staining except male and female blastocyst. A white arrow points a green speckle represent Barr body or X inactive chromosome in female blastocyst and these spots are not found in male blastocyst (data not shown). In zygotes, the female pronucleus (F) is smaller and closer to the polar body than the male pronucleus (M).

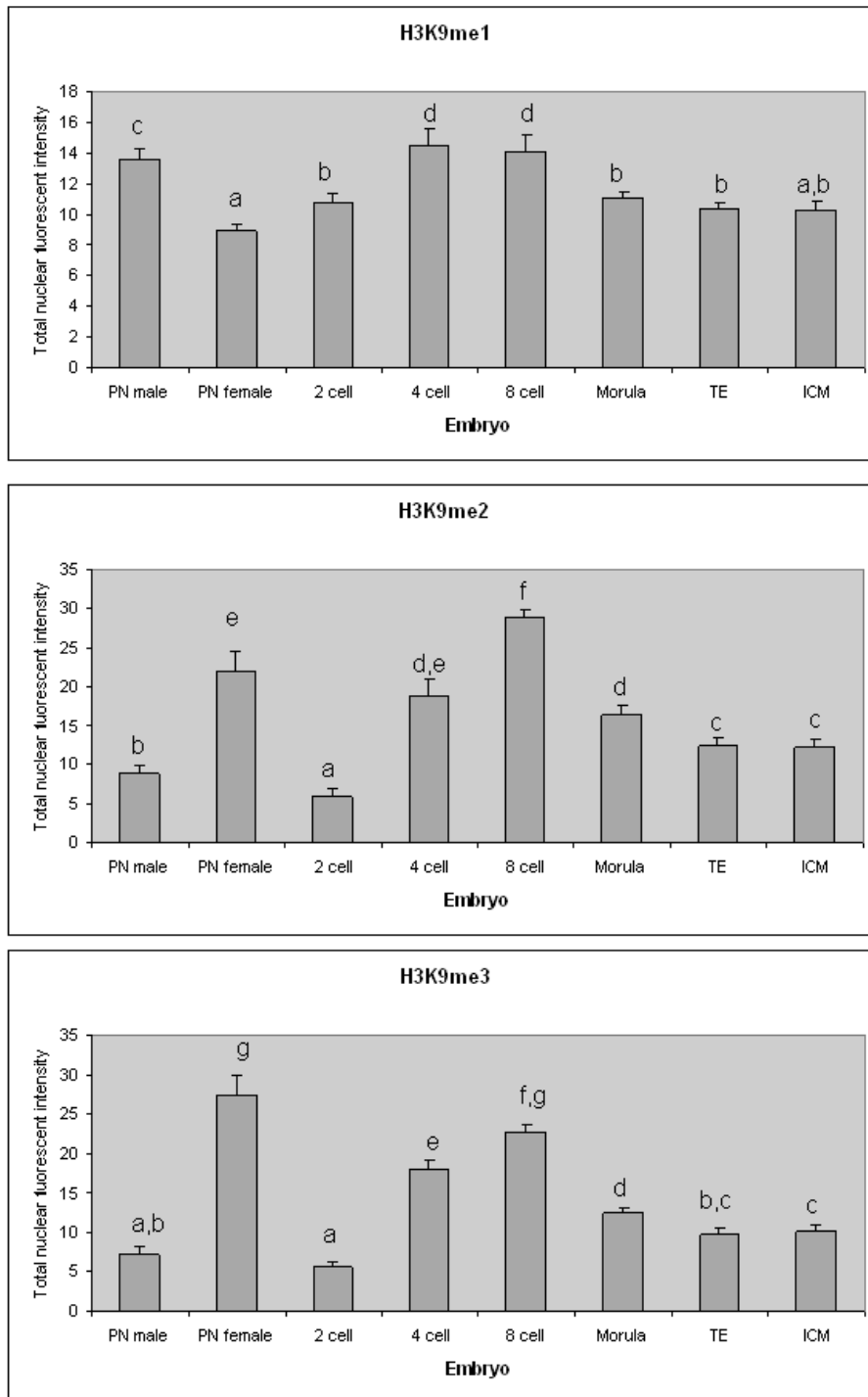


Figure 3.11 Semi-quantification of total nuclear fluorescence intensity of H3K9me1, H3K9me2 and H3K9me3 in preimplantation embryos. Thirty embryos per stage were used for analysis. Total nuclear fluorescence intensity is shown relative to the embryo stage indicated. Statistical differences ($P < 0.05$) are shown as a-g. Y axis is total nuclear intensity and x axis is embryonic stage. Total nuclear fluorescence intensity is shown in mean \pm standard error. Statistic difference was analysed using ANOVA and Student's t test. Bar represent standard error of mean (SEM)

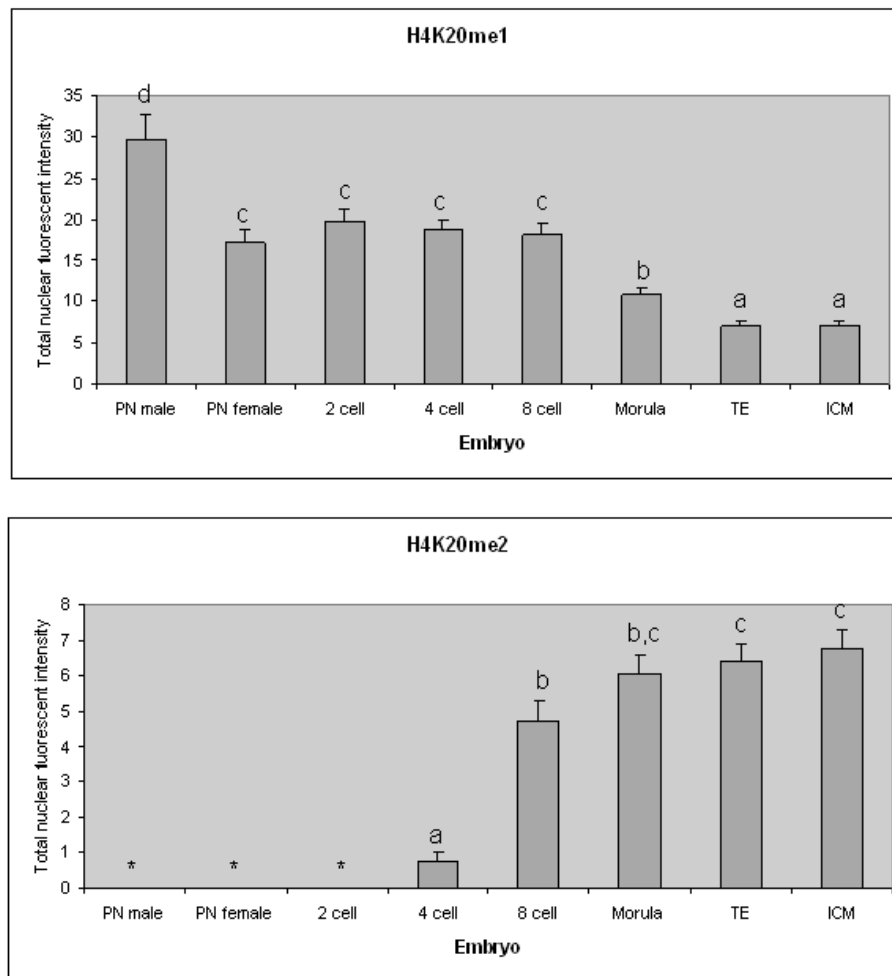


Figure 3.12 Semi-quantification of total nuclear fluorescence intensity of H4K20me1 and H4K20me2 in preimplantation embryos. Thirty embryos per stage were used for analysis. Total nuclear fluorescence intensity is shown relative to the embryo stage indicated. Statistical differences ($P < 0.05$) are shown as a-g. Y axis is total nuclear intensity and x axis is embryonic stage. Total nuclear fluorescence intensity is shown in mean \pm standard error. Statistic difference was analysed using ANOVA and Student's t test. Bar represent standard error of mean (SEM)

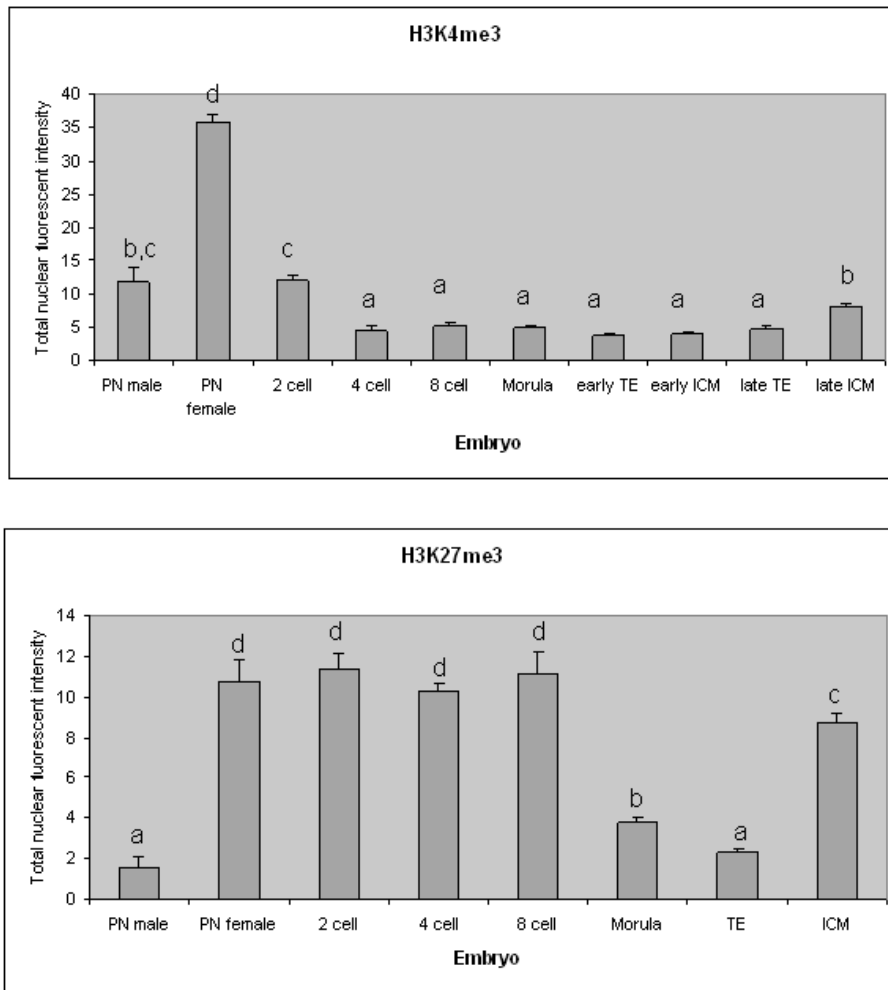


Figure 3.13 Semi-quantification of total nuclear fluorescence intensity of H3K4me3 and H3K27me3 in preimplantation embryos. Thirty embryos per stage were used for analysis. Total nuclear fluorescence intensity is shown relative to the embryo stage indicated. Statistical differences ($P < 0.05$) are shown as a-g. Y axis is total nuclear intensity and x axis is embryonic stage. Total nuclear fluorescence intensity is shown in mean \pm standard error. Statistic difference was analysed using ANOVA and Student's t test. Bar represent standard error of mean (SEM)

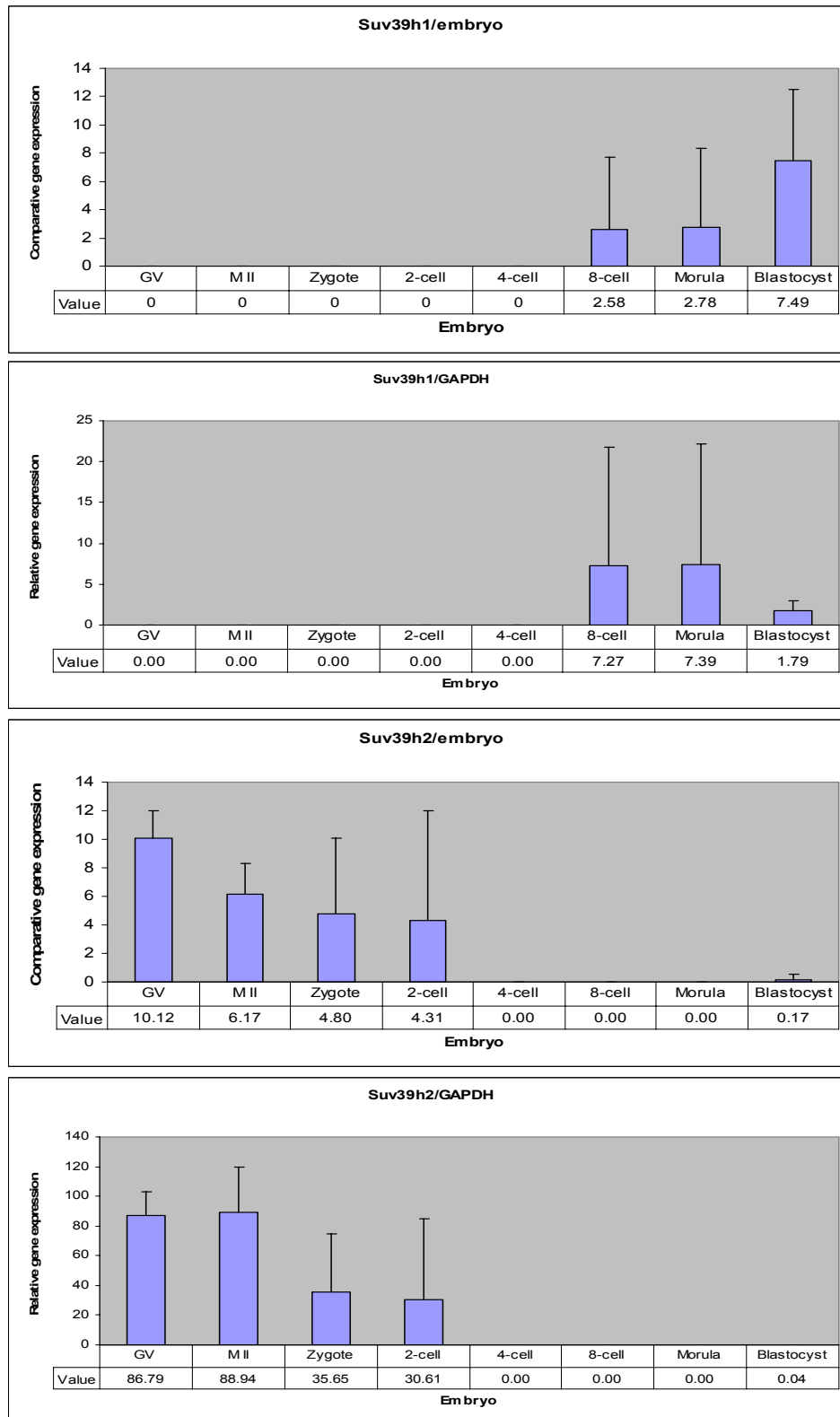


Figure 3.14 Comparative and relative gene expression of Suv39h1 and Suv39h2 in preimplantation embryos. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalisation with reference gene) or relative to Gapdh (normalised with Gapdh). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage.

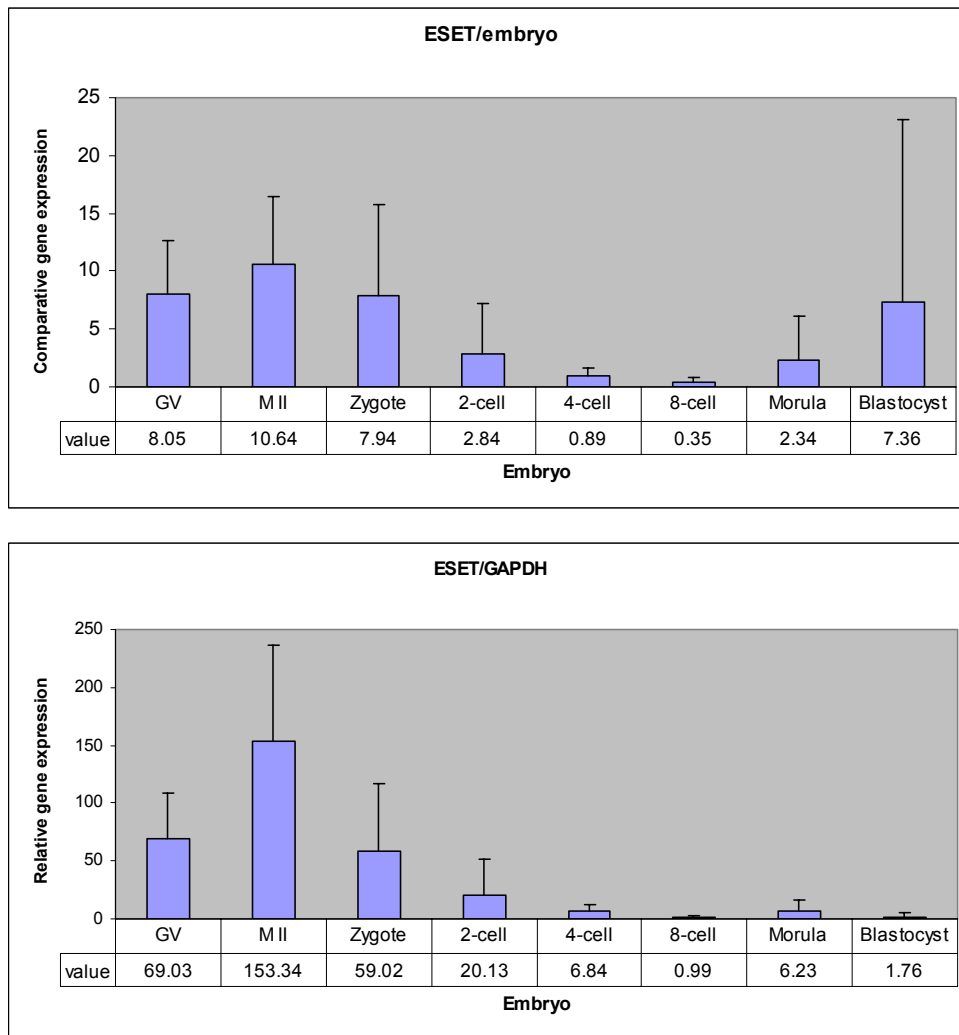


Figure 3.15 Comparative and relative gene expression of Eset in preimplantation embryo. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalised) or relative to Gapdh (normalised with Gapdh). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage.

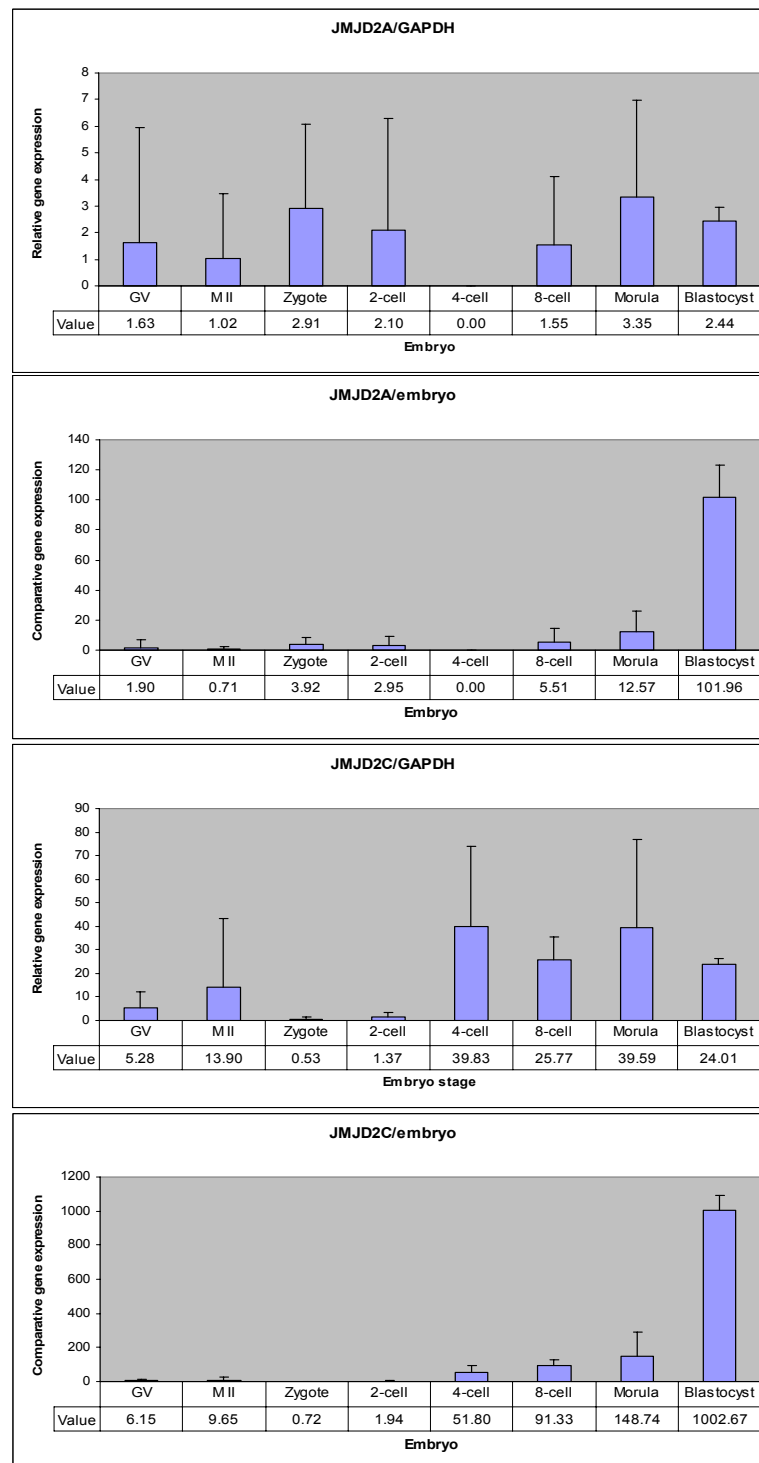


Figure 3.16 Comparative and relative gene expression of *Jmjd2a* and *Jmjd2c* in preimplantation embryo. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalisation with reference gene) or relative to *Gapdh* (normalised with *Gapdh*). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage.

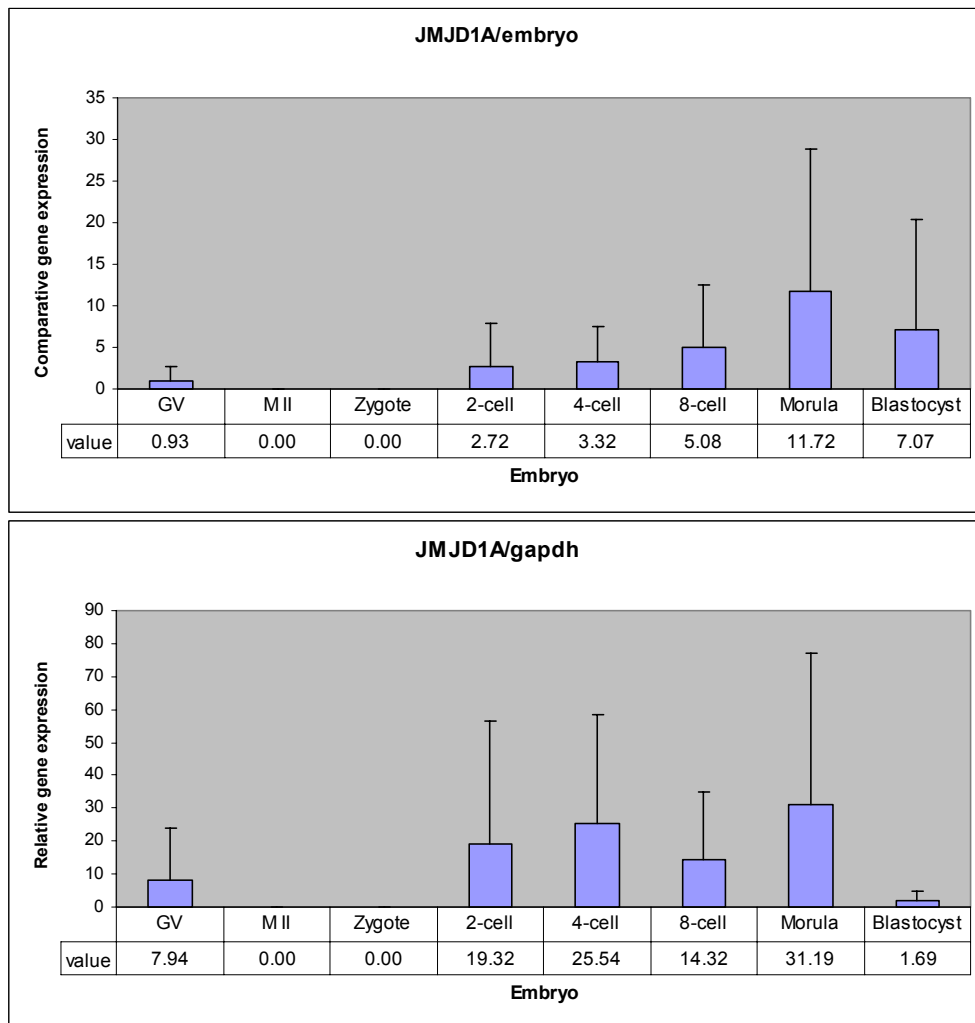


Figure 3.17 Comparative and relative gene expression of *Jmjd1a* in preimplantation embryo. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalisation with reference gene) or relative to *Gapdh* (normalised with *Gapdh*). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage.

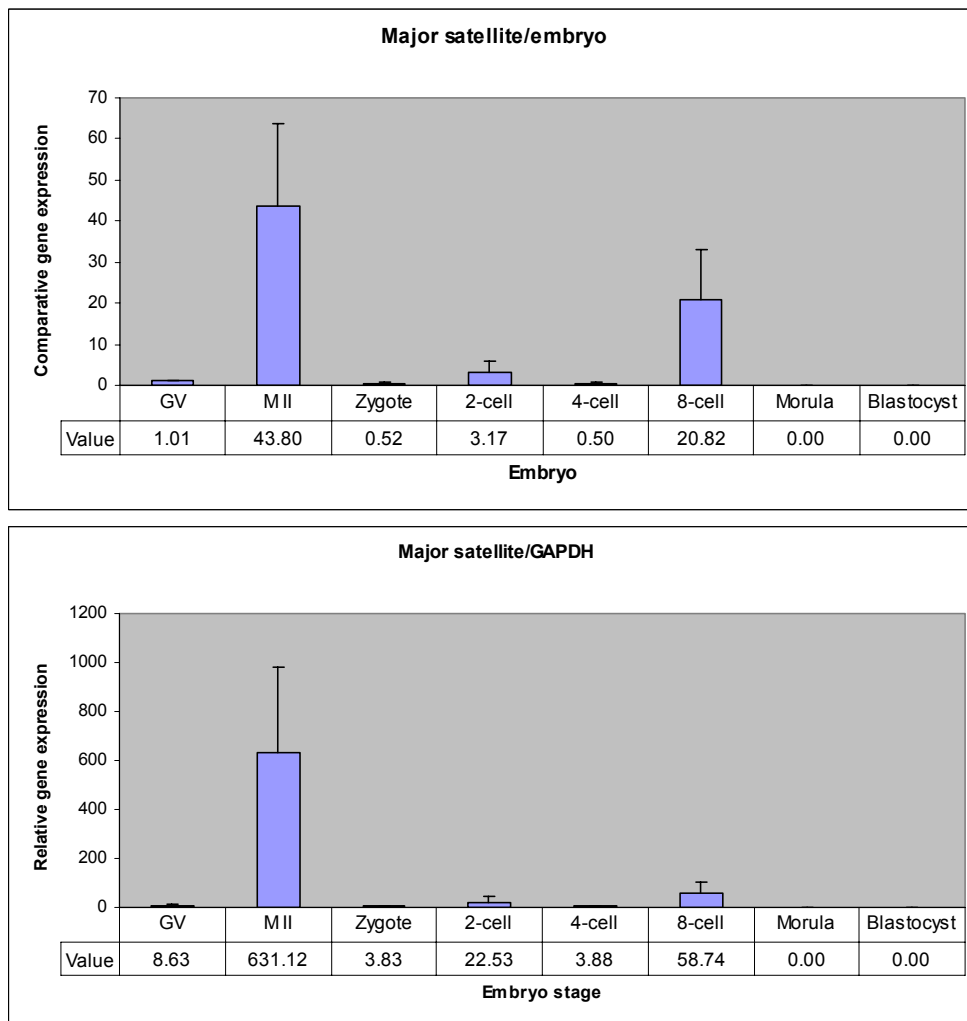


Figure 3.18 Comparative and relative gene expression of major satellite in preimplantation embryo. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalisation with reference gene) or relative to Gapdh (normalised with Gapdh). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage. RT negative samples were used to exclude DNA contamination for analysis of satellite transcripts.

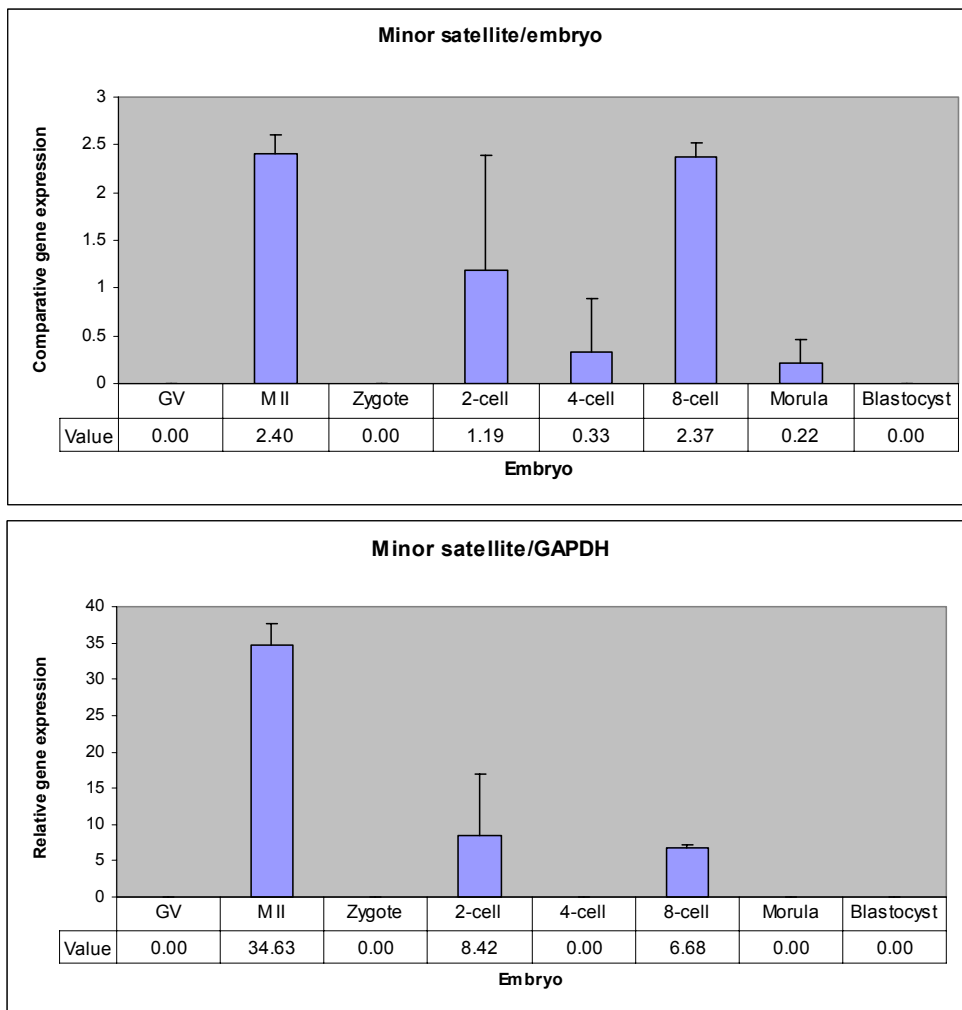


Figure 3.19 Comparative and relative gene expression of minor satellite in preimplantation embryo. RT-QPCR of histone methyltransferase mRNA expression in the embryo stages indicated gene expression. The value is gene expression ratio either per embryo (non-normalisation with reference gene) or relative to Gapdh (normalised with Gapdh). Error Bar represents standard deviation. Y axis is gene expression ratio and x axis is embryonic stage. RT negative samples were used to exclude DNA contamination for analysis of satellite transcripts.

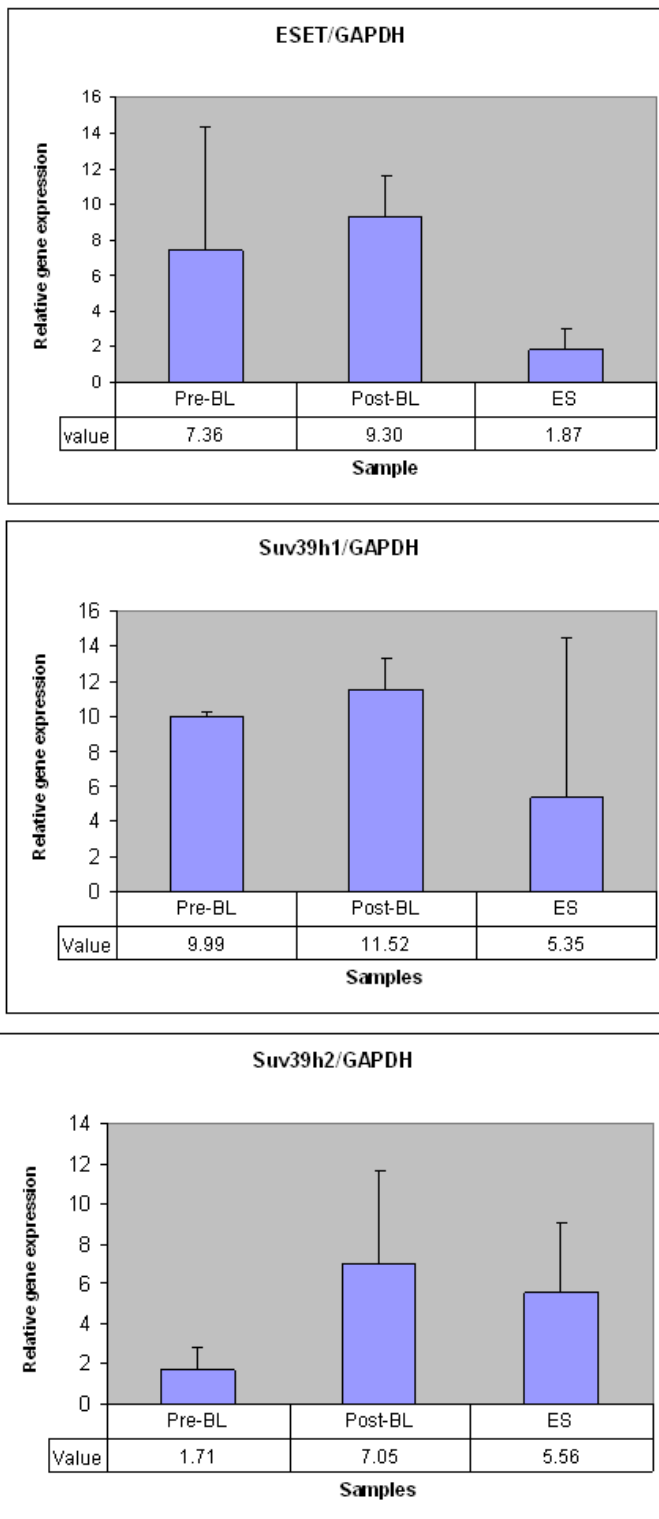


Figure 3.20 Relative gene expression of Eset, Suv39h1 and Suv39h2 compared between blastocysts and ES cells. The value is a gene expression ratio relative to Gapdh (per Gapdh). Pre-BL=preimplantation blastocyst, Post-BL= in vitro implantation blastocyst, ES=ES cell. Y axis is gene expression ratio and x axis is embryonic stage. Pair Wise Fixed Allocation Randomisation Test was used to calculate statistical differences. Bar represent standard error of mean (SEM)

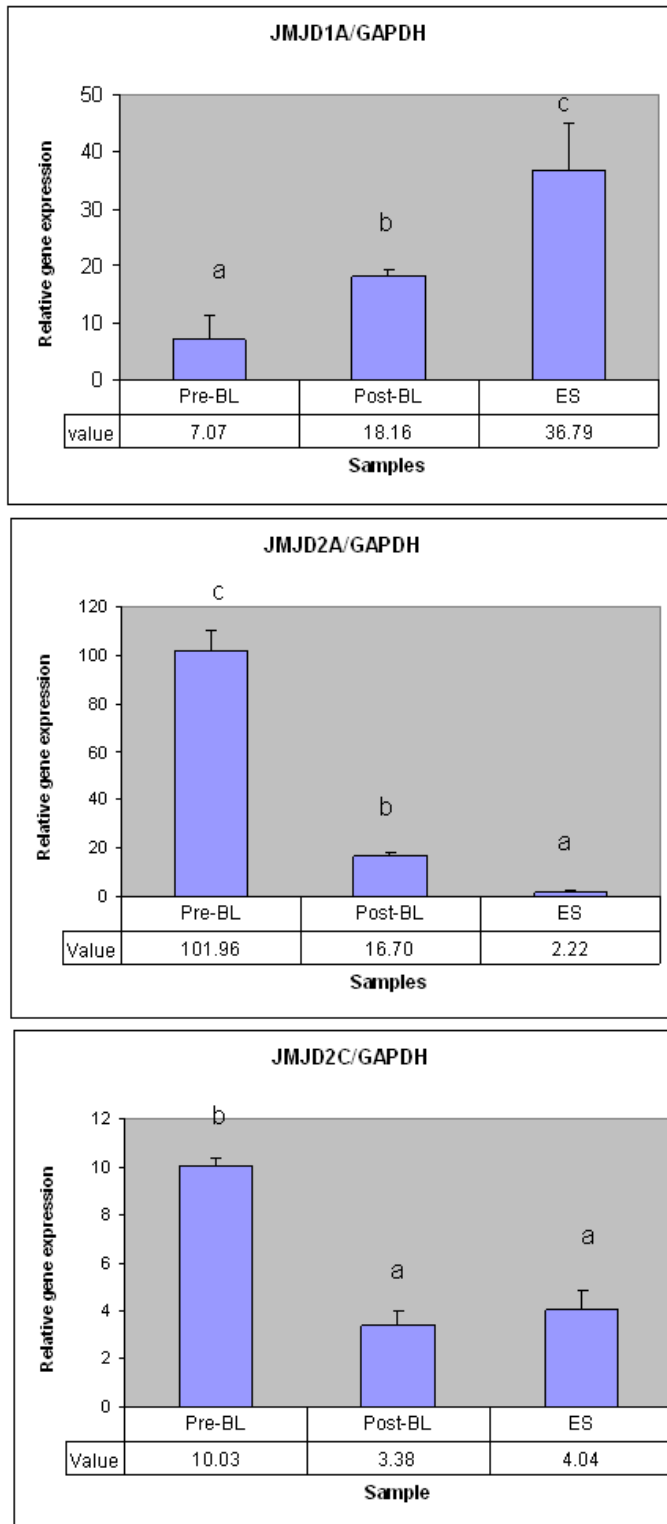


Figure 3.21 Relative gene expression of Jmjd1a, Jmjd2a and Jmjd2c compared between blastocyst and ES cell. The value is gene expression ratio relative to Gapdh (per Gapdh). Pre-BL=preimplantation blastocyst, Post-BL= in vitro implantation blastocyst, ES=ES cell. ^{a-c} represent statistical difference (P<0.05) between samples. Y axis is gene expression ratio and x axis is embryonic stage. Pair Wise Fixed Allocation Randomisation Test was used to calculate statistical differences. Bar represent standard error of mean (SEM).

CHAPTER 4

Heterochromatin Maturation in Mouse Development and ES cells

Chapter 4

Heterochromatin maturation in mouse development and ES cells.

4.1 Introduction

Heterochromatin describes the highly condensed and transcriptionally silent chromatin of the genome. Rich in repetitive sequences and low in gene density, it has a characteristic chromatin signature. One important key feature of heterochromatin is its ability to extend its domain and self propagate through cell division. When heterochromatin spreads across domains, gene repression may directly result either from the association of heterochromatin markers such as histone methylation, heterochromatin protein 1 (HP1) and DNA methylation with gene promoters or from the relocation of active genes to the vicinity of heterochromatin (Grewal and Jia, 2007, Elgin and Reuter, 2006) (see Figure 4.1A and 4.1B).

4.1.1 Epigenetic regulation heterochromatin formation

The formation of heterochromatin is likely to be regulated by epigenetic processes such as DNA and histone methylation. Domains of constitutive heterochromatin possess an abundance of DNA methylation (Bird, 2002), histone methylation marks such as H3K9me3 (Peters et al., 2003) and H4K20me3 (Kourmouli et al., 2004) as well as HP1 (Kourmouli et al., 2005). Studies on mouse pericentric heterochromatin show that the formation of heterochromatin requires sequential histone modifications and HP1 proteins (Kourmouli et al., 2004, Schotta et al., 2004, Kourmouli et al., 2005). First, repetitive sequence which already contains H3K27me1 and H3K9me1 recruits the methyltransferase *suv3-9h* to complete trimethylation of H3K9; next HP1 α and HP1 β bind to H3K9me3-containing nucleosomes to stabilise chromatin; and finally, *suv4-20h* enzymes are recruited that modify histone H4 to form H4K20me3.

In contrast to constitutive heterochromatin, trimethylation of histone 3 lysine 27 (H3K27me3) is considered to be a feature of facultative heterochromatin. It is associated with transcriptional silencing by polycomb group (PcG) proteins especially in X chromosome inactivation (Okamoto and Heard, 2006). Moreover, this facultative heterochromatin also contains DNA methylation, dimethylation of histone 3 lysine 9 (H3K9me2) (Peters et al., 2003), monomethylation of histone 4 lysine 20 (H4K20me1) (Sims et al., 2006) and HP1 (Chadwick and Willard, 2004).

4.1.2 Function of heterochromatin markers H4K20me3 and HP1 α

H4K20me3 is not only found at pericentric heterochromatin, but is also observed at telomere and subtelomere regions (Blasco, 2007a). Telomeres become shorter in aged cells, and this is a well-known marker for senescence (Blasco, 2007b). The abrogation of Suv4-20h enzymes results in decreasing H4K20me3 at telomeres and subtelomeres, concomitantly with an increase in telomere length and sister chromatid recombination (Benetti et al., 2007). These results suggest that H4K20me3 or Suv4-20h enzyme may control telomere length and DNA recombination. Recently, H4K20me3 together with H3K9me3 and HP1 were found to control imprinted genes by localising on the silenced imprinted gene promoter and a non-expressed pseudogene (Pannetier et al., 2008, Delaval et al., 2007, Regha et al., 2007). Suv4-20h knock-out mice show abnormality in placental and foetal growth (Schotta et al., 2008), these growth abnormalities may result from aberrant expression of imprinted genes such as *Igf2* which are controlled by H4K20me3 (Schotta et al., 2008). Moreover, H4K20me3 is also reported to be related to the progression of many cancers in animals and human (Fraga et al., 2005, Pogribny et al., 2006).

At centromeric and telomeric heterochromatin, HP1 is required for the proper maintenance of chromosomal and genome integrity (Fanti and Pimpinelli, 2008). Moreover, HP1 at pericentric and centromeric heterochromatin plays an important role in recruiting the cohesin complex and kinetochore proteins that are crucial for chromosome segregation at mitosis and prevention of aneuploidy (Zhang et al., 2007, Nonaka et al., 2002). HP1 also associates with cancer in human; HP1 α , but not HP1 β or HP1 γ , is down-regulated in highly invasive or metastatic breast cancer cells compared

to poorly invasive or non-metastatic cancer cells (Kirschmann et al., 2000, Norwood et al., 2006) and this also correlates to dimerisation of HP1 α (Norwood et al., 2006). Furthermore over expression of HP1 α in highly invasive/metastatic cells that have low levels of HP1 α leads to a less invasive phenotype (Kirschmann et al., 2000). Taken together these lines of evidence may imply that the HP1 α could modulate molecular properties of carcinoma cells needed for invasion or metastasis. One hypothesis is that HP1 α normally silences genes required for metastasis, making HP1 α a candidate metastasis suppressor. Depending on whether HP1 α is down-regulated during tumour progression, HP1 α could be used as a predictive marker for metastatic breast cancer.

4.1.3 Hypothesis

Recent reports suggest that the epigenetic profile and heterochromatin organisation in embryos is unique and different from somatic cells (Martin et al., 2006b, Probst et al., 2007, Martin et al., 2006a). The hypothesis of the present study was that heterochromatin is immature in the early embryo and matures concomitantly with development. I have tested this hypothesis by recording the dynamics of various epigenetic markers in the developing embryo.

4.2 Materials and Methods

4.2.1 Samples

Embryos and mouse embryonic fibroblast (MEF) cells were collected from B6CBAF1 mice. ES cells were obtained either from recent isolation in the lab (termed the ‘noo1’ line) or from the Institute of Stem Cell Research, MRC Centre for Regenerative Medicine, University of Edinburgh (E14tga-line). For each antibody, approximately 30 embryos per stage were used for immunofluorescence. The techniques of embryo collection, *in vitro* implantation, ES cell isolation, cell culture, immunofluorescence, western blotting, RNA isolation, cDNA synthesis and PCR are described in Chapter 2 (Materials and Methods).

4.2.2 Experimental design

The experimental design is summarised in Figure 4.2. Embryo and adult tissue were collected and analysed by immunofluorescence of whole embryo and sections, and western blotting of embryo extracts. Heterochromatin was detected by immunostaining with various markers: H4K20me₃, H3K9me₃, HP1 α , 5MeC for constitutive heterochromatin, and H3K9me₂, H4K20me₁, H3K27me₃ for facultative heterochromatin, and HP1 β for both types of heterochromatin. Chi-squared analysis was used to calculate statistical differences between the numbers of fluorescent positive cells in E14.5 embryos compared to E17.5 embryos, and the difference between ES cell differentiation and *in vitro* implantation. Statistical analysis was performed using the online statistic software VassarStats (Lowly, 2009) (<http://faculty.vassar.edu/lowry/odds2x2.html>).

4.3 Results

4.3.1 Epigenetic signature of mouse somatic heterochromatin

Mouse somatic cells such as fibroblasts stained for DAPI showed a characteristic pattern of 10-20 bright dots in the nucleus indicating areas of higher DNA density (Figure 4.3). These regions of higher chromatin density correspond mainly to pericentromeric heterochromatin, which in the acrocentric chromosomes of the mouse is organised into chromocentres that join several chromosomes (Martens et al., 2005). These dots also stain brightly for DNA methylation, H3K9me3, H4K20me3 and HP1 α (Figure 4.3) (Peters et al., 2002, Schotta et al., 2004, Kourmouli et al., 2004, Kourmouli et al., 2005), consistent with the classic epigenetic profile of constitutive heterochromatin (Martens et al., 2005). Uniquely in the mouse, the pericentromeric chromatin organisation allows a direct microscopic observation of the most abundant constitutive heterochromatin compartment in the nucleus. I have taken advantage of this aspect to follow the formation of heterochromatin through development in terms of the appearance of chromocentres and the epigenetic markers, as detected with antibodies. I chose antibodies that have been demonstrated in the literature (Martens et al., 2005, Schotta et al., 2004, Peters et al., 2002) as well as in our hands (Figure 4.3) to stain these epigenetic epitopes in somatic chromatin.

4.3.2 Immature chromatin in preimplantation embryo

In our studies, all heterochromatin epigenetic markers (DNA methylation, H3K9me3, H4K20me3, HP1 α , HP1 β , H4K20me1, H3K9me2 and H3K27me3) were found in most but not all developmental stages of preimplantation mouse development (Figure 4.4). H3K9me2, H3K9me3 and H4K20me3 were concurrently reduced in two-cell embryos, with the exception of H4K20me3 which was completely erased after the two-cell stage (Figure 4.4). The substantial reduction of H4K20me3 was also observed in another report (Kourmouli et al., 2004), and seems to be correlated with the presence of the corresponding methylases Suv4-20h for which the mRNA level was undetectable in early preimplantation embryos, but was later found in periimplantation blastocysts

(Figure 4.4). The Suv4-20h level may bear on another substrate, H4K20me2 which was clearly observed in late preimplantation embryos (see Chapter 3, Figure 3.7).

Surprisingly, HP1 α which is commonly found in constitutive heterochromatin of somatic cells (Schotta et al., 2004, Dialynas et al., 2007), was undetectable in all developmental stages before implantation. This observation was supported by van der Heijden et al (2005) and Beaujean, N. (personal communication), but inconsistent with Houliard et al (2006). Unlike previous reports for somatic cells (Wu et al., 2006, Schmiedeberg et al., 2004, Gilbert et al., 2003, Ayoub et al., 2008), in which HP1 β is predominantly found in heterochromatin, the distribution of HP1 β in our preimplantation embryos was throughout the whole nucleus (Figure 4.4) and was comparable to previous reports (Martin et al., 2006b, Martin et al., 2006a). The lack of H4K20me3, HP1 α and Suv4-20h suggested that heterochromatin in the preimplantation embryo was more immature than in somatic cells.

4.3.3 Heterochromatin matures post implantation

The next question was when the mature heterochromatin was established. *In vivo* and *in vitro* implantation embryos at different stages of development were immunostained and observed. As it is difficult to control the exact postimplantation time of *in vivo* implantation embryos from pregnant mice, *in vitro* implantation was initially used as an alternative model for the study of very early implantation.

Intriguingly, after *in vitro* implantation, H4K20me3 and HP1 α - which previously were not found in the preimplantation blastocyst - were both detected. At approximately 24 to 48 hours, H4K20me3 was firstly detected, but only in mural trophoblast (mTE) not in polar trophoblast (pTE) or inner cell mass (ICM) cells. The ICM cells were defined as cells that were positive for Oct4 (Figure 4.6). HP1 α however, was hardly detectable at this stage. After four days of culture, both H4K20me3 and HP1 α were strongly detected both in giant trophoblast and ICM-derived cell colonies (Figure 4.7B). TE cells showed a speckle pattern of H4K20me3 and HP1 α specific for heterochromatin but not the ICM colony which seemed to have an equal distribution in both euchromatin and heterochromatin (Figure 4.7B). After initial implantation into the petri dish, further *in vitro* growth results in loss of the normal embryo organisation and differentiating

cells become difficult to identify. Positional developmental signals might also be lost to the cells, resulting in abnormal epigenetic patterns as well as disorganised tissues. For this reason, it was necessary to follow *in utero* embryo development. Similarly, *in vivo* implantation results showed that H4K20me3 was not found in E3.5 (preimplantation) but was early detected early in E4.5 (postimplantation), when it was only found in mural TE, not in polar TE and ICM cells (Figure 4.8).

Next, I investigated when the establishment of H4K20me3 and HP1 α occurred in postimplantation embryos. Embryos at different stages of development E5.5, E6.5 or E7.5 (early implantation) were stained with the H4K20me3 antibody using a whole mount method. However, H4K20me3 was not detected in interphase nuclei (Figure 4.9A). To confirm that our whole mount immunofluorescence for postimplantation embryo is suitable for postimplantation embryo, the embryos were stained with anti-H3K9me3 antibody, and the results showed that H3K9me3 was detected both in epiblast (derived from ICM) and extraembryonic ectoderm (derived from pTE). This demonstrated that our protocol allows antibody penetration through the embryo in both epiblast and extraembryonic ectoderm (Figure 4.9B).

For embryos retrieved later during gestation, antibody staining was performed on thin sections. Postimplantation embryos E8.5, E14.5 or E17.5 were fixed and embedded in paraffin or frozen for cryosection. In paraffin sections, H4K20me3 was undetectable in most embryonic cells but it was visible in decidual cells of the placenta derived from the maternal uterus (Figure 4.10A). The control antibody H3K9me3 was detectable in both placental and embryonic tissue (Figure 4.10B). However, one common problem of using paraffin sections is that some antigens may lose antibody sensitivity after processing. Although antigen retrieval steps (see Chapter 2 Material and Methods) were performed, the recovery efficiency may not be complete and success will vary with individual antibodies or antigens. For this reason cryosections were also performed to confirm the results.

Although cryosections preserve antigen accessibility, they can result in poor tissue morphology. To aid accurate identification of embryonic tissue, alternate sections of embryos were taken through a haematoxylin and eosin (H&E) staining protocol for both

paraffin section and cyosection. Cryosections from E8.5 embryos showed that heterochromatin markers H4K20me3 and HP1 α were not detected while H3K9me3 was clearly positive (Figure 11). Later stages of embryo E14.5 and E17.5 were cryosectioned to investigate the signal of H4K20me3 and HP1 α . Notably, H4K20me3 became strongly detectable in E14.5 embryos (Figure 4.12, left) and this stage simultaneously showed the appearance of HP1 α (Figure 4.13, left). The number of positive cells for H4K20me3 and HP1 α (defined as having a high positive signal and more than 3 dots/cell) were counted and the cell counts from E14.5 embryos were compared with those from E17.5 embryos (Figure 4.12 and 4.13, left and right respectively). The results showed that H4K20me3 was found in 50.0% of cells (250/500) in E14.5 embryos, which increased to 80.0% (400/500) (statistical significance, $P < 0.001$) in E 17.5 cells (Figure 4.13). A similar pattern was found for HP1 α for which the number of positive cells of E17.5 was significantly higher ($P < 0.001$) than in E14.5, with 75.8% (379/500) and 45.6% (228/500) positive cells, respectively.

The overall amount of H4K20me3 was confirmed using western blotting. Unexpectedly, anti-histone H4 antibody (control antibody) from different commercial sources (Abcam, Santa Cruz and Upstate), but not anti-H4K20me3 antibody, could not bind the histone H4 in whole embryo and placenta tissue. However both anti-H4 and anti-H4K20me3 antibody could bind their epitopes in mouse embryonic fibroblasts (MEFs) that were derived from the same stage of embryo tissue (E14.5). Surprisingly, H4K20me3 in embryo and placenta tissues (E14.5 and E17.5) was shown to have a higher apparent molecular weight (14 KDa) compared with MEF derived from E14.5 (10 KDa apparent MW) (Figure 4.14). Taken together, it is probable that MEFs which had been isolated from embryos lost some histone modifications during *in vitro* culture, and that this modification might have sterically prevented the antibody from binding to the histone H4 epitope. The results may suggest that increases in molecular weight of H4K20me3 protein may have been the result of other modifications occurring at the same nucleosomes where H4K20me3 appears *in vivo* but that these might be lost after *in vitro* culture. However; the large difference in molecular weight of proteins detected in extracts from *in vivo* and *in vitro* cells may also reflect the fact that anti-H4K20me3 antibodies may be not specific enough to be analysed by western blot using *in vivo*

embryos, as it might be possible could bind unknown non-specific proteins in developing embryos.

Based on the absence/presence of H4K20me3 and HP1 α in preimplantation and postimplantation embryos, I suggest that heterochromatin is immature in preimplantation embryos and only becomes mature post implantation. This immature heterochromatin could plausibly cause chromatin to be globally more open which might allow embryonic-specific transcription complexes access to chromatin, thus reactivating zygotic genes. Pericentric chromatin is poor in protein encoding genes, however, many of the early transcribed embryonic genes are repetitive sequences such as Murine Endogenous Retrovirus (MuERV-L), intracisternal A particle (IAP) and other mobile elements, which are commonly inactivated by heterochromatin in adult cells (Svoboda et al., 2004, Evsikov et al., 2004, Peaston et al., 2004, Ma et al., 2001, Puschendorf et al., 2006). In addition, mature heterochromatin may function as a gene silencing compartment to ectopic genes that are moved to within its proximity (Grewal and Jia, 2007, Grewal and Elgin, 2007)

4.3.4 Dynamics of H4K20me3 and HP1 α in ES cells

There is considerable evidence that epigenetics plays an important role in the regulation of gene expression patterns in ES cells. For example, the promoter regions of active pluripotency genes such as Oct4 and Nanog, are marked by acetylation of H3 and H4 during active gene transcription, whereas after differentiation these genes are marked with repressive DNA and histone methylation (Gan et al., 2007, Azuara et al., 2006). One important piece of evidence is the finding that H3K27me3 may repress cell lineage specific genes in ES cells by forming a bivalent chromatin structure, where this repressive mark coexists with activating marks H3K9ac or H3K4me3 (Azuara et al., 2006, Bernstein et al., 2006). The bivalent modification patterns disappear after the differentiation of ES cells (Azuara et al., 2006, Bernstein et al., 2006). This suggests that the presence of the epigenetic facultative marker H3K27me3 might be functionally important for maintaining pluripotency by preventing the expression of lineage-control genes in ES cells.

Interestingly, I found that ES cells displayed two types of distributions for H4K20me3

as well as HP1 α : either diffuse or in foci. The correlation between these HP1 α and H4K20me3 patterns from immunofluorescence experiments was shown in Figure 4.14. Cells can be classified into 3 types; Types I (Figure 4.15, left panel) had diffuse HP1 α and very weak signal of H4K20me3; in Type II (Figure 4.15, middle panel) HP1 α was punctate but H4K20me3 was diffuse, and in Type III (Figure 4.15, right panel) both HP1 α and H4K20me3 were punctate. Allocation of each cell type was done after manually counting 1,000 cells from randomly captured fluorescence images. The percentage of each cell type population was 10%, 54%, 36%, for Type I, II and III respectively. Importantly I did not observe cells in which H4K20me3 was punctate and HP1 α was diffuse (i.e. the inverse of type II). These results may suggest that HP1 α binds to heterochromatin before H4K20me3 and may be involved in recruiting H4K20me3 in ES cells. This is consistent with previous proposals in *Drosophila* and mammals that the HP1 α is required to recruit Suv4-20h to catalyse H4K20me3 (Schotta et al., 2004, Kourmouli et al., 2005). However, after ES cell differentiation by withdrawal of LIF for 7 days, only type III cells were found. Our results support the suggestion that although ES cells have all heterochromatin markers, the heterochromatin is still highly dynamic. Furthermore, if I assume that the diffuse staining corresponds to a less localised epigenetic marker, then this type of heterochromatin was only found in ES cells but not differentiated cells and it may make ES cell chromatin as more accessible for the transcription machinery more than in differentiated cell.

4.3.5 H4K20me3 is more associated with cell differentiation *in vitro* implantation than in ES cell differentiation

In the present study, H4K20me3 was not detected until postimplantation and the hypothesis that this mark may be associated with cell differentiation was tested. The ES cell differentiation (ESD) and *in vitro* implantation (IVP) methodologies were used as models for studying cell differentiation. I analysed the correlation between H4K20me3 and differentiation by observing the signal of H4K20me3 at pericentric heterochromatin relative to the expression of Oct4 in nuclei. The specific hypothesis was that H4K20me3 may increase in signal after cells lose Oct4 during differentiation. The results showed that after differentiation, cells could be divided into four categories: (A) strong Oct4 but weak H4K20me3, (B) strong Oct4 and H4K20me3, (C) weak Oct4 but

strong H4K20me3 and (D) both Oct4 and H4K20me3 weak. The results are shown in Figure 4.16 for IVP and Figure 4.17 for ESD. In cells with low Oct4, H4K20me3 was increased in only 26% (60/230) in ESD cells but 64% (135/210) in IVP cells. It seems likely that a low level of Oct4 at IVP was correlated ($P < 0.0001$) with H4K20me3, rather than in ESD. Moreover, 54% (95/175) of ESD cells and 45% (90/200) of IVP cells increased the levels of H4K20me3 while the Oct4 level was still clearly detectable, suggesting that during the differentiation H4K20me3 increased before the significant reduction of Oct4.

4.3.6 Global heterochromatin shows plasticity in early differentiation

Western blotting was used to analyse global chromatin modification changes during ES cell differentiation to embryoid bodies. After induction to embryoid bodies (day 7), the facultative heterochromatin markers H3K9me2, H4K20me1, H3K27me3 were significantly decreased. Constitutive heterochromatin H3K9me3 increased but HP1 β decreased and H4K20me3 and HP1 α were not significantly changed (Figure 4.18). However, using flow cytometry, which can measure the fluorescence intensity in each cell within populations of more than 10,000 cells (Figure 4.19), I showed that H4K20me3 and HP1 α seem preferentially enriched in cells that have a lower signal of Oct4.

These results revealed that during the early events of ES cell differentiation, global level of constitutive heterochromatin markers were slightly increased or stable while facultative heterochromatin markers were dramatically decreased. It is possible to speculate that maintaining the level of constitutive heterochromatin marker such as H4K20me3 may help ES cell to protect centromeric function and chromosome stability (Schotta et al., 2008) while the decreased facultative heterochromatin may facilitate activation of lineage genes that are normally repressed by H3K27me3 and H3K9me2 (Bernstein et al., 2006).

4.3.7 *In vitro* culture induce maturation of constitutive heterochromatin

These results and those published by several other reports (Dialynas et al., 2007, Kourmouli et al., 2005, Benetti et al., 2007, Martens et al., 2005) have revealed the

presence of H4K20me3 and HP1 α in ES cell lines. By contrast, H4K20me3 and HP1 α were not found in ICM or epiblast. It is possible that H4K20me3 and HP1 α may be established in ES cells in culture after many passages. To test this, new ES-like cells (named noo01) were isolated from blastocysts, and epigenetic and chromatin modifications were observed in the early passages of ES cells and compared to ICM and epiblast. These ES-like cells exhibited similar morphology to ES cells (Figure 4.20A) in that they were positive for Oct4 and Nanog protein (Figure 4.21D and E) and were able to form embryoid bodies (Figure 4.20B) and beating cardiomyocytes after differentiation. Remarkably by the early passage (passage 1-3) these ES-like cells had already established H4K20me3 and HP1 α (Figure 4.21) at levels that were comparable to established ES cells (E14tg2a) (Figure 4.21D and E). H4K20me3 and HP1 α were not observed in ICM cells (Figure 4.6A) or epiblast (Figure 4.8A).

To study in more detail when cells from the ICM establish H4K20me3 after culture, blastocysts were placed into ES cell medium and observed from 24 hour to 96 hours. The results showed that trophectoderm differentiated to trophoblast and attached to the plate at approximately 24-48 hours. At this time H4K20me3 was visible in the trophoblast, but not in the ICM, and was specific to heterochromatin (Figure 4.6B). After 4 days culture, the ICM colonies outgrew and giant trophectoderm developed (Figure 4.7B). ICM-derived cells established H4K20me3 and HP1 α but in diffuse form rather than punctuate form (Figure 4.7B). Conversely, giant trophectoderm cells had HP1 α and H4K20me3 in a punctuate form (Figure 4.7B).

One possible explanation for this is that factors in the culture medium such as LIF or factors present in serum may induce H4K20me3 in ES cells and embryos. To test this hypothesis, ES cells were cultured in medium supplemented with serum (FBS) or with serum replacement (Knock-out serum replacement, KOSR, Invitrogen). No difference was found in either the pattern or amount of H4K20me3 in ES cells cultured with serum or in KOSR, as measured by immunofluorescence or western blot (Figure 4.22). In contrast there was a significant change in other modifications and heterochromatin proteins such as H3K9me2, H3K9me3, H4K20me1 and HP1 β (Figure 4.22). Secondly, *in vitro* implantation embryos cultured with or without LIF were compared. Implantation embryos established H4K20me3 by 24 to 48 hours when supplemented

with or without LIF. Again, no difference in the pattern of H4K20me3 *in vitro* implantation embryo with or without LIF was detected by immunofluorescence. Thirdly, I used Trophectodermal Stem (TS) cell medium (commonly used for isolating TS cells from blastocysts) instead of ES cell medium for *in vitro* implantation. Culturing embryos for 4 days in TS cell medium resulted in a smaller colonies than those grown in ES cell medium. It was expected that these cells were TS-like cells derived from the polar trophectoderm. Almost all of the cells in TS cell medium established H4K20me3 and HP1 α (Figure 4.7A).

Collectively, the data suggest that culturing embryos either in ES cell medium or TS cell medium can induce H4K20me3 and HP1 α establishment. Although serum and LIF are not factors required for induction of H4K20me3 and HP1 α , it could be another component in medium, for example a difference in nutrients between embryo and ES cell medium. Another possibility is loss of signals from other cells *in vivo* which may lead ES cells to established heterochromatin marker *in vitro*.

4.4 Discussion

These analyses of the changes in cell nuclear chromatin organisation in mouse embryos have revealed dynamic changes in heterochromatin patterns during preimplantation and post implantation development that continue even into late developmental stages. In addition, epigenetic differences between ES cells and the ICM cells have been identified. These results call into question how epigenetic mechanisms help govern the fate of cells both in normal development and during tissue culture. Greater understanding is needed of how epigenetic modifications on histones and DNA can maintain and propagate heritable cell type characteristics. In turn this may provide cell nuclear differentiation markers to assess the epigenetic profile of cells in development and disease as well as the effects of tissue culture and *in vitro* assisted reproduction.

4.4.1 The difference between ICM and ES cells

Mouse ES cells are isolated from the ICM of blastocysts, hence both cell types contain pluripotent transcription factors such as Oct4, Nanog and Sox2. However, some studies suggest that ES cells exhibit some properties that are not normally shown by embryos. For example, no pluripotent cell type in the embryo is capable of long-term self-renewal *in vivo*. In terms of genetic markers, ES cells express some specific markers such as Kit, DAZL and Ddx4 which have very low or no transcription in the ICM (Horie et al., 1991, Zwaka and Thomson, 2005, Clark et al., 2004, Toyooka et al., 2003). In terms of chromatin composition, the results presented here clearly show that the ICM differs from ES cells with respect to the lack of H4K20me3 and HP1 α to form mature heterochromatin. Moreover, previous studies showed that the chromatin marker H2A.Z is also not detected in the ICM but becomes positive after *in vitro* implantation (Rangasamy et al., 2003) and is also found in ES cells (Creyghton et al., 2008). Recently transcription profiles of the human ICM and ES cells has revealed these two cell types are significantly different (Reijo Pera et al., 2009) as well as the profile of H3K4me3/H4K27me3 on gene promoter also reveal the difference between mouse ICM and ES cells (Dahl et al.).

4.4.2 Mechanism of heterochromatin maturation

It has been shown in previous studies that H4K20me3 marks somatic heterochromatin, dependent on HP1 and Suv4-20h enzymes (Schotta et al., 2004, Kourmouli et al., 2005). The supporting evidence from the present study shows that first, H4K20me3, HP1 α and Suv4-20h were absent from preimplantation embryos, but eventually become highly detectable postimplantation embryo; second, ES cells show a diffuse or punctuate staining pattern, possibly more dynamic of HP1 α and H4K20me3 localisation, in which the pattern of H4K20me3 seems to depend on HP1 α . Taken together, it is possible to speculate that loss of H4K20me3 in preimplantation embryo may result from a lack of HP1 α and Suv4-20h enzymes. After implantation or establishment of ES cells, Suv4-20h and HP1 α were highly detectable, suggesting that Suv4-20h and HP1 α may be very important for establishment of mature heterochromatin by recruiting H4K20me3 in development. Although active histone demethylation might be another potential

mechanism, there is no published report describing a H4K20me3-demethylase. It was thought that the demethylase Jmjd2a was a potential candidate for a H4K20me3 demethylase as it has a domain specific for H4K20me3, but there is no functional proof that the activity can remove H4K20me3 (Lee et al., 2008).

4.4.3 Chromatin and cell differentiation.

The present experiments confirm and extend earlier observations suggesting an association between the appearance of H4K20me3 and the initiation of differentiation of the pluripotent cells. H4K20me3 was not seen in Oct4-positive cells, however, soon after implantation, it became clearly visible. A previous study (Biron et al., 2004) suggested that H4K20me3 may be associated with cellular differentiation of neural and muscle cells in postimplantation mouse embryos. Similar results have been observed in other species (Karachentsev et al., 2007), for instance in *Drosophila* in which the antibody against H4K20me3 failed to detect the epitope at any stage of early development until mid-gastrulation. An interpretation that might be considered is that H4K20me3 is associated with early differentiation of the epiblast, which in mice occurs soon after implantation embryos. In contrast to the postimplantation embryo, H4K20me3 was found in ES cells, and did not undergo a significant change after early differentiation. This suggests that the epigenetic state of chromatin which regulates early differentiation in postimplantation embryos may be different from that in ES cells.

It is believed that at specific stages in development there may be biologically important differences in the openness of chromatin, for example that chromatin in preimplantation embryos is more open than in ES cells and differentiated cells (Table 4.1). A number of different studies have suggested that chromatin might be less compact and more transcription-permissive than that of in differentiated cells (Kobayakawa et al., 2007, Meshorer et al., 2006, Efroni et al., 2008, Martens et al., 2005). The differences in the presence and pattern of H4K20me3, HP1 α and HP1 β described in this study may account for these differences in chromatin structure.

4.4.4 Heterochromatin, tissue maturation and aging

The present study indicated that a proportion of the H4K20me3 increases in late development and might be related to an ageing mechanism. A similar result from a previous report using mass spectrometry showed that liver and kidney cells from senescent animals acquired more H4K20me3 than young animals (Sarg et al., 2002). Moreover, two studies in human cell lines in which H4K20me3 is very low or undetectable show that H4K20me3 is visible or increases either in late passage cells or non-growing cells (Sarg et al., 2002, Shumaker et al., 2006). Furthermore, previous studies on global and specific gene expression levels in human neural development show that H4K20me3 is up-regulated in adult mature cerebellum compared to immature cerebrum from foetuses and infants, and is not related to gene transcription (Stadler et al., 2005). For all these reasons, it might be suggested that H4K20me3 is linked to tissue maturation or ageing.

My work leads to a conclusion that constitutive heterochromatin undergoes very slow changes in composition from embryo to late foetal development. These changes may correlate to different stages of cell lineage specification. In particular, the appearance of H4K20me3 seems associated with terminal differentiation and tissue maturation in development or the continued maintenance of cells in the ES/epiblast lineage. Although its effect on heterochromatin structure and function is as yet unknown, other work suggests that H4K20me3 containing heterochromatin is essential for maintenance of perinatal, non-cancerous cell states and can confer heterochromatin maintenance capability (Schotta et al., 2008, Benetti et al., 2007, Pogribny et al., 2007). I propose that constitutive heterochromatin, while contributing to maintaining cell type, also retains the ability to adapt to sequential cell fate changes during cell lineage specification. As these changes come to an end, a necessary gradual stabilisation follows by increasing the maintenance capabilities of heterochromatin, which relies on the essential acquirement of H4K20me3 and HP1 α .

Table 4.1 The presence and pattern of heterochromatin markers in mouse embryo development and in ES cells.

	H4K20me3	HP1α	HP1β	Chromatin
Preimplantation embryo	Absent or Very weak	Absent or Very weak	Diffuse	Highly open
Postimplantation embryo	Speckled	Speckled	Speckled	Closed
Undifferentiated ES cells	Speckled/Diffuse	Speckled/Diffuse	Speckled	Open
Differentiated ES cells	Speckled	Speckled	Speckled	Closed

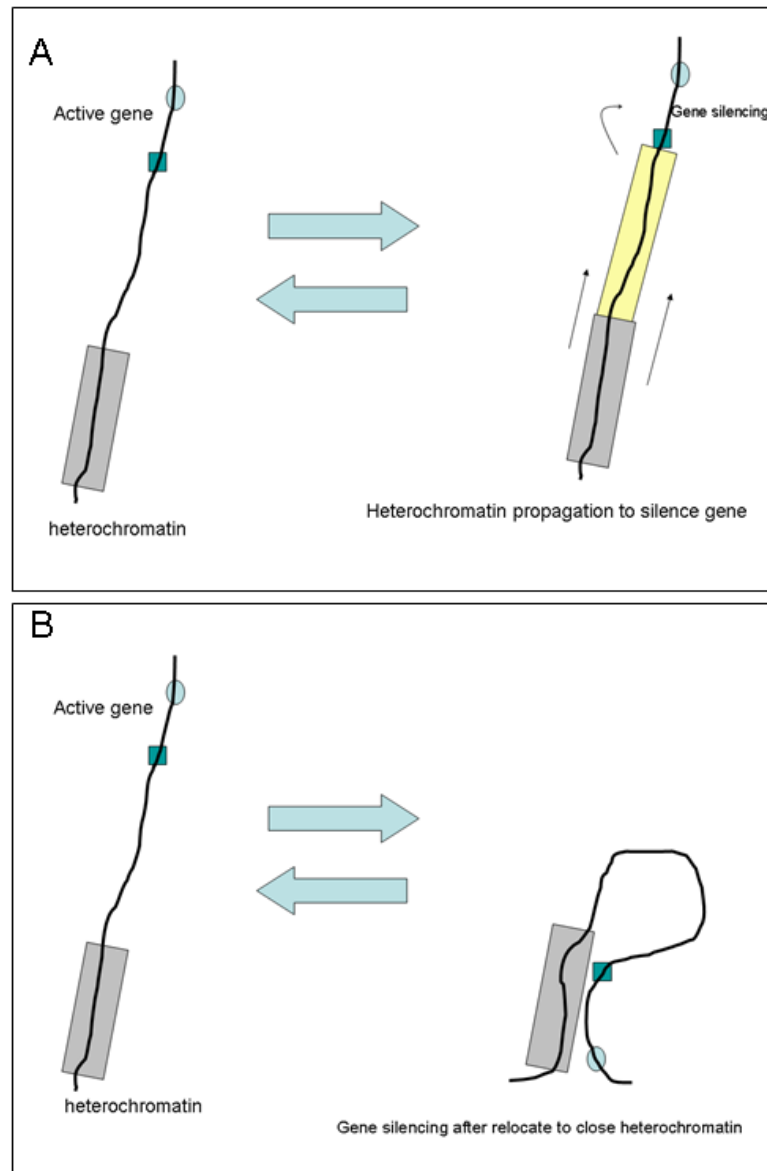


Figure 4.1 Model for heterochromatin spreading and gene relocation affecting gene expression during development and differentiation. (A) Heterochromatin propagates to neighbouring gene causing gene silencing. (B) Active gene moving in the vicinity of heterochromatin can result in silencing of the gene.

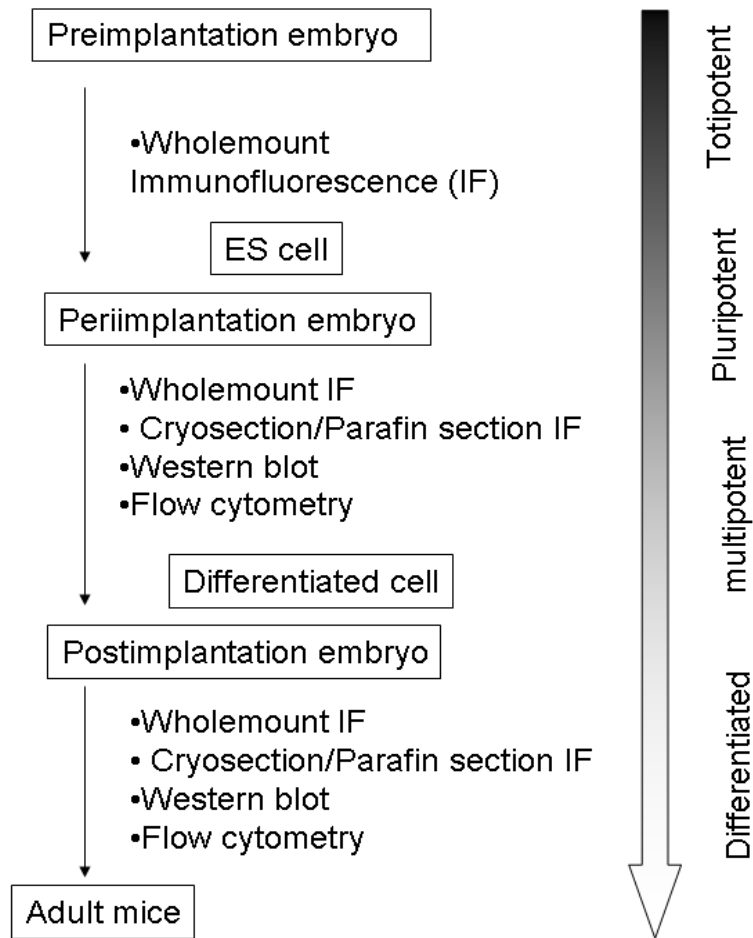


Figure 4.2 Experimental designs of the study. Embryos and ES cells were collected based on developmental stage and differentiation status, respectively. Immunocytochemistry was observed with fluorescence microscopy, flow cytometry and western blotting.

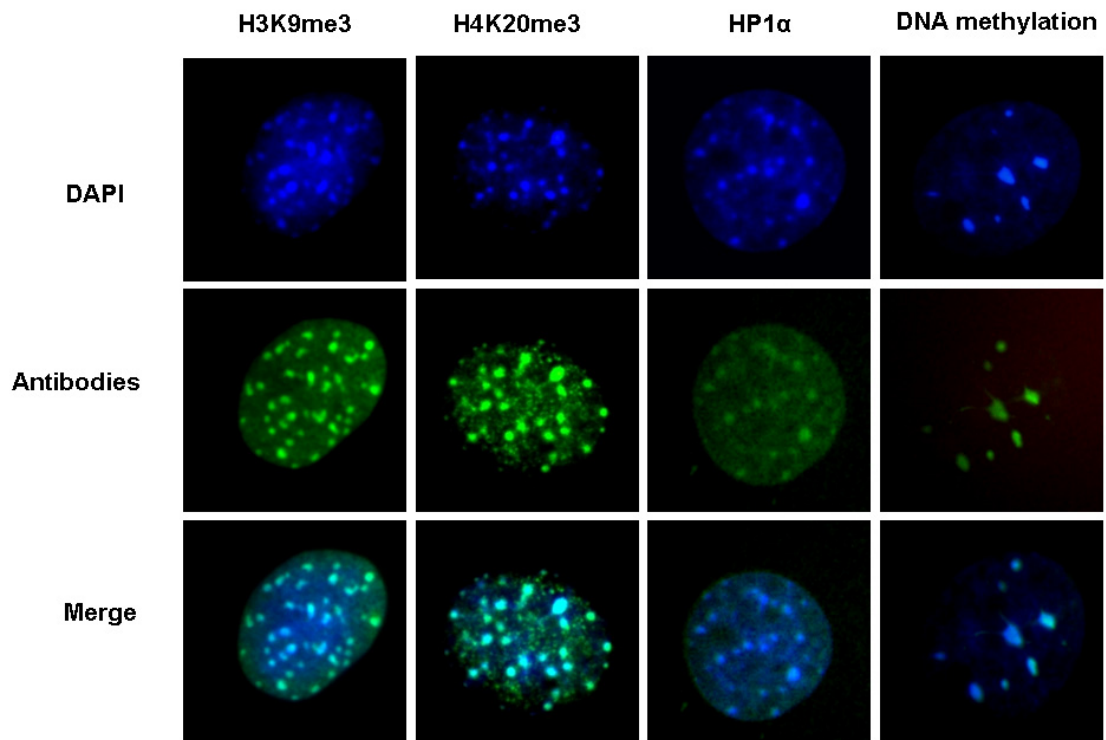


Figure 4.3 Heterochromatin markers in somatic cells. Mouse embryonic fibroblast cells were stained with heterochromatin markers H3K9me3, H4K20me3 HP1 α and DNA methylation (green), DNA was counterstained with DAPI (blue).

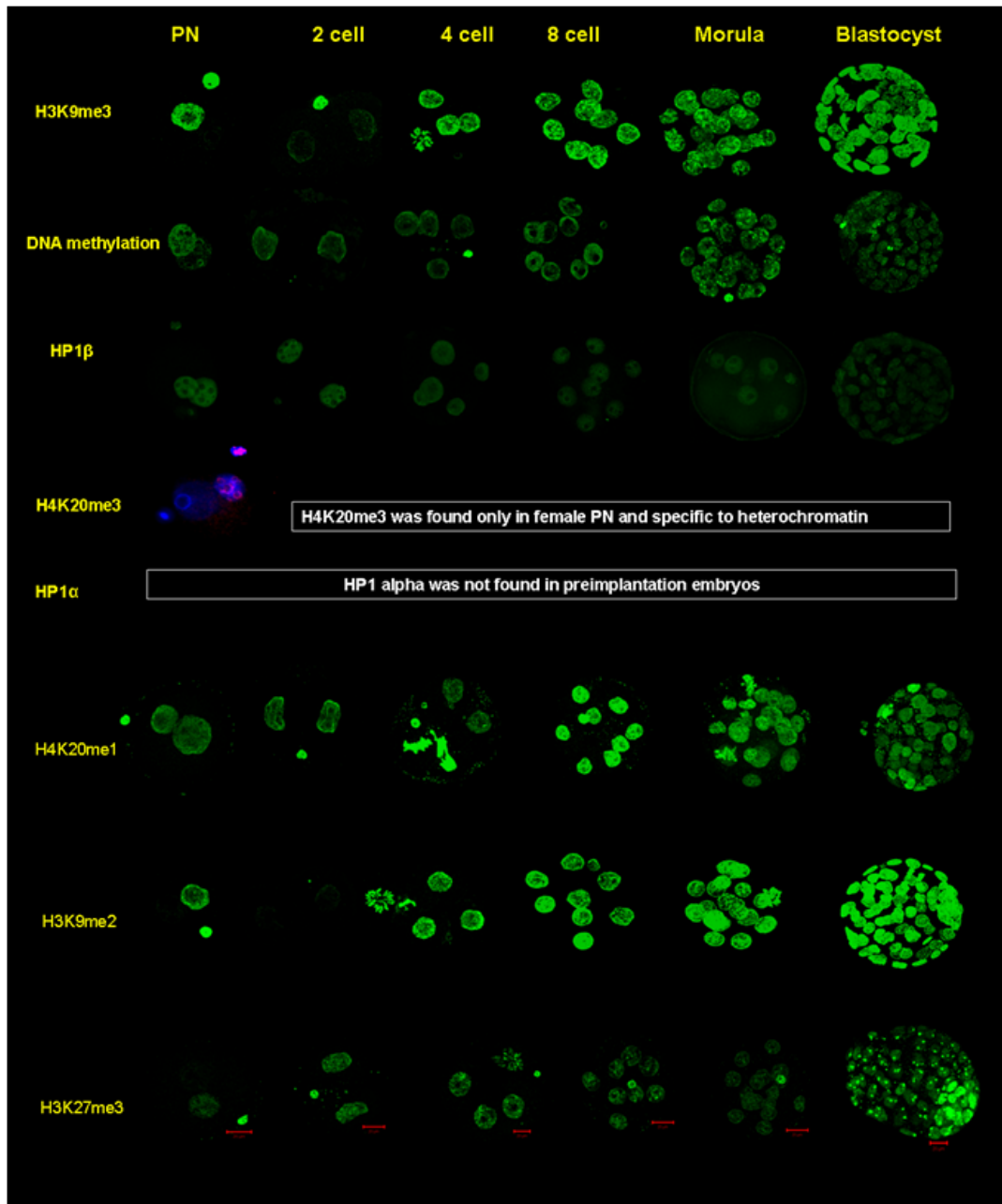


Figure 4.4 Immunofluorescence microscopy images of heterochromatin markers in preimplantation embryo nuclei. Primary antibodies were against H3K9me3, H4K20me3, 5-methyl cytosine, HP1 α , HP1 β , H3K9me2, H3K27me3 and H4K20me1. Positive signal was visualised using secondary fluorescent antibodies (FITC =green, TRITC = red). Immunofluorescence was observed using confocal microscope. Most positive antibodies were shown in green colour except H4K20me3 which is shown in red. Embryos were counterstained with DAPI (blue). All antibodies showed positive staining, except for H4K20me3 which was detected only in zygote (female chromatin) and HP1 α which were undetectable in preimplantation embryo nuclei. The scale bar (red) represents 20 μ m. 30 embryos per stage for each antibody were used.

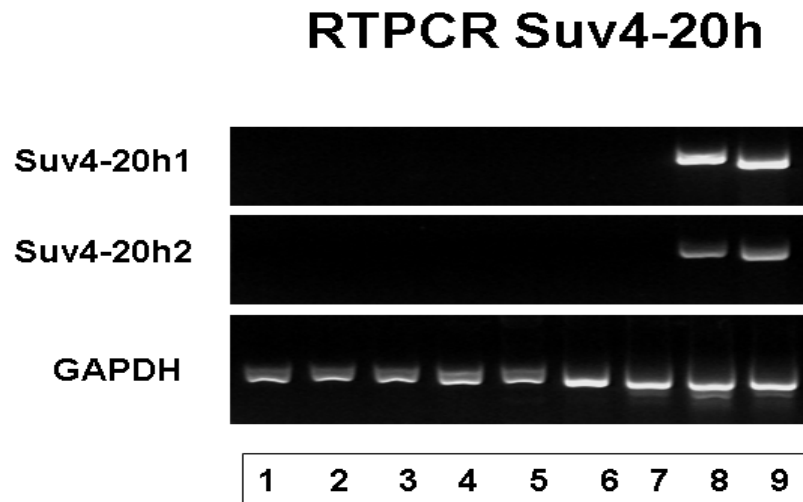


Figure 4.5 The mRNA level of Suv4-20h1 and Suv4-20h2. mRNA level of Suv4-20h1 and Suv4-20h2 enzymes which are H4K20me3 methyltransferases, detected by RT-PCR and separated by gel electrophoresis. Both Suv4-20h1 (655 bp) and Suv4-20h2 (800 bp) were not found in oocyte or early implantation embryos, but were detected in blastocyst. Lane 1 = immature oocyte, 2 = mature oocyte, 3 = zygote, 4 = two-cell embryo, 5 = four-cell embryo, 6 = eight-cell embryo, 7 = morula, 8 = preimplantation blastocyst, 9 = *in vitro* early postimplantation blastocyst (24 hours). Gapdh mRNA (458 bp) was used as a loading control. Ten embryos per stage were used and the PCR was repeated twice.

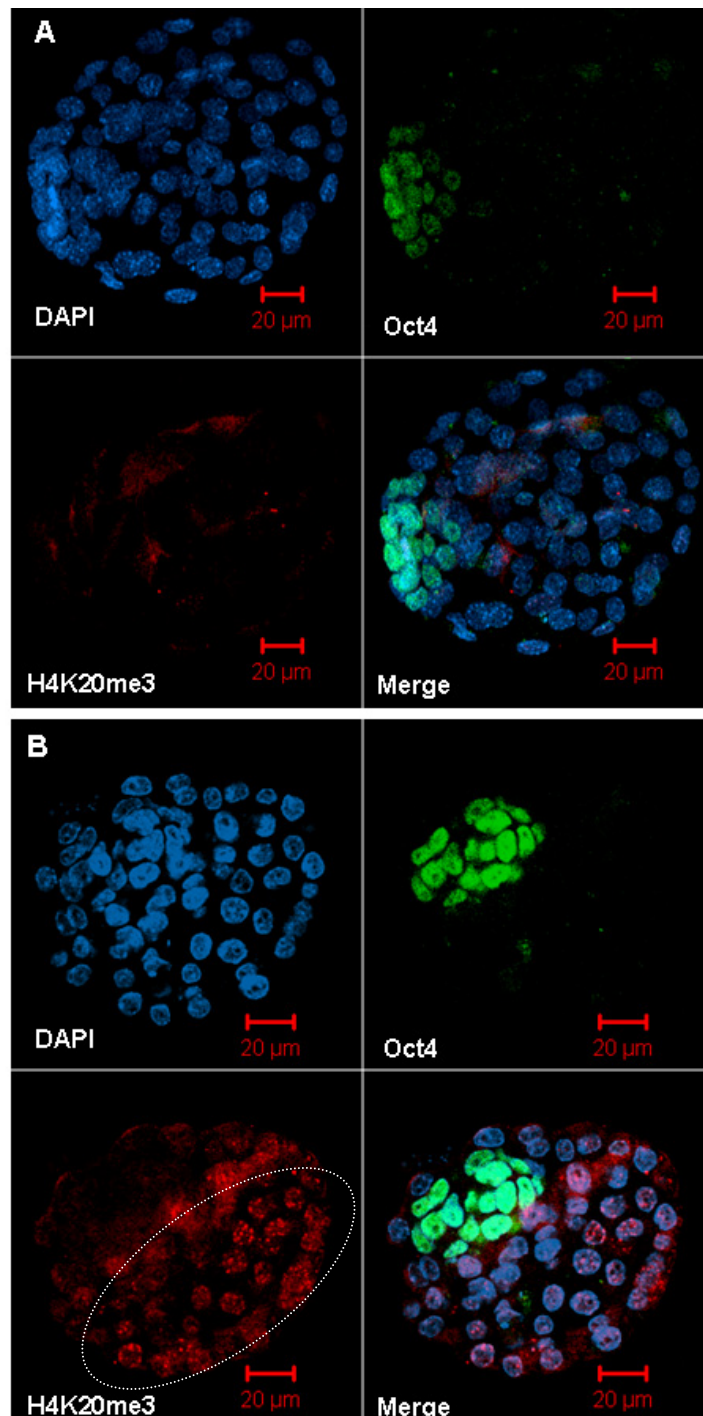


Figure 4.6 Immunofluorescence confocal microscopy images of *in vitro* implantation embryos. (A) H4K20me3 (red) was undetectable before implantation. (B) H4K20me3 was found at 48 hours post *in vitro* implantation in trophoctoderm only (inside the oval). ICM was stained with anti-Oct4 antibody (green) and DNA was counterstained with DAPI (blue). The scale bar (red) represents 20 μm .

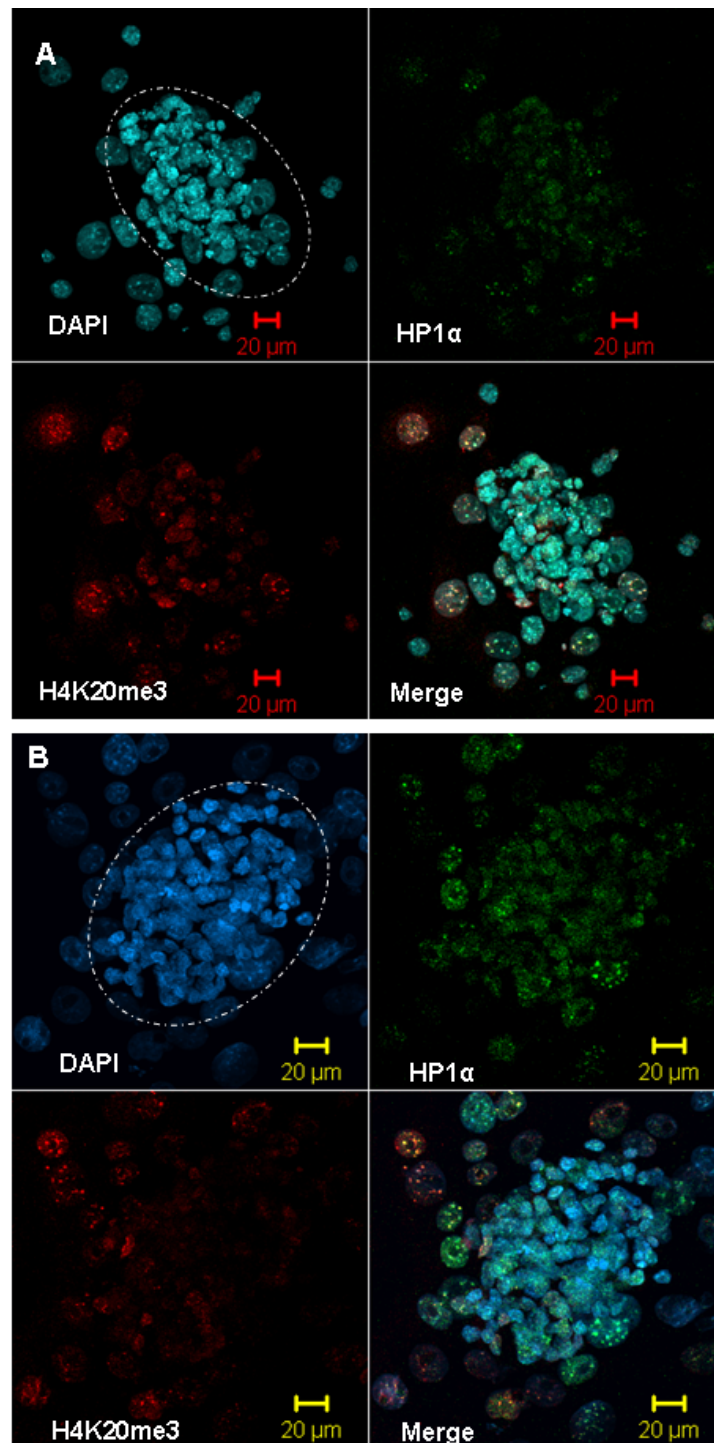


Figure 4.7 Effect of culturing *in vitro* implantation embryos for 4 days. Embryos were immunostained for HP1 α (green), H4K20me3 (red) and DNA was counter stained with DAPI (blue). (A) Embryos cultured with TS cell medium, shown inside the oval is preTS cell colony, and outside is trophoblast. (B) Embryos cultured in ES cell medium. Inside the oval is a preES cell colony, outside is trophoblast. The scale bar (red and yellow) represents 20 μ m.

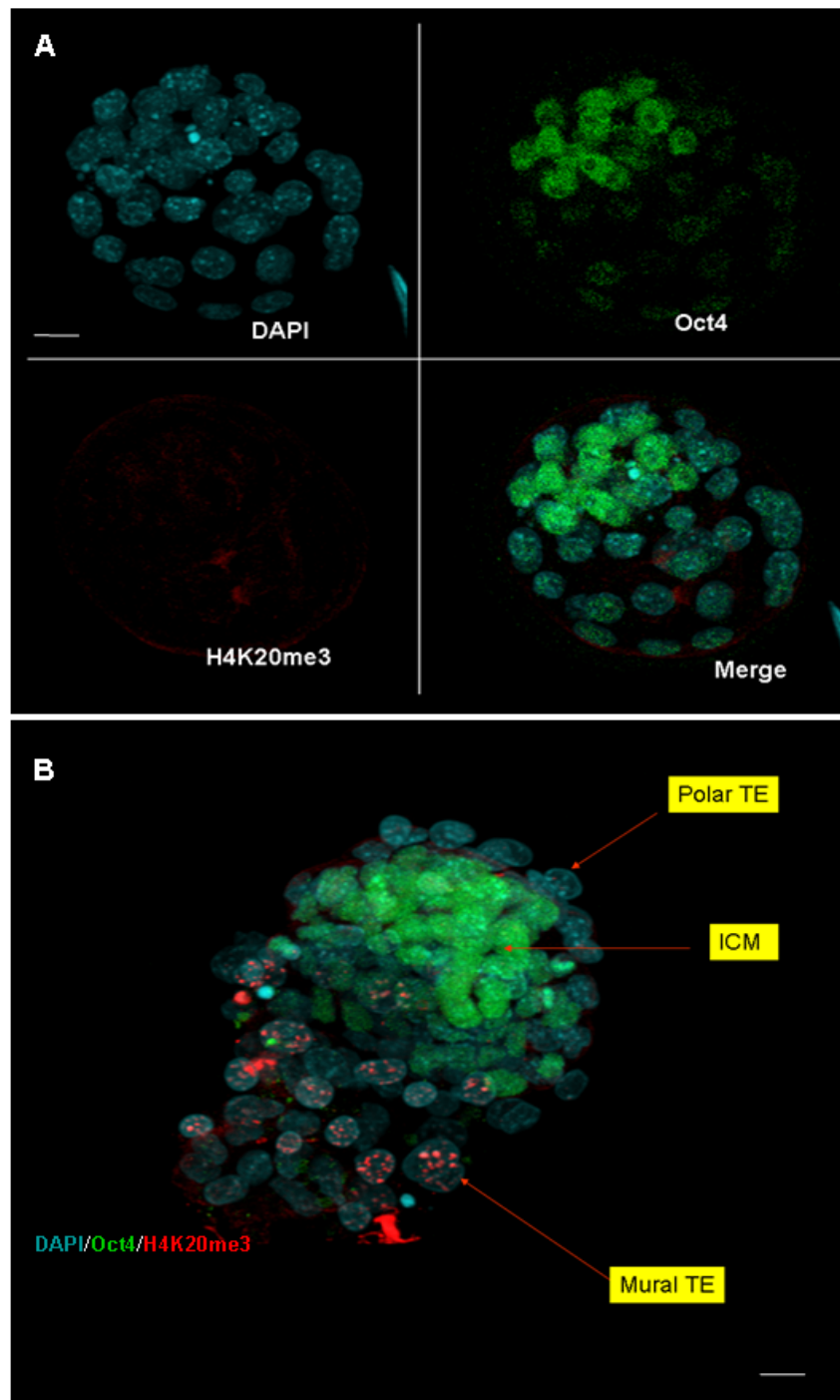


Figure 4.8 Immunofluorescence confocal microscopy images of *in vivo* preimplantation (A) and postimplantation embryos (B). Embryos were immunostained for H4K20me3 (red), Oct4 (green) and DNA was counterstained with DAPI (blue). H4K20me3 was found in postimplantation embryos (E4.5) but not preimplantation embryos (E3.5). Only mural trophoblast (TE) was positive for H4K0me3 but not ICM and polar TE. The scale bar (white) represents 20 μ m.

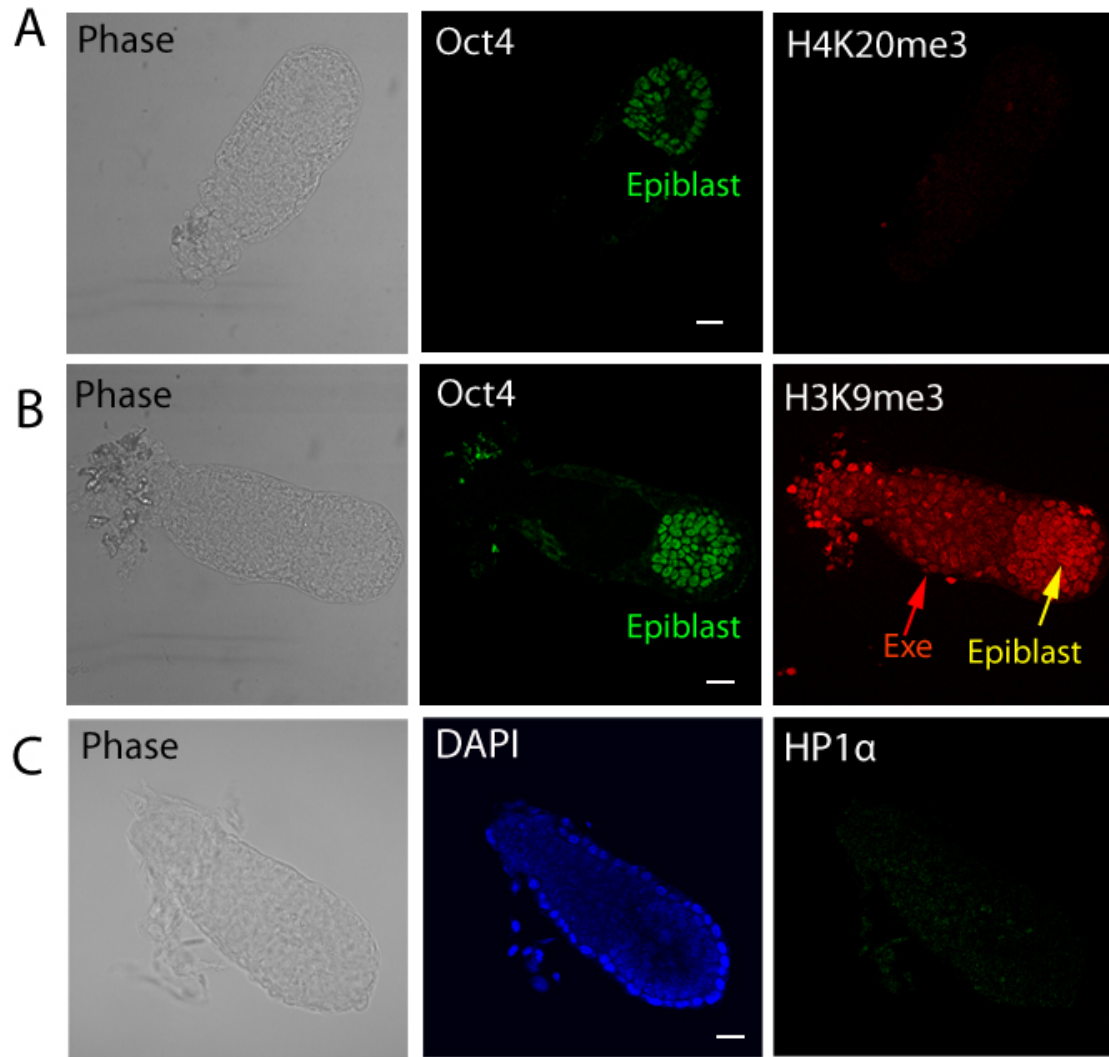


Figure 4.9 H3K9me3, not H4K20me3 and HP1 α , was found in early postimplantation embryos. Immunofluorescence and phase contrast images of *in vivo* postimplantation embryos (E6.5). (A) Embryo was double stained with anti-Oct4 (green) and H4K20me3 (red) antibodies. Epiblast cells were positive for Oct4 but negative for H4K20me3. (B) Embryos were stained with anti-Oct4 (green) and anti-H3K9me3 (red), the whole embryo was positive H3K9me3 while epiblast positive for Oct4. Exe=extraembryonic ectoderm. (C) Embryo was stained for HP1 α and counterstained with DAPI (blue); embryos were negative for HP1 α . The scale bar (white) represents 20 μ m.

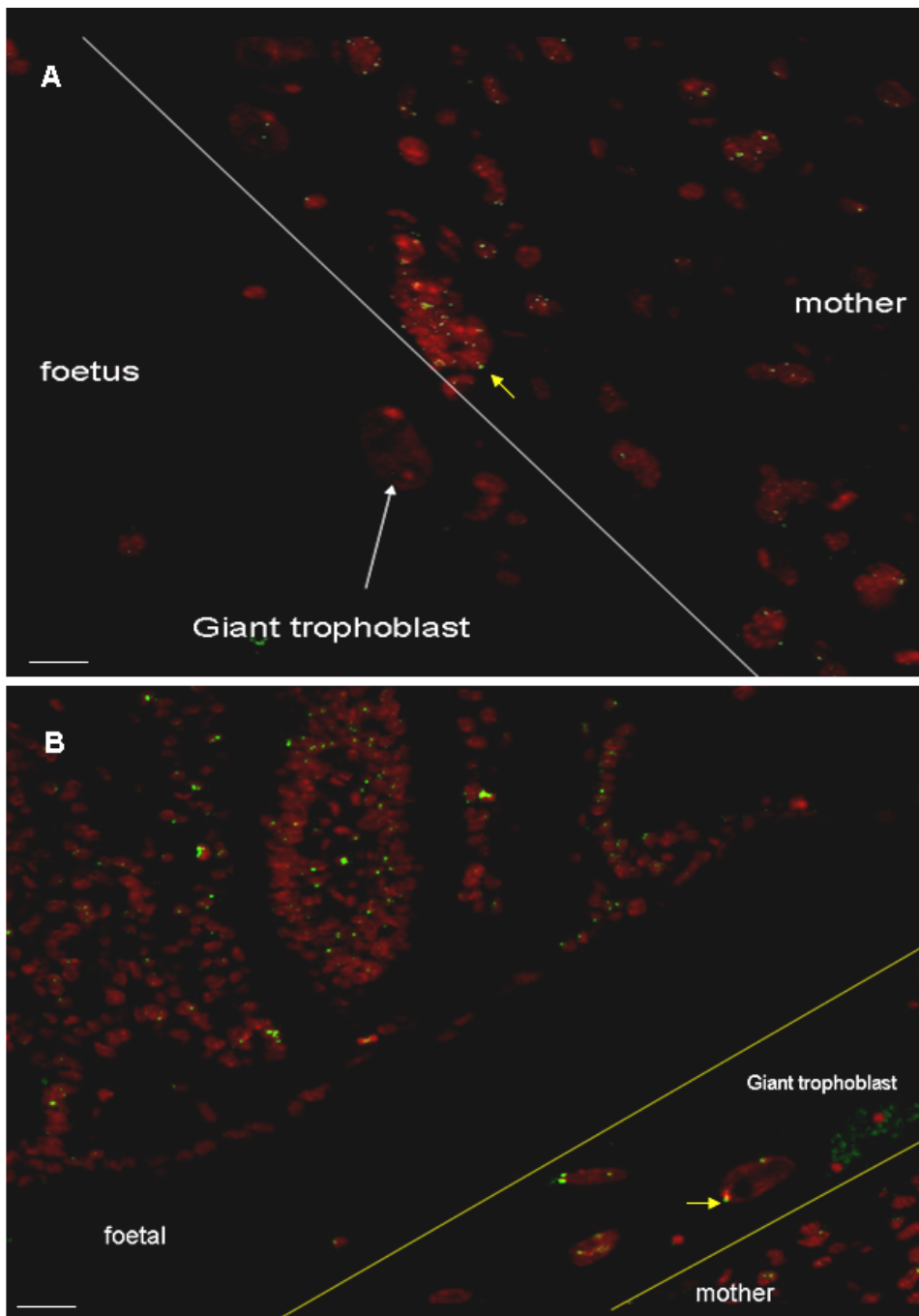


Figure 4.10 Immunofluorescence images of postimplantation embryo (E8.5) from paraffin section. (A) H4K20me3 was detected only in maternal decidual cells and not in embryonic cells. (B) H3K9me3 (control antibody) was found both in maternal decidual cells and in embryonic cells. Green = antibodies (H4K20me3 or H3K9me3), Red = DAPI. The yellow arrow points to one of the heterochromatin foci positive for H4K20me3 which also colocalises with DAPI. The scale bar (white) represents 20 μm.

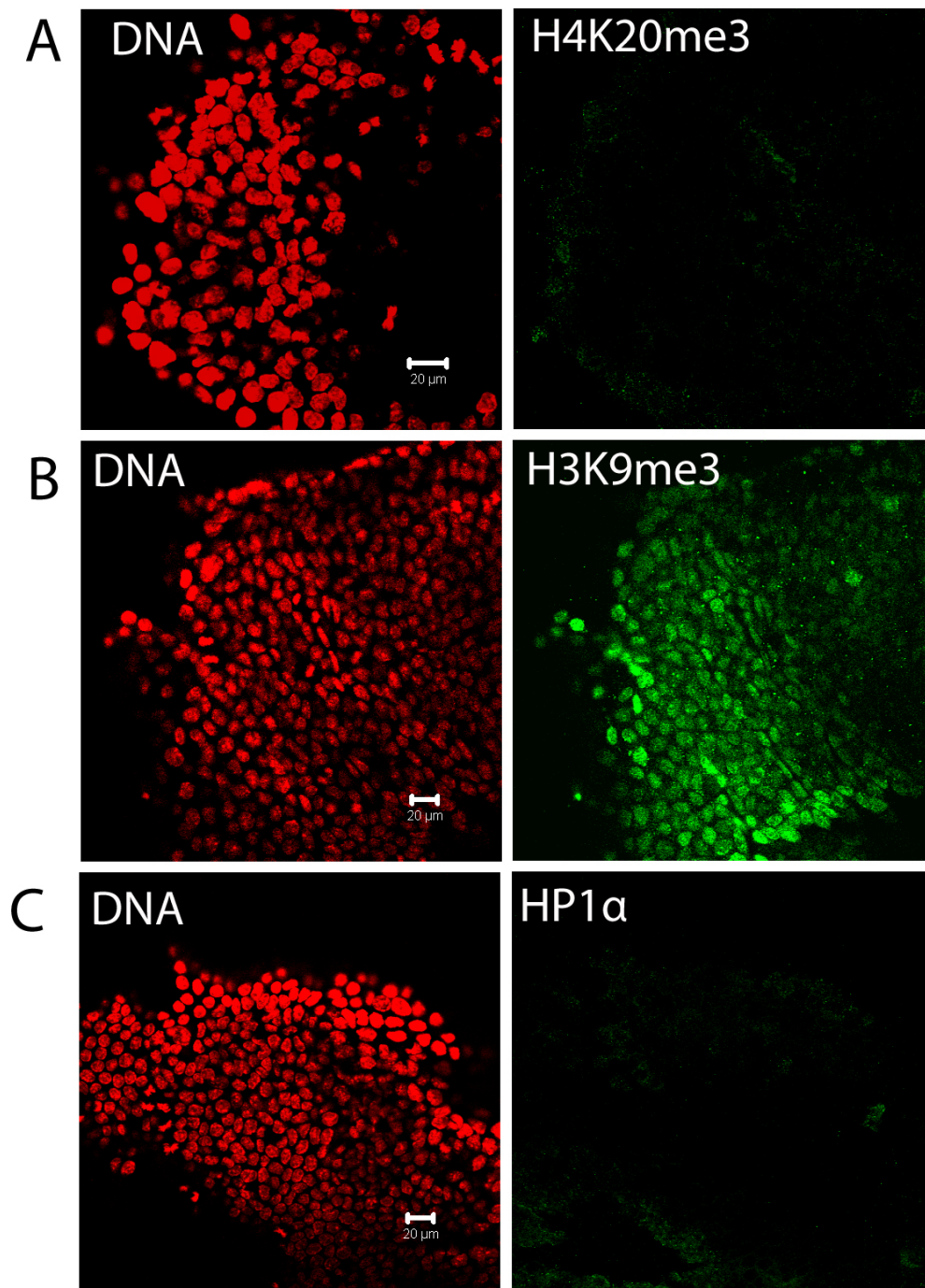


Figure 4.11 Heterochromatin marker H4K20me3 and HP1 α was not detected in E8.5 embryos. E8.5 embryos were cryosectioned and stained with heterochromatin markers (green) H4K20me3 (A), H3K9me3 (B) and HP1 α (C). Only H3K9me3 was detected. DNA was counterstained with DAPI (pseudo-coloured red). The scale bar (white) represents 20 μ m.

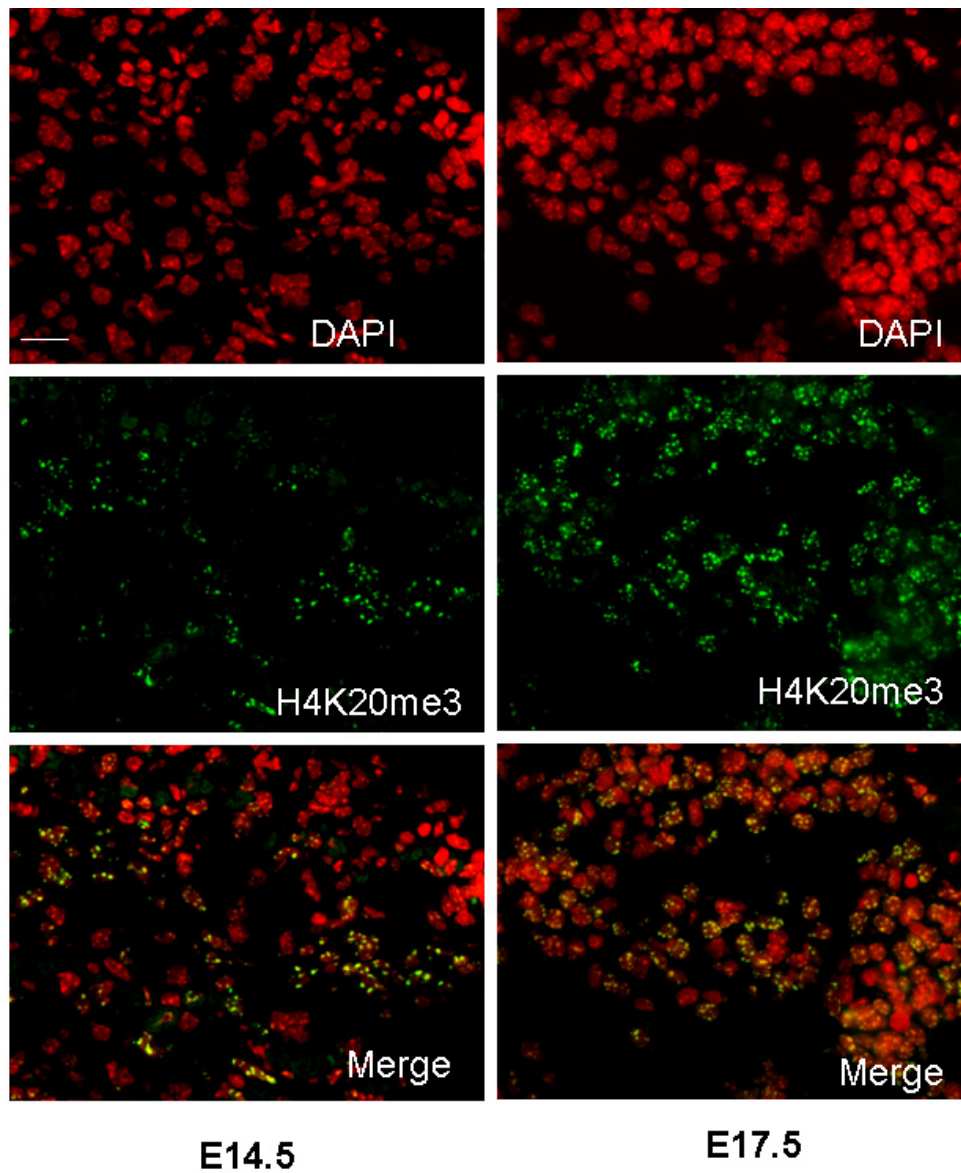


Figure 4.12 Immunofluorescence microscopy images of postimplantation embryos (cryosection of foetal liver tissue). E14.5 and E17.5 embryos are shown in left and right panels respectively. Green = H4K20me3, Red = DAPI. H4K20me3 positive cells increased from E14.5 to E17.5. The scale bar (white) represents 20 μ m.

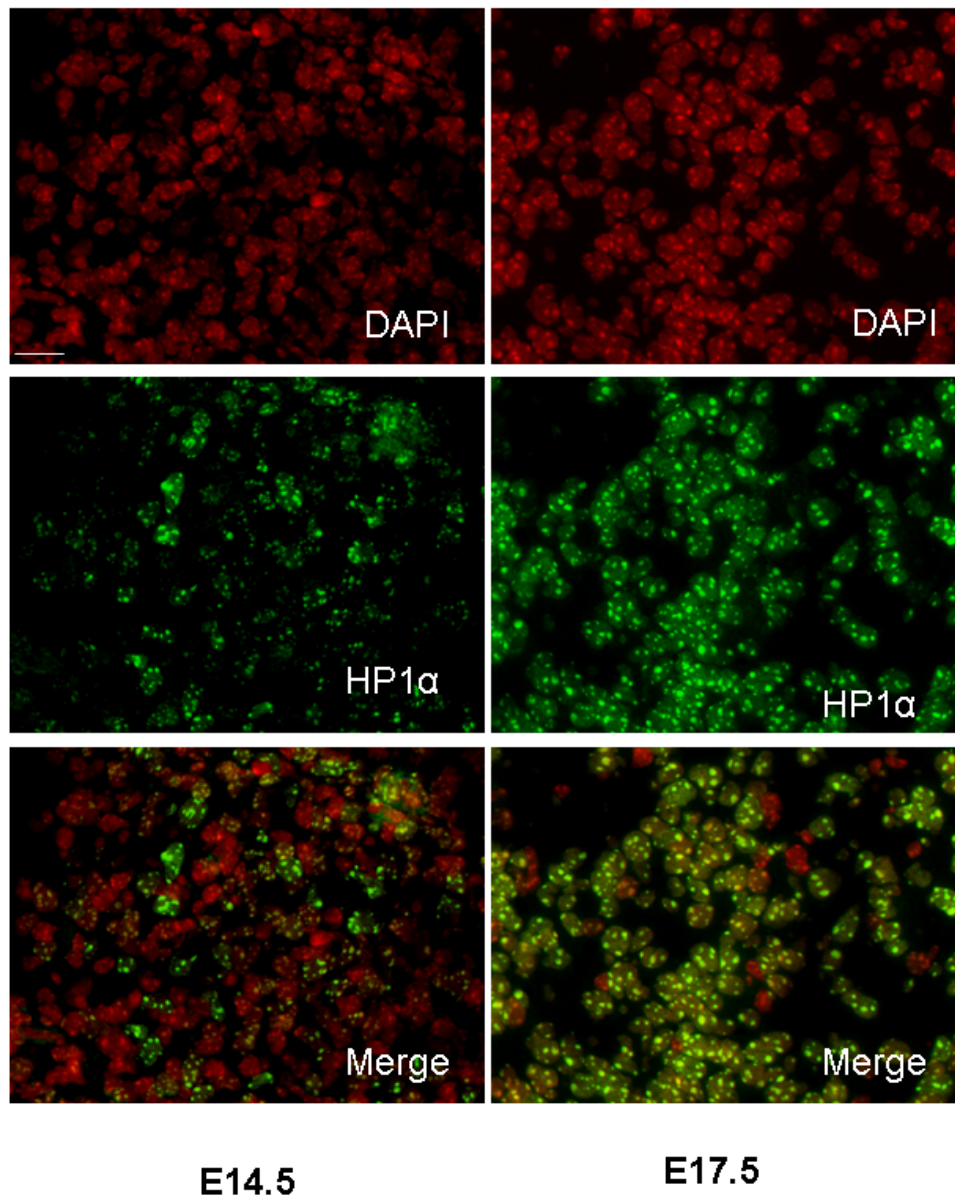


Figure 4.13 Immunofluorescence images of postimplantation embryos (cryosection of foetal liver tissue). E14.5 and E17.5 embryos are shown in left and right panels respectively. Green fluorescent signal represents HP1 α and red represents DAPI-DNA counterstaining. HP1 α positive cells increased from E14.5 to E17.5. The scale bar (white) represents 20 μ m.

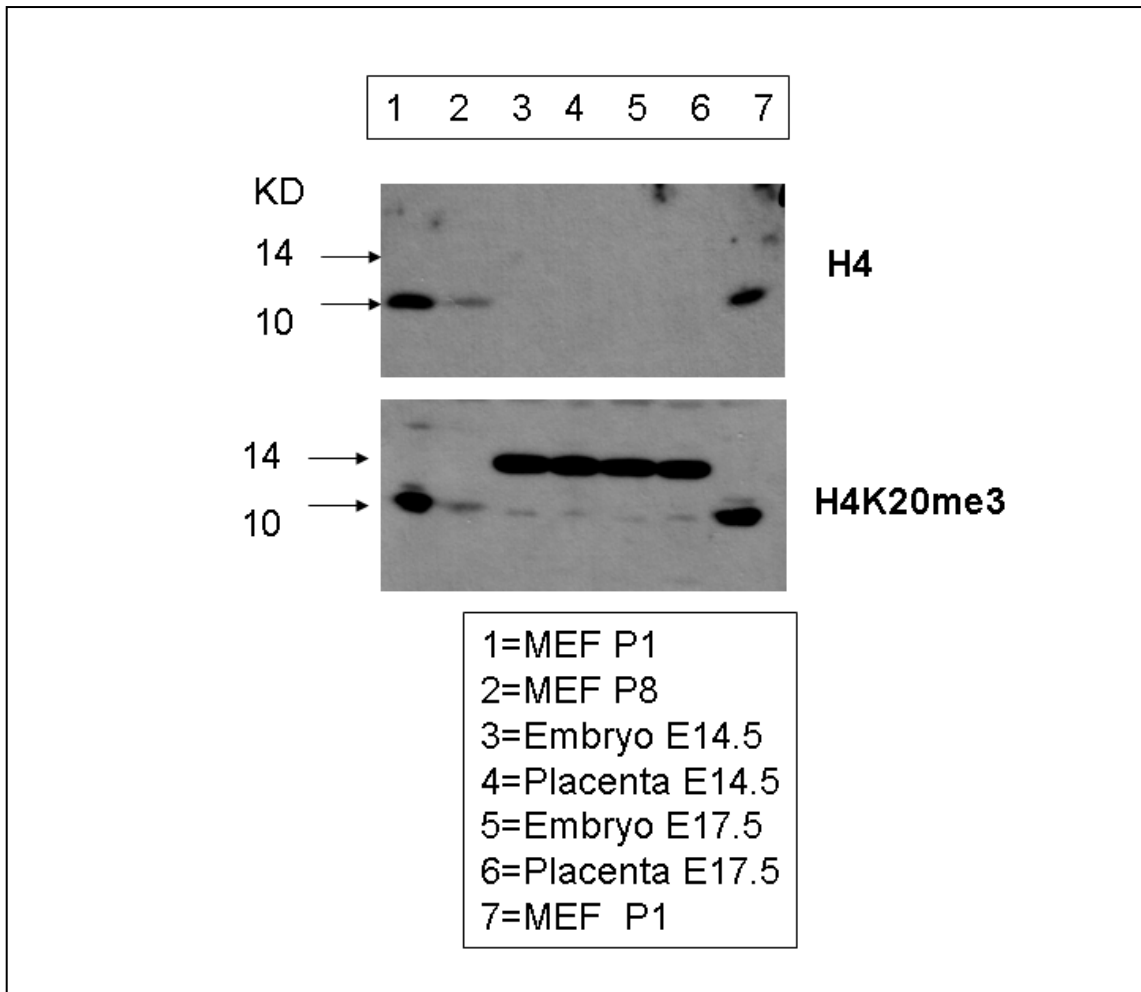


Figure 4.14 Western blots of postimplantation embryo extract SDS-PAGE. Protein was detected using antibodies against H4K20me3 and H4 (loading control) Lane number 1 = MEF first passage (P1); 2 = MEF eighth passage (P8); 3 = embryo E14.5; 4 = placenta E14.5; 5 = embryo E17.5; 6 = placenta E17.5; 7 = MEF first passage (P1).

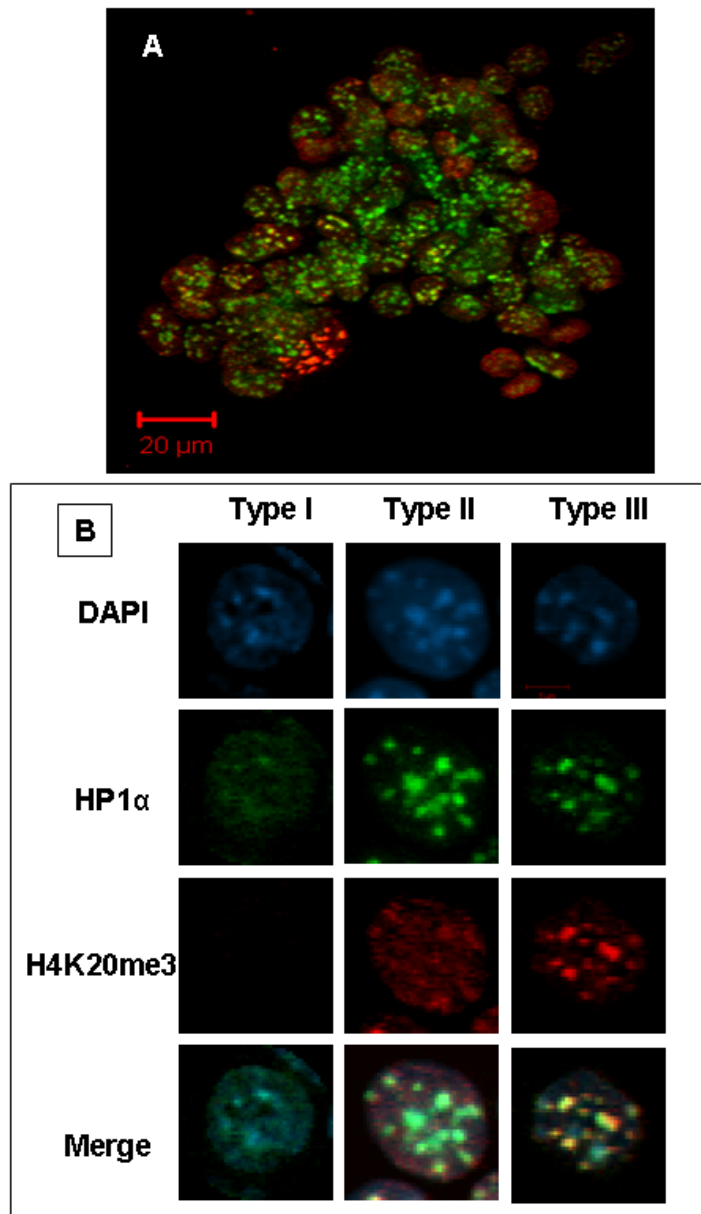


Figure 4.15 Dynamics of H4K20me3 and HP1 α in ES cells. ES cell line (E14tg2a) was immunostained using anti- H4K20me3 (red) and anti-HP1 α (green) in ES cells. These images show the correlation between H4K20me3 and HP1 α in ES cells. (A) Heterogeneous patterns of HP1 α and H4K20me3 in ES cells. (B) Cells were classified into three types: Type I (left panel) diffuse HP1, weak or absent H4K20me3; Type II (middle panel) punctate HP1 α but diffuse H4K20me3, and Type III (right panel) both HP1 α and H4K20me3 were diffuse. Blue = DAPI, green = HP1 α , Red = H4K20me3. The scale bar (red) represents 20 μ m.

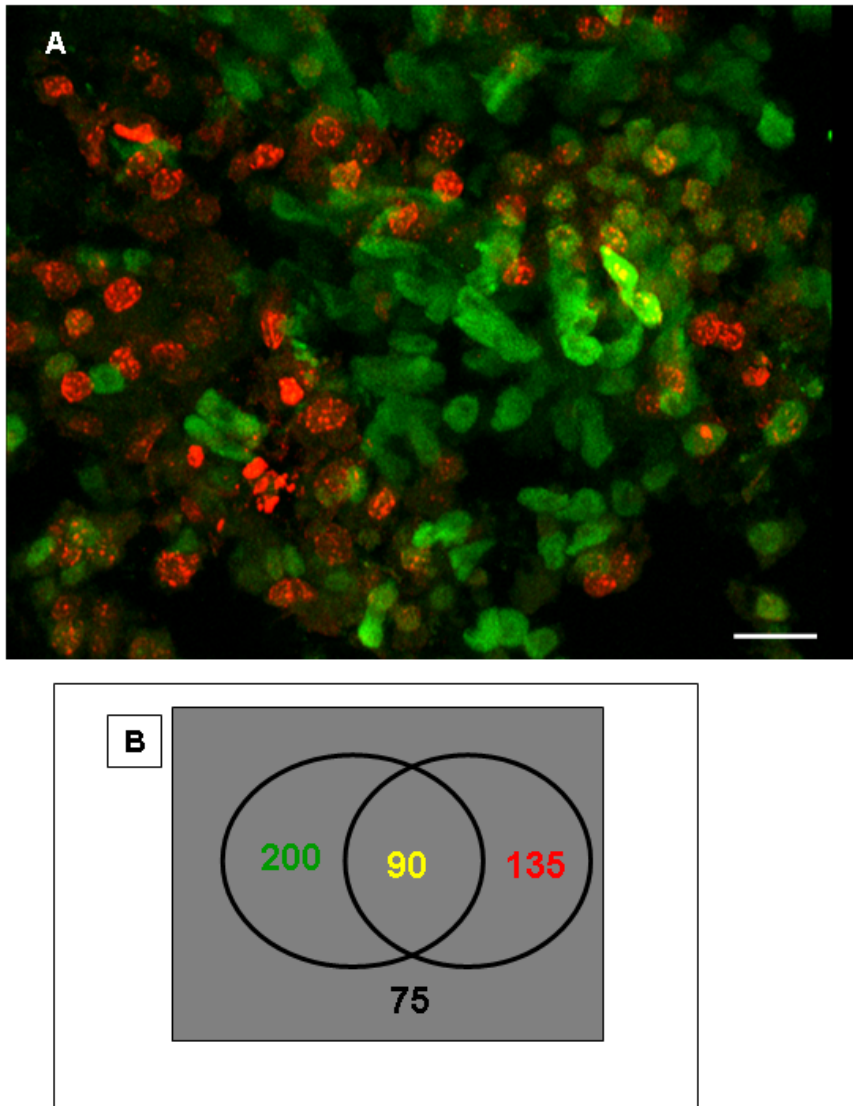


Figure 4.16 Correlation between H4K20me3 and Oct4 of *in vitro* postimplantation embryo (14 days postimplantation). (A) Cells were immunostained using anti-Oct4 antibody (green) and anti-H4K20me3 antibody (red). (B) Cells were counted based on the presence of fluorescence markers, green = Oct4; red = H4K20me3; yellow = Oct4 and H4K20me3; and black = negative for Oct4 and H4K20me3. The scale bar (white) represents 20 μm .

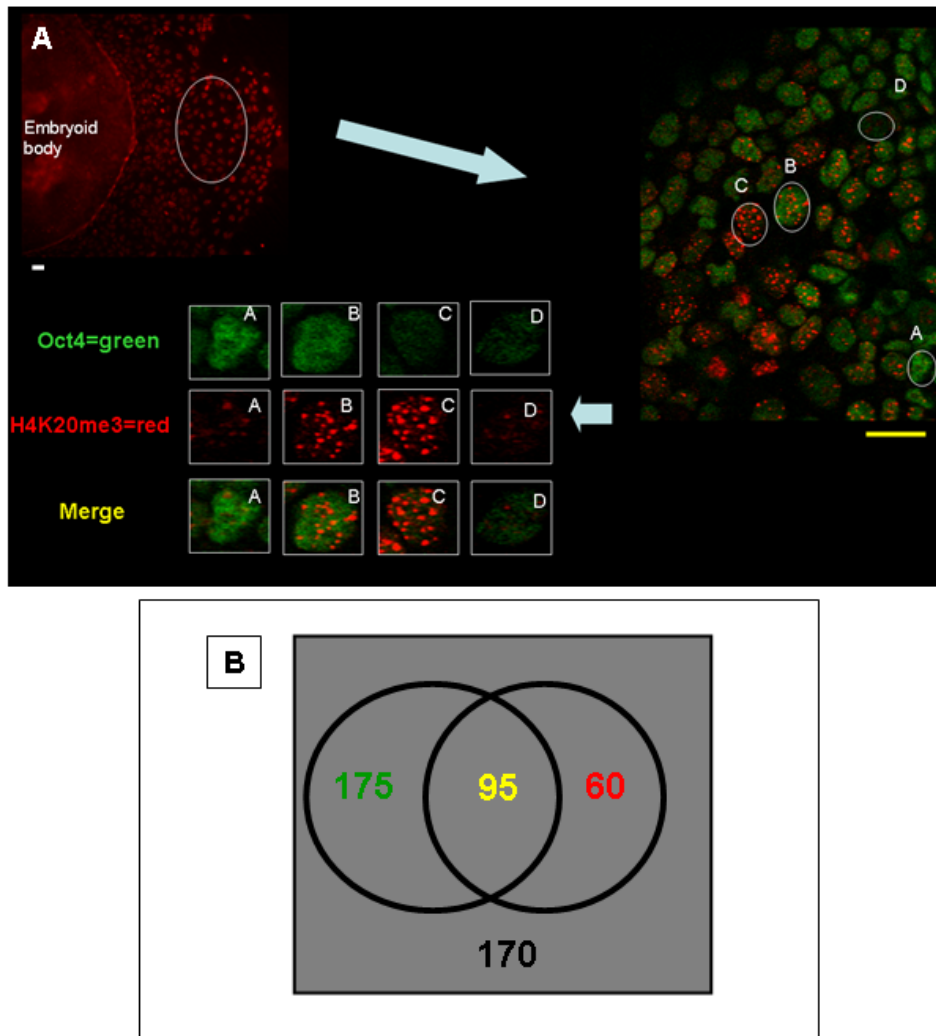


Figure 4.17 Correlation between H4K20me3 and Oct4 of *in vitro* ES cell differentiation (14 day post-differentiation). (A) Cells were immunostained using anti-Oct4 Oct4 (green) and anti-H4K20me3 (red). (B) Cells were counted based on the presence of fluorescence markers, green=Oct4, red=H4K20me3, yellow=Oct4 and H4K20me3, and black=negative for Oct4 and H4K20me3. The scale bar (white) represents 20 μ m.

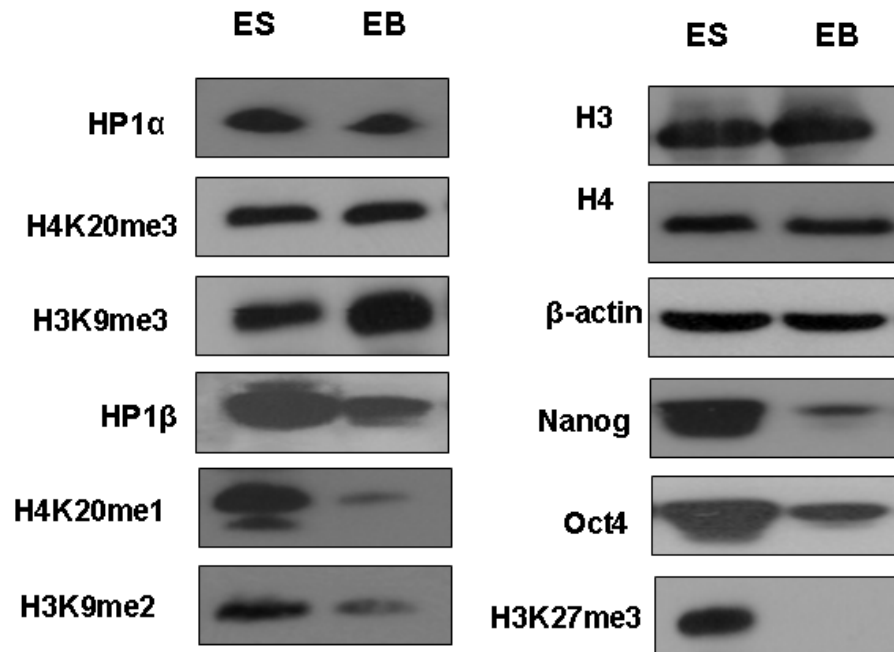


Figure 4.18 Proteomic profile of epigenetic marker during ES cell differentiation (Day 7 post-differentiation). Western blot analysis was performed to detect changes in heterochromatin markers HP1 α , HP1 β , H3K9me3, H4K20me3, H4K20me1, H3K9me2, H3K27me3 in ES cells and embryoid bodies. Anti-H3, anti-H4 and anti- β -actin antibodies were used as loading controls. Pluripotent markers Oct4 and Nanog were used to analyse ES cell and embryoid bodies. After early differentiation, H3K9me3 increases but H4K20me3 and HP1 α are stable. H4K20me1, H3K27me3, and H3K9me3 significantly decrease. Oct4 and Nanog dramatically decrease, confirming loss of pluripotency in differentiated cells. Loading control H3, H4 and β -actin are stable confirming equal loading of protein between lanes.

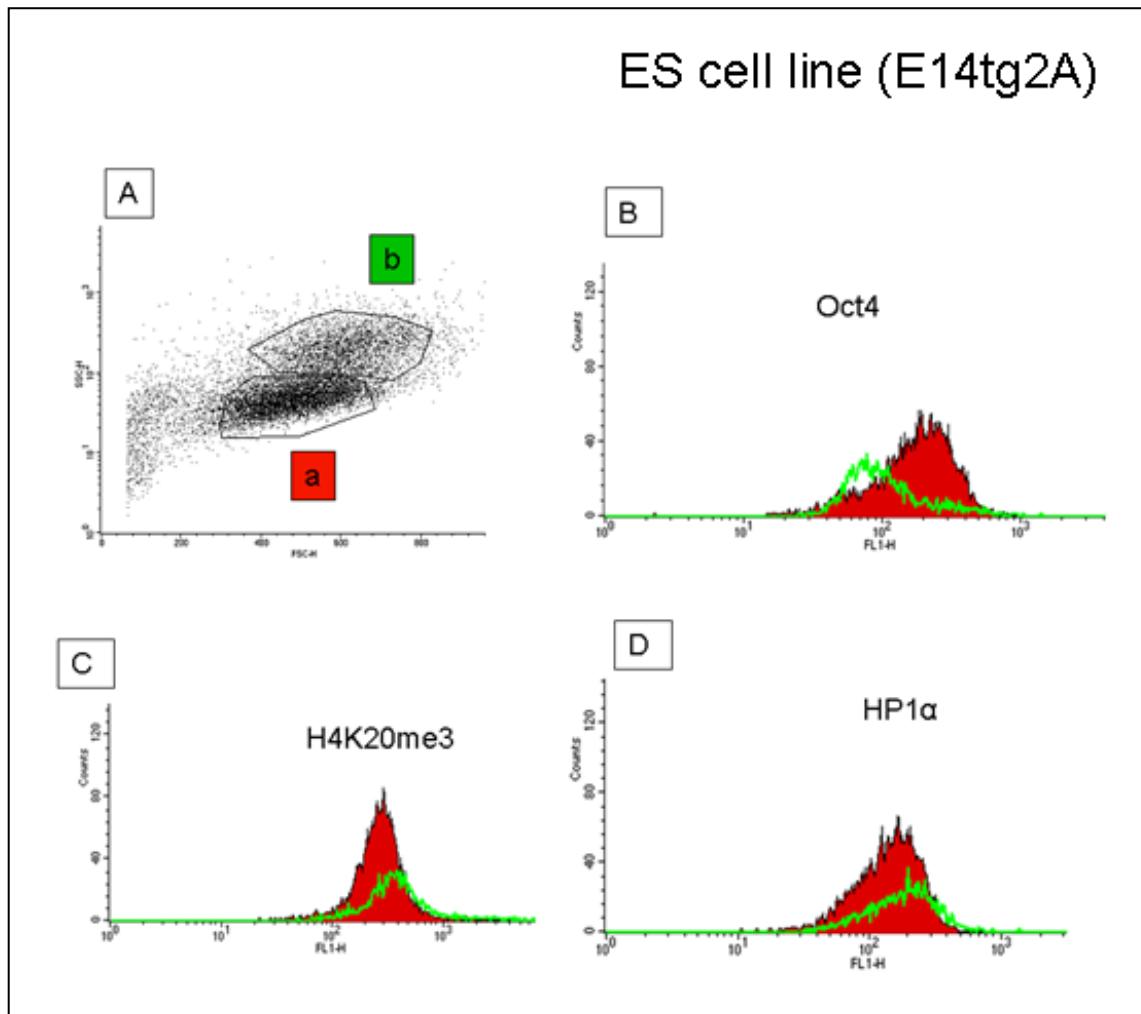


Figure 4.19 Heterogeneous populations of ES cells. Flow cytometry revealed ES cell heterogeneity after immunostaining with Oct4, HP1 α and H4K20me3 antibodies. (A) ES cells were separated into two populations “a” (red) and “b” (green) by granularity (x-axis) and size population (y-axis). Images B-D, x-axis = fluorescent intensity, y-axis=cell number. Two population (a=green and b=red) of ES cells was observed after staining with Oct4 (B), H4K20me3 (C) and HP1 α (D). Population “a” (red) tended to have a higher signal of Oct4 (B) but lower signal of H4K20me3 (C) and HP1 α (D) compare to population “b” (green).

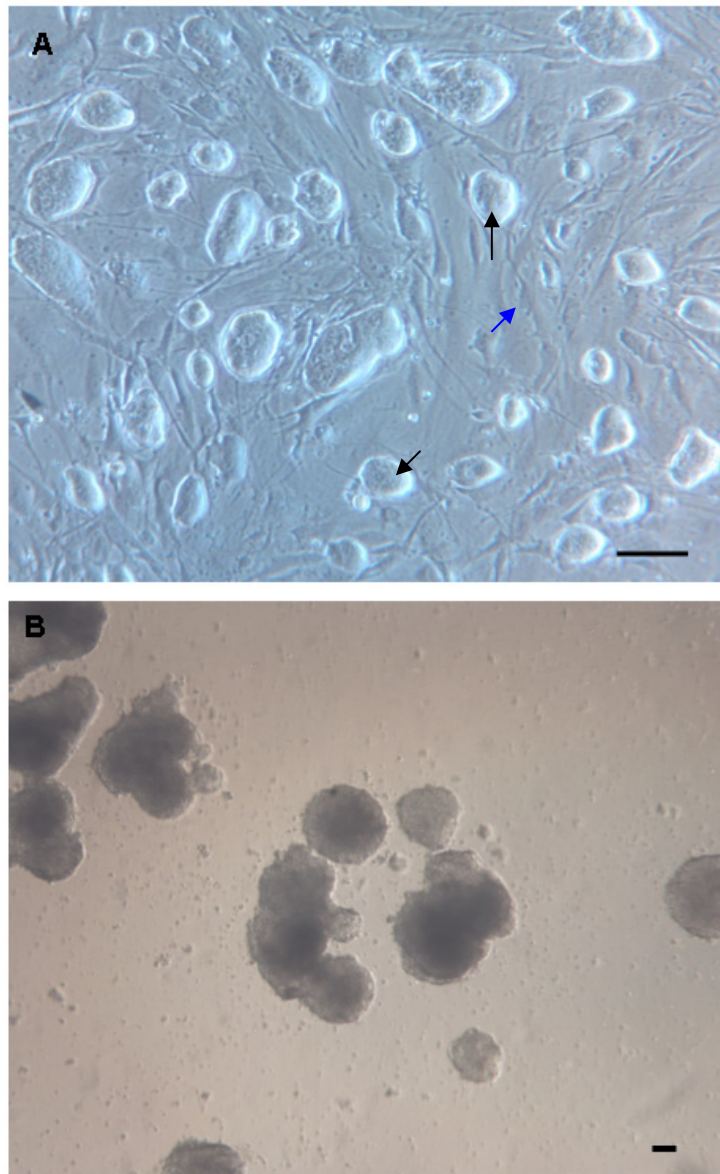


Figure 4.20 ES-like cells (noo01). Image of new ES-like cells (noo01) and their embryoid bodies. (A) The morphology of new ES-like cells were isolated from ICM of blastocyst and cultured with MEF-feeder cell. (B) ES-like cells were able to differentiate into embryoid bodies. Black arrows represent ES cell-like colonies and blue arrow shows a feeder cell (inactivated mouse embryonic fibroblast). The scale bar (black) represents 20 μm .

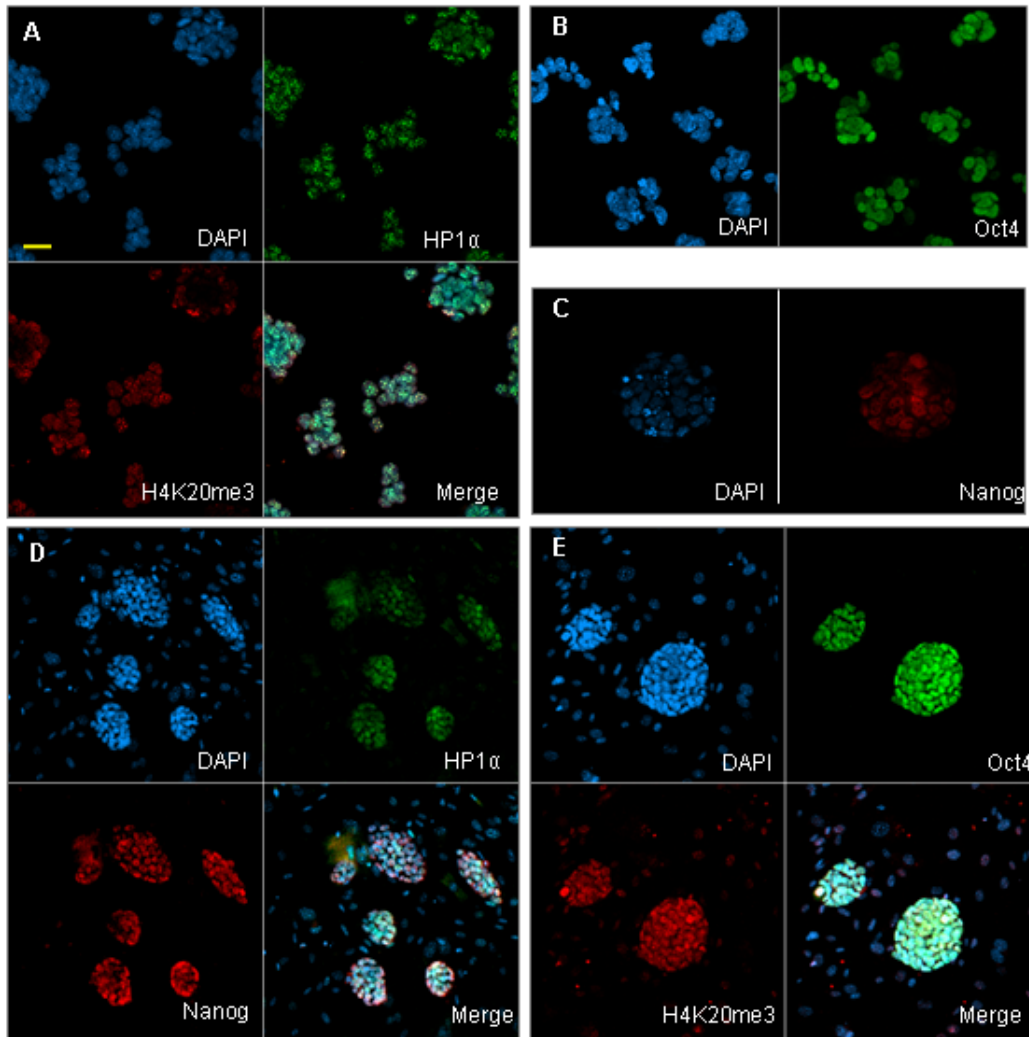


Figure 4.21 Immunofluorescence microscopy images of ES cells. Established ES cells (E14tg2a) are shown in images A to C and ES-like cells (noo-01) are shown in images D to E. All cells were DNA counterstained with DAPI (blue), and pluripotency was confirmed using Oct4 and Nanog antibodies. (A) Cells were positive for H4K20me3 (red) and HP1 α (green). (B-C) Pluripotency of E14tg2a cell line was confirmed using markers Oct4 (green) and Nanog (red). (D) noo-01 cells were positively stained for HP1 α (green) and Nanog (red). (E) ES-like cells were positive for both Oct4 (green) and H4K20me3 (red). Both ES cells and ES-like cells show similar profiles of chromatin and pluripotent markers. The scale bar (yellow) is 20 μ m.

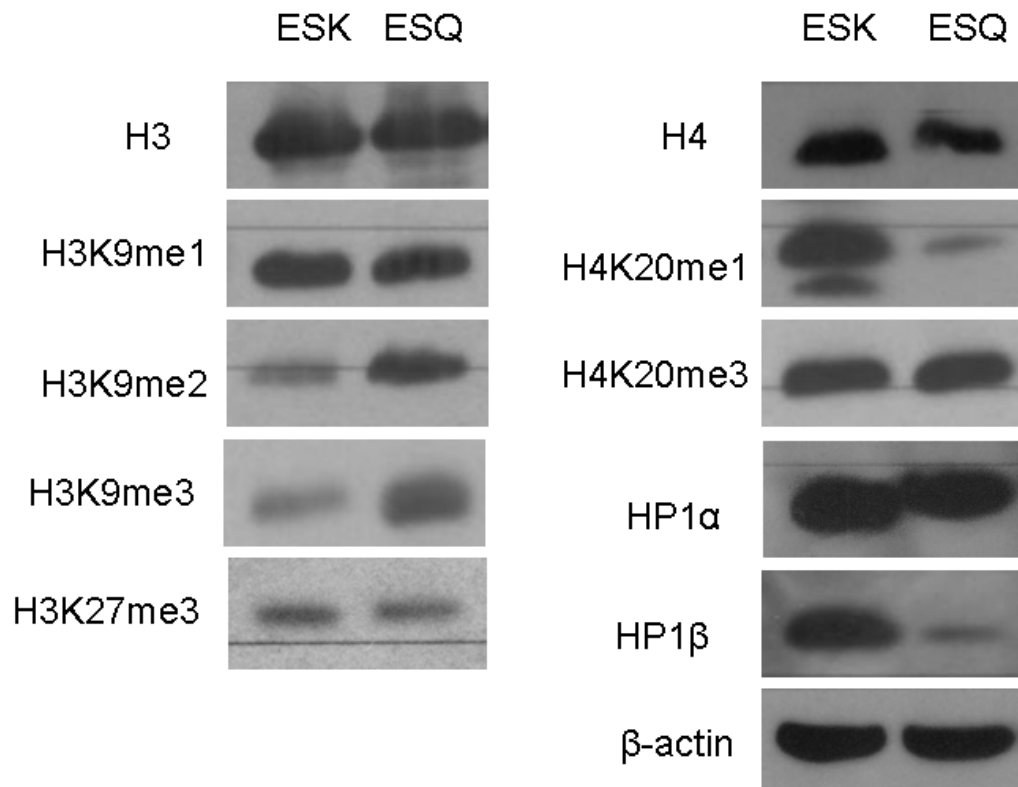


Figure 4.22 Western blot of ES cell cultured in medium contained serum compare with serum free medium. Expression of H4K20me3 and HP1 α were not different in cells cultured in medium containing serum and serum replacement. ESK=ES cell cultured with medium contained **K**nockout serum replacement (serum free). ESQ=ES cell cultured medium contained with ES cell-**Q**ualified foetal bovine serum. H3K9me1.

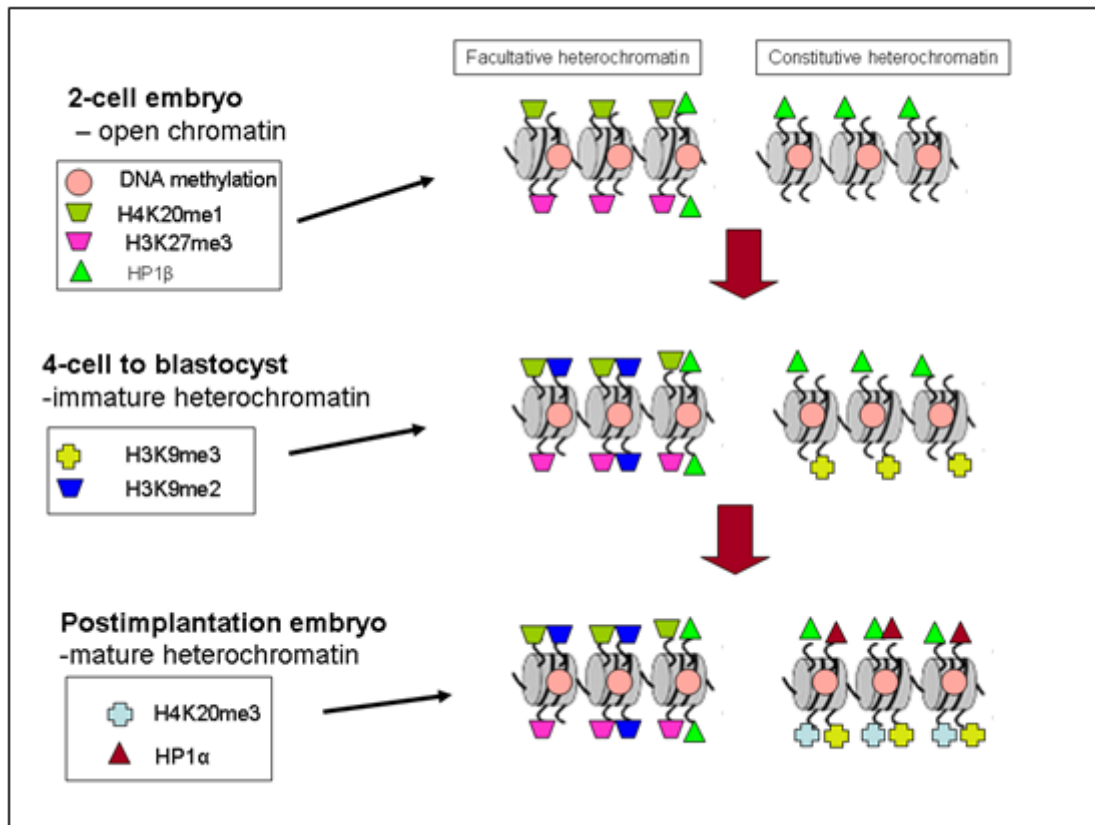


Figure 4.23 Model for immature heterochromatin in early development. At the two-cell stage the only chromatin modifications present in embryos are DNA methylation, H4K20me1, H3K27me3, and HP1 β , which may cause the onset of zygotic gene activation. In four-cell embryos, chromatin is further modified with H3K9me3 and H3K9me2. Heterochromatin matures postimplantation by enrichment of H4K20me3 and HP1 α .

CHAPTER 5

Role of histone demethylases in mouse preimplantation development

Chapter 5

Role of histone demethylases in mouse preimplantation development as epigenetic reprogramming modulators.

5.1 Introduction

Reprogramming of the parental epigenetic marks and chromatin organisation to an embryonic chromatin state is a critical event during preimplantation development (Reik, 2007). At fertilisation in mammals, an asymmetry of parental epigenetic marks is set up; in which the paternal chromatin contains only few histone methylations while the maternal chromatin is packaged with histones that are fully modified (van der Heijden et al., 2005, van der Heijden et al., 2006, Santos et al., 2002, Santos et al., 2005, Adenot et al., 1997, Kim et al., 2003). Subsequently, active DNA demethylation is believed to occur only in the male chromatin of the zygote (Santos et al., 2002, Mayer et al., 2000, Yamazaki et al., 2007) and histone demethylation (H3K9 and H4K20) has been detected later at the two-cell stage (Kourmouli et al., 2004, Liu et al., 2004). The mechanisms of DNA and histone demethylation in the embryo still remain unknown. Epigenetic asymmetry and demethylation are believed to help reset adult chromatin to the embryonic state, which may lead to activation of zygotic gene expression (Minami et al., 2007). These ZGA genes are mainly repeat sequence related genes that are normally silenced by H3K9me3, H4K20me3 and DNA methylation (Martens et al., 2005).

Incomplete epigenetic reprogramming may cause developmental arrest as shown by many studies using nuclear transfer (Mann et al., 2003, Kang et al., 2001, Young et al., 2001). Embryos reconstructed by nuclear transfer mainly fail at the two-cell stage (in mouse) and show abnormalities in the onset of zygotic gene activation (ZGA) (Inoue et al., 2006, Suzuki et al., 2006, Sebastiano et al., 2005). Epigenetic asymmetry of parental genomes is never occurs in cloned embryos (Merico et al., 2007, Yoshida et al., 2007)

which coincides with aberrant DNA methylation patterns (Shi and Haaf, 2002) and the remnants of the somatic form of chromocentre and histone methylation (Martin et al., 2006a, Martin et al., 2006b, Merico et al., 2007). Moreover, incomplete epigenetic reprogramming may be related to developmental failure and abnormal development in embryos derived from assisted reproductive biotechnology such as *in vitro* embryo production and intracytoplasmic sperm injection (ICSI) (De Rycke et al., 2002, Wrenzycki et al., 2005).

5.1.1 Histone demethylases

Histone demethylases have only relatively recently been discovered (Whetstine et al., 2006). There are two classes of enzymes which have been reported with histone lysine demethylase activity. The first class of enzyme is an amine oxidase homologue and LSD1 and LSD2 are the only enzymes which belong to this class (Karytinis et al., 2009). LSD1 and 2 contain a flavin adenine dinucleotide (FAD)-dependent amine oxidase domain (Forneris et al., 2008, Marmorstein and Trievel, 2009, Culhane and Cole, 2007). These enzymes can convert H3K4me2 to H3K4me1, and H3K4me1 to unmethylated H3K4. However, LSD1 is unable to demethylated H3K4me3 (Forneris et al., 2008, Marmorstein and Trievel, 2009, Culhane and Cole, 2007).

The second class contains the proteins which have Jumonji (JmjC)-domains. JMJC-mediated demethylation requires multiple co-factors to act co-ordinately to hydroxylate the methyl group and demethylate mono-, di- or tri-methylation of H3K9, H3K36 and H3K27 (Nottke et al., 2009, Marmorstein and Trievel, 2009, Shin and Janknecht, 2007b). The nomenclature of the demethylase enzymes found in mammals is summarised in Table 5.1.

5.1.2 Objectives of the study

Currently the role of histone demethylases in mouse development is poorly understood, and only two demethylase genes, *Lsd1* and *Jmjd1a* have been studied in gene deletion experiments in mice (Wang et al., 2007, Okada et al., 2007). Some of these enzymes such as the *Jmjd2* protein family may be responsible for histone demethylation postfertilisation, but there is no current evidence to support this. Furthermore, the

research information available for the Jmjd2 family has been limited. Several studies show that Jmjd2 genes have been related to cancer development (Vinatzer et al., 2008, Italiano et al., 2006), but only one recent publication has described Jmjd2c in mouse preimplantation development (Wang et al., 2009). The specific aim of the this current study was to investigate the function of histone demethylase enzymes Jmjd1a, Jmjd2a and Jmjd2c in mouse preimplantation embryos using siRNA-mediated knock-down methodology. I hypothesise that Jmjd1a, Jmjd2a and Jmjd2c are essential for normal preimplantation development.

Table 5.1 Summary of histone lysine (K) demethylase (KDM) nomenclature and substrate specificity for mice and human.

New standardised family name	Family member (mammalian)	Alternative (previous) name	Substrate	References
KDM1	KDM1	LSD1, AOF2, BHC1110	H3K4me1/2	(Shi et al., 2004)
KDM2	KDM2A	JHDM1A, FBXL11	H3K36me1/2/	(Yamane et al., 2006, He et al., 2008)
	KDM2B	JHDM1B, FBXL10		
KDM3	KDM3A	JHDM2A, JMJD1A, TSGA	H3K9me1/2	(Chen et al., 2006)
	KDM3B	JHDM2B, JMJD1B		
KDM4	KDM4A	JMJD2A, JHDM3A,	H3K9me2/3, H3K36me2/3	(Klose et al., 2006, Cloos et al., 2006)
	KDM4B	JMJD2B		
	KDM4C	JMJD2C, GASC1		
	KDM4D	JMJD2D		
KDM5	KDM5A	RBP2, JARID1A,	H3K4me2/3	(Klose et al., 2007, Scibetta et al., 2007, Kim et al., 2008, Akimoto et al., 2008)
	KDM5B	PLU-1, JARID1B		
	KDM5C	SMCX, JARID1C,		
	KDM5D	SMCY, LARID1D		
KDM6	KDM6A	UTX	H3K27me2/3	(Agger et al., 2007)
	KDM6B	JMJ3		

5.2 Materials and Methods

Methods describing embryo collection, immunofluorescence and siRNA-mediated knock-down are described in Chapter 2 Materials and Methods. siRNA injections were repeated at least three times using more than 20 embryos per one injection time. Commercially validated Oct4-siRNA (Ambion) was used as a positive control, this siRNA has also been confirmed by Professor Wilmut's lab to reduce Oct4 expression in embryos.

5.2.1 Relative gene expression

Quantitative analysis of relative gene expression and statistical differences were calculated using REST 2008 software (see Equation 1) (Pfaffl et al., 2002). Ct value and reaction efficiency were obtained from Rotor-gene 6000 software using comparative analysis. The REST software uses the Pair Wise Fixed Allocation Randomisation Test to calculate statistical value (Pfaffl et al., 2002, Horgan and Rouault, 2000). For comparison between control and treatment embryos (gene knock-down), multiple reference genes were used for normalisation between samples. Normalisation of gene expression was calculated using the geometric mean of multiple reference genes, (see Equation 2) (Vandesompele et al., 2002, Pfaffl et al., 2002).

$$R = \frac{(E_{\text{target}})^{Ct_{\text{control}} - Ct_{\text{sample}}}}{(E_{\text{ref}})^{Ct_{\text{control}} - Ct_{\text{sample}}}} \quad \text{(Equation 1)}$$

R = gene expression ratio, E_{target} = real-time PCR efficiency of target gene transcript; E_{ref} = real-time PCR efficiency of reference gene transcript and Ct = cycle number

5.2.2 Isolation of inner cell mass (ICM) and trophectoderm (TE)

Trophectoderm (TE) cells were mechanically dissected with a mini-surgery blade (Swann Morton) while ICM was isolated using immunosurgery. The immunosurgery technique was slightly modified from the original report (Solter and Knowles, 1975). The zona pellucida was removed by incubation of blastocysts into acid tyrodes (Sigma). Zona free blastocysts were then incubated with rabbit anti-mouse antibody (Sigma,

1:100) for 30 minutes in 37 °C incubator and then transferred to a drop of guinea pig complement to remove TE for 30 minutes in 37 °C incubator.

5.3 Results

5.3.1 Validation of siRNA

siRNAs used in this experiment were predesigned commercially. , Before embryo microinjection experiments were carried out the specificity of siRNA was tested in ES cells by transfection. The target gene expression was measured after 24 hours culture. The results showed that Jmjd2a, Jmjd2c and Jmjd1a siRNAs were specific and caused a significant reduction in targeted gene expression (Figure 5.1).

5.3.1 Jmjd2c regulates cell fate during the morula to blastocyst transition

Jmjd2c is one of the most interesting demethylases and has been shown to be involved in carcinogenesis (Vinatzer et al., 2008), in the regulation of androgen receptor-dependent gene expression (Wissmann et al., 2007) and in maintenance of pluripotency in stem cells (Loh et al., 2007). In ES cells, Jmjd2c prevents ES cell differentiation by inhibiting histone methylation (H3K9me3) at the Nanog promoter (Loh et al., 2007). In the present studies (see Chapter 3) Jmjd2c was found in maternal oocytes and its expression rapidly decreases after fertilisation. Zygotic gene expression of Jmjd2c was clearly evident at the four-cell stage (Figure 3.16 Chapter 3). Jmjd2c was preferentially expressed in ICM rather than TE cells (Table 5.3).

The effect of depleting Jmjd2c after injection of Jmjd2c-siRNA is shown in Table 5.2. The results revealed that approximately 24 % (24/100) of embryos arrested at the zygote stage, whilst most embryos arrested at morula (65 %; 65/100). The transition from morula to blastocyst was only 51 % (35/68), which is significantly lower than for control embryos (95% in non-injected group and 98% in the injected negative control siRNA embryos). The arrested embryos underwent degeneration during the next 24 hours (Figure 5.2B and C) and the remaining embryos which progressed to blastocyst

stage seemed to be morphologically normal. Unlike the ES cell study by Loh et al (2007) which reported a decrease in Nanog expression after interruption of *Jmjd2c* gene, my QPCR results show that expression of Sox2 is reduced in morula while Oct4 and Nanog did not significantly change. However, expression of *Cdx2* (a marker for TE) decreased.

5.3.2 *Jmjd2a* is essential for very early development

Apart from its demethylase function, *Jmjd2a* is known to activate the androgen receptor in prostate cancer (Shin and Janknecht, 2007a). The role of *Jmjd2a* in development has never been described. My studies reveal that *Jmjd2a* has two waves of gene expression; first at zygote to two-cell stage and later at eight-cell to blastocyst (Figure 3.16 chapter 3). At blastocyst stage the expression of *Jmjd2a* in ICM was highly significant relative to the TE (see Table 5.3).

After *Jmjd2a*-siRNA injection (Table 5.2), 79 % (79/100) of zygotes developed to two-cell embryo but most of these (65 %; 37/57) failed to reach the four-cell embryos stage (Figure 5.2A). The transition rate from four-cell to eight-cell was 50 % (10/20) compare to 100 % in control embryos (non-injection and negative control siRNA injection). Both control and siRNA-injected eight-cell embryos reached the morula, but only 40 % (4/10) of remaining siRNA-injected embryos developed to blastocyst. Overall, the developmental rate from zygote to blastocyst was very low at only 6 % (4/72) in *Jmjd2a*-depleted embryos compared to the control embryos in which 85-90 % developed to blastocyst.

5.3.3 *Jmjd1a* is essential for early development and specific for the trophectoderm

Recent studies indicate that *Jmjd1a* has an important role in nuclear hormone receptor-mediated gene activation (Urbanucci et al., 2008), male germ cell development (Okada et al., 2007) and regulating metabolic gene expression and obesity resistance (Tateishi et al., 2009). Moreover, *Jmjd1a* demethylates H3K9Me2 at the promoter regions of *Tcl1*, *Tcfcp2l1*, and *Zfp57* and positively regulates the expression of these pluripotency-associated genes (Loh et al., 2007)

My studies (Figure 3.17 in Chapter 3) revealed that *Jmjd1a* was not found in mature oocytes but gene expression occurs after fertilisation. However the expression level was significantly decreased from morula to blastocyst. Furthermore, the expression of *Jmjd1a* was specific to the TE as it was not found in the ICM (see Table 5.3). After *Jmjd1a*-siRNA injection (Table 5.2, Figure 5.3A) most embryos (67 %; 49/73) arrested at the two-cell stage. Embryo development rate from two-cell to four-cell was 53 % (26/49), four-cell to eight-cell was 30 % (8/26), eight-cell to morula was 87 % (7/8) and morula to blastocyst was 14 % (1/7). Overall the developmental rate from zygote to blastocyst was only 1 % (1/73).

5.3.4 Preliminary studies of histone methylation in *Jmjd1a* and *Jmjd2a* deficient embryos

In the present study, asymmetry of H3K9me2 and H3K9me3 was found in normal zygotes and at the two-cell stage (5 embryos for each marker) (see Chapter 3) which is similar to previously published studies (Santos et al., 2005, van der Heijden et al., 2005). I hypothesised that *Jmjd1a* (H3K9me2 demethylase) or *Jmjd2a* (H3K9me3 demethylase) may be involved in this process. *Jmjd1a* depleted embryos were stained H3K9me2 and *Jmjd2a* depleted embryos were stained for H3K9me3 and compare to normal embryos. Surprisingly, this pronuclear asymmetry of parental chromatin disappeared in either *Jmjd1a* or *Jmjd2a* deficient zygotes or two-cell embryos (Figure 5.3-5.6). Consistent with this observation, semi-quantitative measurement showed that the fluorescence intensity between in female and male PN of zygotes seemed to be equal in *Jmjd1a*/*Kdm3a* as well as *Jmjd2a* embryos. The intensity ratio (female/male) for H3K9me3 in the control zygote was 3.80 and higher than in *Jmjd2a* deficient zygotes (ratio=0.98). Similarly, the fluorescence intensity ratio in control embryos (2.50) was more than two times higher than in *Jmjd1a* depleted embryos (1.09).

5.3.5 Zygotic gene expression in *Jmjd1a* and *Jmjd2a* deficient embryos

The majority of *Jmjd2a* and *Jmjd1a* deficient embryos failed to develop after the two-cell stage and four-cell stage. I hypothesised that removal of the repressive histone mark by *Jmjd2* and *Jmjd1* may help to activate some ZGA genes that are repressed by H3K9 methylation, and interruption of demethylase may cause down regulation of ZGA. In

this experiment, the expression of three ZGA genes (Zcan4d, Elf1a, MuERV) were measured using QPCR which was normalised relative to three reference genes (Gapdh, Actin, Rpl1a). Relative gene expression levels are shown in Table 5.7 (Jmjd1a deficient embryos) and Table 5.9 (Jmjd2a deficient embryos). The raw data expression values for each gene before normalisation are shown in Table 5.6 and 5.8. The results showed that both Jmjd2a and Jmjd1a expression were reduced after each siRNA injection; Jmjd1a expression was reduced by 50% and Jmjd2a was undetectable. Among the three ZGA genes in the present studies, mRNA level of MuERV was decreased more than Elf and Zcan4d (Table 5.7 and 5.9). Expression of MuERV was reduced 47% in Jmjd2a deficient embryos and 12% in Jmjd1a deficient embryo compared to control.

MuERV is a transposon related gene (Evsikov et al., 2004) which is normally silenced by H3K9me3, H4K20me3 or DNA methylation in somatic cells (Martens et al., 2005), but which expresses in zygotes and two-cell embryos (Kigami et al., 2003). Histone demethylase may help activate gene expression by removing or preventing methylation at gene promoter, and interruption of histone demethylase may lead to reduction of ZGA gene expression and subsequently, failure in preimplantation development as found in the present study.

5.4 Discussion

One important step for creating new life is the reprogramming of the parental epigenetic signatures and chromatin organisation by the maternal oocyte during preimplantation development. This process includes an asymmetry of parental epigenetic marks at the zygote and two-cell stage, the active DNA demethylation in the zygote and histone demethylation (H3K9 and H4K20) at the two-cell stage by unknown mechanisms. In the present study I discovered that the mechanism responsible for histone methylation asymmetry in the zygote and histone demethylation at the two-cell stage may involve histone demethylases Jmjd2a and Jmjd1a. Moreover, cell fate decisions during blastocyst formation could be regulated by demethylases Jmjd2c, Jmjd2a and Jmjd1a.

These results provide new insights into this area of early developmental biology and might be useful in improving success rates in assisted reproductive technology.

5.4.1 Jmjd2 might control epigenetic asymmetry of parental genome

Previous reports (van der Heijden et al., 2005, Santos et al., 2005) and my data show that H3K9me3 is strongly detected after fertilisation in female chromatin, whereas the signal from paternal chromatin is very weak or undetectable. This asymmetry between parental genomes has been suggested to be important for inducing ZGA from the paternal chromatin (Minami et al., 2007). The mechanism of epigenetic asymmetry may be simply explained by the lack of male chromatin modifications due to removal of histone proteins during spermatogenesis, while in the female all histone methylation remains during oogenesis (van der Heijden et al., 2005, van der Heijden et al., 2006). In contrast, my preliminary data shows that interruption of histone demethylase Jmjd2a could induce histone methylation in paternal chromatin to be equivalent to female chromatin. The majority of Jmjd1a and Jmjd2a-deficient embryos arrested at the zygote and two cell stage (Table 5.2). I propose that Jmjd2a may specifically prevent histone methylation at male chromatin, but Jmjd2a is possibly prevented access to female chromatin due to this chromatin already being occupied by heterochromatin markers H3K9me3 and H4K20me3.

5.4.2 Histone demethylases and zygotic gene activation

It is well established that ZGA genes mainly begin to be expressed in the two-cell embryo (Minami et al., 2007, Schultz, 2002). These genes mostly contain repetitive sequences (Evsikov et al., 2004), and gene activity is repressed by histone and/or DNA methylation in somatic cells (Martens et al., 2005). Notably, for three genes in my study, ZGA genes expression occurred simultaneously with global nuclear histone demethylation (H3K9me3, H3K9me2 and H4K20me3). The co-incidence between histone demethylation and ZGA was supported by the finding that Jmjd2a and Jmjd1a knock-down suggested a possible involvement in ZGA, and one of ZGA genes MuERV was affected after depletion of Jmjd2a. Hence, additional studies need to be performed to investigate whether histone demethylases Jmjd2a and Jmjd1a can affect other ZGA gene expression.

5.4.3 Histone demethylase is important for blastocyst formation

The knock-down embryo experiments in this study demonstrated that three demethylases *Jmjd2c*, *Jmjd2a* and *Jmjd1a* are critical for blastocyst formation. Depletion of these individual genes caused significant failure of blastocyst formation. Both *Jmjd2c* and *Jmjd2a* were highly expressed in ICM while *Jmjd1a* was found in TE. These results may suggest that depletion of *Jmjd2c* or *Jmjd2a* genes may allow the development of embryos that cannot form an ICM proper, whereas *Jmjd1a* may cause TE formation. Both the present study and another recent report (Wang et al., 2009) showed that *Jmjd2c* depleted embryos fail to form blastocysts. The present studies also suggested that *Jmjd2c* may regulate the pluripotency gene *Sox2* but not *Oct4* and *Nanog*; however, this observation was different from recent study (Wang et al., 2009) which believed that *Jmjd2c* may control *Oct4*, *Nanog* and *Sox2*.

5.4.4 Redundancy or functional difference of *Jmjd1* and *Jmjd2* family

All *Jmjd2* and *Jmjd1* proteins have the ability to remove H3K9me3 and H3K9me2, but their non-demethylase function is still poorly known. The present studies showed that a temporal expression of *Jmjd1a*, *Jmjd2a* and *Jmjd2c* was found during early preimplantation development (Figure 3.17 and 3.18 in Chapter 3), which were comparable to published microarray data (Hamatani et al., 2004, Zeng et al., 2004). Using published microarray data (<http://www.ncbi.nlm.nih.gov/geo/>) (Hamatani et al., 2004, Zeng et al., 2004) and results from *in silico* expression analysis (Kato and Kato, 2007) of mouse preimplantation embryos together with the result of the present studies, I was able to draw a hypothesis of temporal expression of *Jmjd2* and *Jmjd1* family genes (Table 5.11). The temporal expressions of *Jmjd2* genes suggest there may be a redundancy of these genes.

In conclusion, histone demethylases *Jmjd2a*, *Jmjd2c* and *Jmjd1a* siRNA knock-down experiments showed that they were essential for preimplantation development. Specifically, *Jmjd2c* was vital for lineage specific (ICM and TE) and was required to form a blastocyst proper. *Jmjd1a* and *Jmjd2a* were very important for the transition from oocyte to embryo especially at two-cell and four-cell embryos. The developmental

failure of demethylase-deficient embryos might result from the loss of asymmetry in parental chromatin of the zygote and aberrant onset of zygotic gene expression.

Table 5.2 Effect of siRNA knock-down of specific genes on preimplantation development

Stage	Non-injected N(%A,%B)	Negative Control siRNA N (%A,%B)	Jmjd2c N (%A,%B)	Jmjd2a N (%A,%B)	Jmjd1a N (%A,%B)
Zygote	66 (100, 100)	67 (100, 100)	100 (100, 100)	72 (100,100)	73 (100, 100)
2-cell	61 (92, 92)	60 (90, 90)	76 (76, 76)	57 (79,79)	49 (67, 67)
4-cell	61 (92, 100)	60 (90, 100)	72 (72, 95)	20 (28,35)	26 (36, 53)
8-cell	61 (92, 100)	60 (90, 100)	71 (71, 99)	10 (14, 50)	8 (11, 30)
Morula	61 (92, 100)	60 (90, 100)	68 (68, 96)	10 (14,100)	7 (10, 87)
Blastocyst	60 (91, 98)	57 (85, 95)	35 (35, 51)	4 (6, 40)	1 (1, 14)

%A= number of embryo each stage/number of zygote, %B=number of embryo each stage/number of embryo in previous stage (ex, 4-cell/2-cell), N=number of embryo. Grey highlight shows the stage that represents the majority of embryos

Table 5.3 Relative gene expression of ICM compared to TE

Gene	Type	RE	Expression ratio (ICM/TE)	SE.
<i>Jmjd2a</i>	TRG	0.76	16.82	11.90 - 23.78
<i>Jmjd2c</i>	TRG	0.81	25.67	20.18 - 34.76
<i>Jmjd1a</i>	TRG	0.8125	0.00*	#

Table 5.4 Non-relative gene expression (no normalisation with reference genes) of ICM compared to TE

Gene	Type	RE	Expression ratio (ICM/TE)	SE
<i>Jmjd2a</i>	TRG	0.76	1.15	1.05 - 1.25
<i>Jmjd2c</i>	TRG	0.81	1.75	1.34 - 2.29
<i>Jmjd1a</i>	TRG	0.8125	0.00*	#
<i>Rpl13a</i>	REF	0.8	0.21	0.19 - 0.23
<i>Actin</i>	REF	0.78	0.02	0.01 - 0.03

ICM=inner cell mass, TE=Trophectoder., TRG=Target gene, REF=Reference gene, CI=Confidential interval, SE=standard error, RE=reaction efficiency, * Gene expression is undetectable in the treatment, # Value can not be calculated. Expression ratio>1 = expression of ICM is higher than TE. Expression ratio<1= expression of ICM is lower than TE.

Table 5.5 Relative gene expression of *Jmjd2c* depletion embryos compared to control embryos.

Gene	Type	RE	Expression Ratio (Treatment/control)	SE
<i>Jmjd2c</i>	TRG	0.76	0.00*	#
<i>Oct4</i>	TRG	0.69	0.99	0.67 - 1.37
<i>Nanog</i>	TRG	0.77	1.54	1.15 - 2.07
<i>Sox2</i>	TRG	0.64	<0.01	0.00 - 0.00
<i>Cdx2</i>	TRG	0.62	0.546	0.38 - 0.79

Table 5.6 Non-relative gene expression (no normalisation with reference genes) of *Jmjd2c* depletion embryos compared to normal embryos.

Gene	Type	RE	Expression Ratio (Treatment/control)	SE
<i>Jmjd2c</i>	TRG	0.76	0.00*	#
<i>Oct4</i>	TRG	0.69	0.54	0.36 - 0.85
<i>Nanog</i>	TRG	0.77	0.84	0.66 - 1.13
<i>Sox2</i>	TRG	0.64	<0.01	0.00 - 0.00
<i>Cdx2</i>	TRG	0.62	0.29	0.22 - 0.38
<i>Hprt1</i>	REF	0.68	1.51	1.08 - 1.84
<i>Gapdh</i>	REF	0.62	0.19	0.13 - 0.29

TRG=Target, gene, REF=Reference gene, CI=Confidential interval, SE=standard error, RE=reaction efficiency. * Gene expression is undetectable in the treatment, # Value can not be calculated. Expression ratio >1= increase in gene expression in treatment compare to control. Expression ratio <1= decrease in gene expression in treatment compare to control.

Table 5.7 Relative gene expression in *Jmjd1a* deficient two-cell embryos compared with control

Gene	Type	RE	Gene Expression Ratio (Treatment/control)	SE
<i>Jmjd1a</i>	TRG	0.89	0.52	0.18-1.36
<i>Elf1a</i>	TRG	0.89	0.89	0.56-4.83
<i>Zcan4d</i>	TRG	0.93	0.93	0.09-2.50
<i>MuERV</i>	TRG	0.89	0.88	0.39-1.04

Table 5.8 Non-relative gene expression in *Jmjd1a* deficient two-cell embryos compared with control

Gene	Type	RE	Gene Expression Ratio (Treatment/control)	SE
<i>Jmjd1a</i>	TRG	0.89	0.48	0.30 - 0.96
<i>Elf1a</i>	TRG	0.88	1.18	0.54 - 2.39
<i>Zcan4d</i>	TRG	0.93	0.46	0.18 - 0.90
<i>MuERV</i>	TRG	0.88	0.63	0.28 - 1.50
<i>Gapdh</i>	REF	0.74	0.74	0.31 - 1.28
<i>Actin</i>	REF	0.75	0.89	0.32 - 1.94
<i>Rpl1a</i>	REF	0.82	0.74	0.60 - 2.77

TRG=Target, gene, REF=Reference gene, CI=Confidential interval, SE=standard error, RE=reaction efficiency,* Gene expression is undetectable in the treatment, # Value can not be calculated. Expression ratio >1= increase in gene expression in treatment compare to control. Expression ratio <1= decrease in gene expression in treatment compare to control.

Table 5.9 Relative gene expression in *Jmjd2a* deficient two-cell embryos compared with control

Gene	Type	RE	Expression Ratio (Treatment/control)	SE
<i>Jmjd2a</i>	TRG	0.86	0.00*	#
<i>Elf1a</i>	TRG	0.90	1.32	0.776 - 3.098
<i>Zcan4d</i>	TRG	0.91	0.78	0.263 - 1.416
<i>MuERV</i>	TRG	0.86	0.53	0.330 - 0.764

Table 5.10 Non-relative gene expression in *Jmjd2a* deficient two-cell embryos compare with control

Gene	Type	RE	Expression Ratio (Treatment/control)	SE
<i>Jmjd2a</i>	TRG	0.86	0.00*	#
<i>Elf1a</i>	TRG	0.90	0.71	0.354 - 1.232
<i>Zcan4d</i>	TRG	0.91	0.42	0.214 - 0.954
<i>MuERV</i>	TRG	0.86	0.28	0.123 - 0.772
<i>Gapdh</i>	REF	0.76	0.43	0.215 - 0.805
<i>Actin</i>	REF	0.77	0.58	0.287 - 1.043
<i>Rpl1a</i>	REF	0.81	0.62	0.430 - 0.788

TRG=Target, gene, REF=Reference gene, CI=Confidential interval, SE=standard error, RE=reaction efficiency,* Gene expression is undetectable in the treatment, # Value can not be calculated. Expression ratio >1= increase in gene expression in treatment compare to control. Expression ratio <1= decrease in gene expression in treatment compare to control.

Table 5.11 Estimated gene expression profile in early implantation embryo from microarray data and qPCR of the present study.

Gene	Oocyte	Zygote	2-cell	4-cell	8-cell
<i>Jmjd2a</i>	+	+++	++	-	+
<i>Jmjd2b</i>	++	++	++	-	+
<i>Jmjd2c</i>	+++	+	+	+++	+++
<i>Jmjd2d</i>	++	-	-	-	-
<i>Jmjd1a</i>	-	-	++	++	+++
<i>Jmj1b</i>	+	+	+	+++	+++
<i>Jmj1c</i>	+	+	++	++	+++

-=absent, +=low expression, ++=moderate expression, +++=High expression

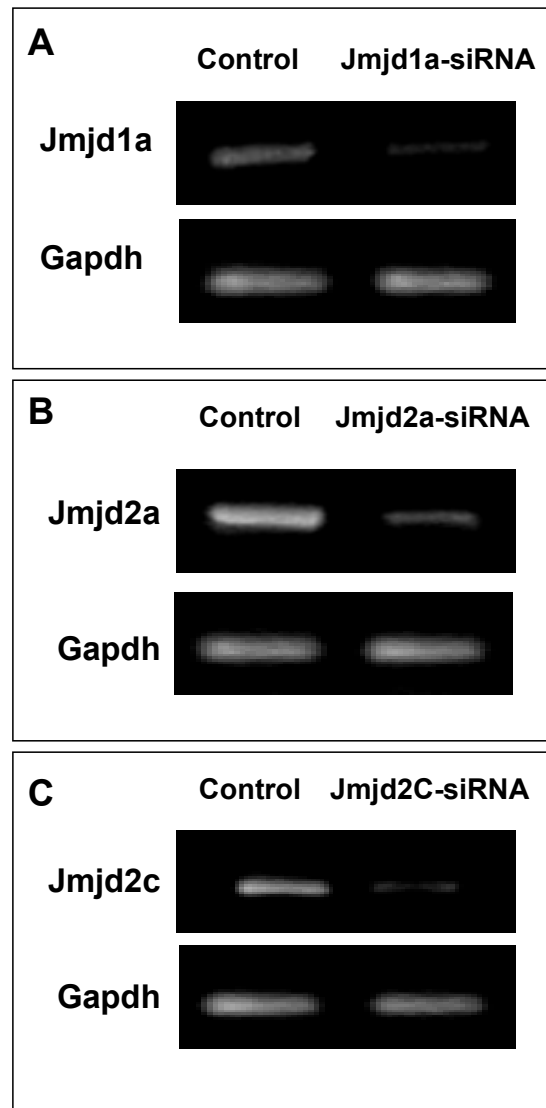


Figure 5.1 Validation siRNA by transfection into ES cells. ES cells were transfected with siRNA to examine gene expression of target gene Jmjd1a (A), Jmjd2a (B) and Jmjd2c (C). Control is ES cells that were incubated in transfection reagent without siRNA. 50 ng of cDNA was used for PCR. PCR Cycle number for Gapdh and Jmjd2c PCR were 28, Jmjd1a was 30 and 35 for Jmjd2a.

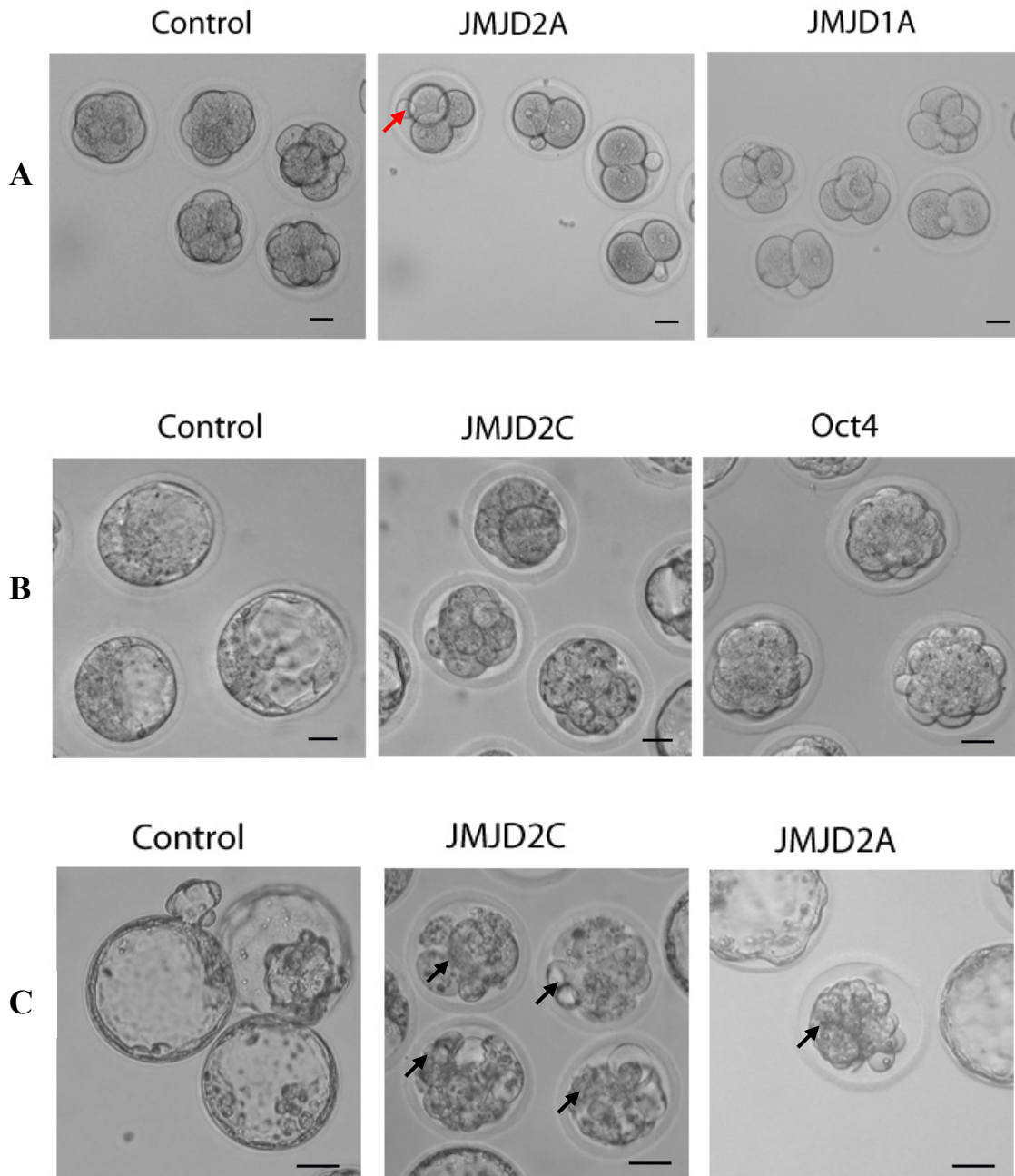


Figure 5.2 *In vitro* development of siRNA-injected embryos. Brightfield microscopy images showing (A) After 3 days culture, *Jmjd2a* (middle) or *Jmjd1a* (right) depleted embryos developed more slowly than control embryo (RNAi negative), with some embryos arrested at three cell stage (red arrow). (B) After 4 days culture, control embryos reached the blastocyst stage whereas *Oct4*-injected embryos (Right) used as a positive control did not develop to blastocyst, similar to *Jmjd2c*-depleted embryo. (C) After 4 days culture, control embryos reached the expanded blastocyst stage while *Jmjd2c*- or *Jmjd2a*-depleted embryos degenerated (black arrow). Scale bar=20 μm.

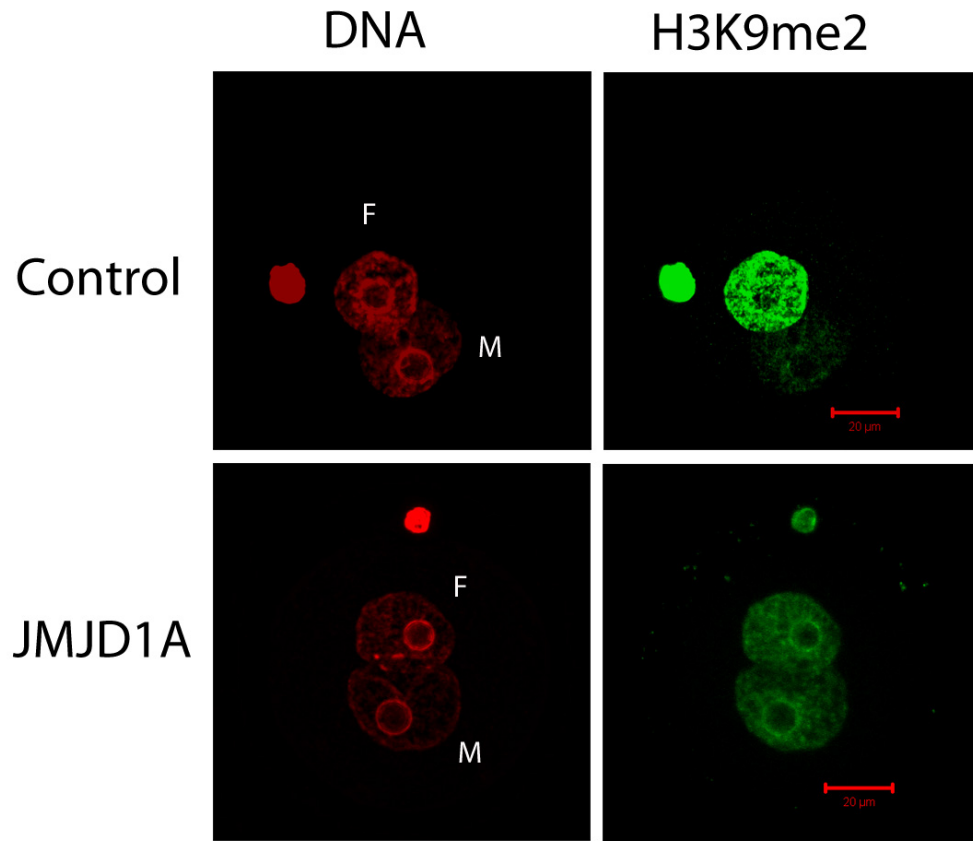


Figure 5.3 Loss of H3K9me2 asymmetry in *Jmjd1a*-deficient zygotes. Fluorescence microscopy images of zygotes stained with H3K9me2 antibodies (detected with fluorescent secondary antibody, green) and DNA was counterstained with DAPI (pseudo-coloured red). Control zygotes (non-injected) showed H3K9me2 pronuclear asymmetry of parental chromatin, in which H3K9me2 was highly enriched in the female pronucleus. By contrast, *Jmjd1a*-siRNA injected embryos lost this asymmetry and male and female pronuclear chromatin appeared to be equally stained. Female pronucleus (F) is smaller and closer to polar body than male pronucleus (M). The scale bar (red) is 20 μm .

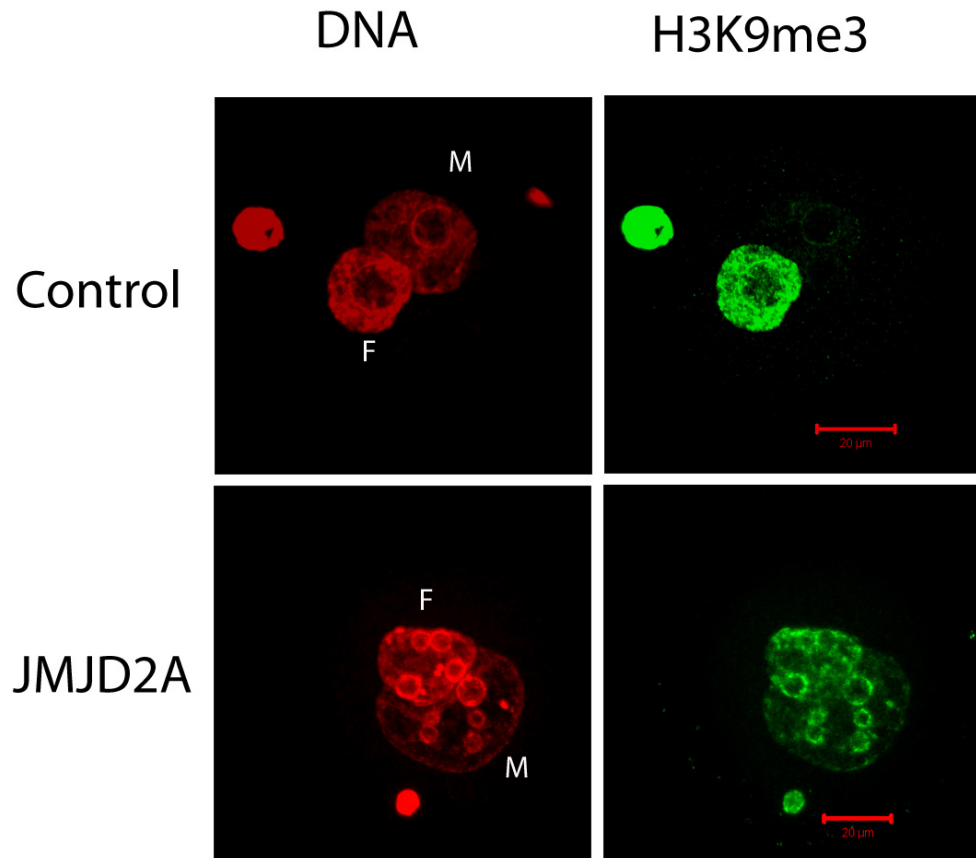


Figure 5.4 Loss of H3K9me3 asymmetry in *jmj2a*-deficient zygotes. Zygotes were immunostained for H3K9me3 (green) and DNA was counterstained with DAPI (pseudo-coloured red). Control zygotes (non-injected) showed H3K9me3 pronuclear asymmetry of parental chromatin, in which H3K9me3 was highly enriched in the female pronucleus. By contrast, *Jmjd2a*-siRNA injected zygotes lost this asymmetry and male and female pronuclear chromatins appear to be equally stained. Female pronucleus (F) is smaller and closer to polar body than male pronucleus (M). The scale bar (red) is 20 μ m.

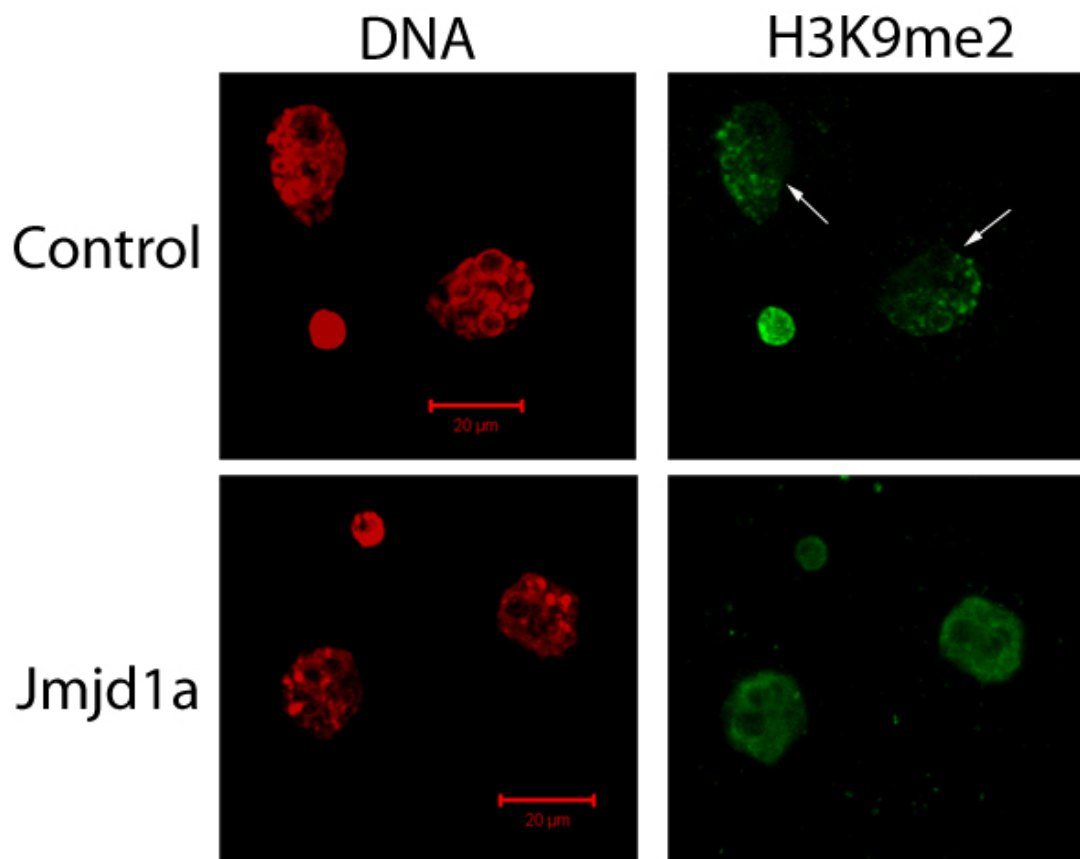


Figure 5.5 Loss of H3K9me2 asymmetry in *jmjd1a*-deficient two-cell embryos. Embryos were immunostained for H3K9me2 (green) and DNA was counterstained with DAPI (pseudo-coloured red). Control embryos (non-injected) showed H3K9me2 asymmetry of parental chromatin, in which H3K9me2 appeared to stain half of the nuclei (white arrow). By contrast, *Jmjd1a*-siRNA injected embryos lost this asymmetry and the whole nucleus seemed to have a uniform distribution of H3K9me2. The scale bar (red) is 20 μm.

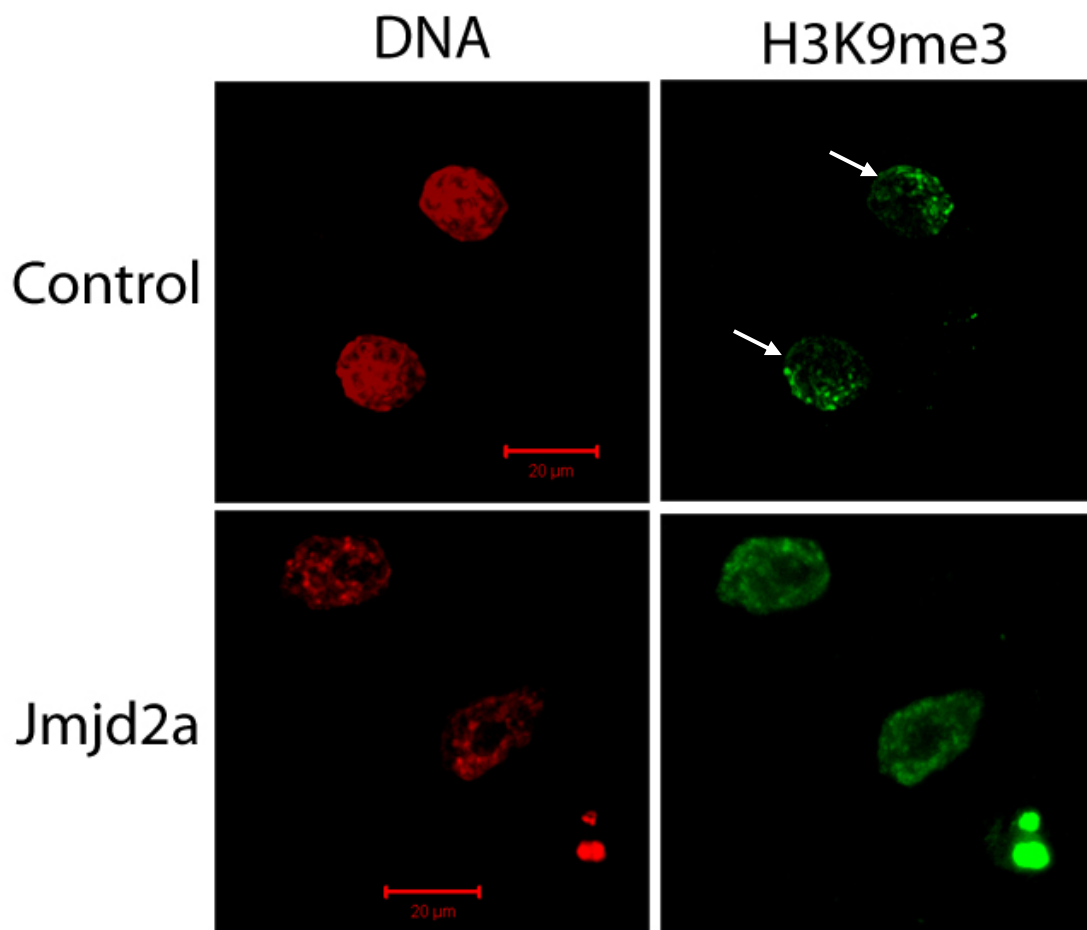


Figure 5.6 Loss of H3K9me3 asymmetry in *jmjd2a*-deficient two-cell embryos. Embryos were immunostained for H3K9me3 (green) and DNA was counterstained with DAPI (pseudo-coloured red). Control embryos (non-injected) showed H3K9me3 asymmetry of parental chromatin, in which H3K9me3 appeared to stain half of the nuclei (white arrow). By contrast, *Jmjd2a*-siRNA injected embryos lost this asymmetry and the whole nucleus seemed to have a uniform distribution of H3K9me3. The scale bar (red) is 20 μ m.

CHAPTER 6

General Discussions

Chapter 6

General Discussions

6.1 Overview and discussions

Epigenetic and chromatin modifications are primary modulators of chromatin structure and nuclear organisation, which can control gene activities in unicellular organisms such as yeast, as well as in the developmental programme of multicellular organisms. Although all cells in one organism have the same genome, each cell type has a different and unique epigenome, which may be unique at individual cell nuclei. Different signatures of epigenetic modifications can create diversity in different cell phenotypes and may also stimulate cell differentiation and development. Under normal circumstances the epigenome differentiates in an irreversible manner except during preimplantation and germ cell development. In these phases, epigenetic and chromatin modifications are extensively reprogrammed. The present study has focussed on reprogramming of epigenetic and chromatin modifications during preimplantation, as well as describing significant epigenetic changes in the postimplantation embryo. In this chapter, the five main discoveries of this thesis are discussed: (i) the dynamics of heterochromatin markers in preimplantation embryos; (ii) heterochromatin maturation in postimplantation development and in ES cells; (iii) the function of H4K20me3 in development; (iv) global epigenome differences between ES cells and inner cell mass, and (v) the importance of demethylases in preimplantation development.

6.1.1 The dynamics of heterochromatin markers in preimplantation embryos

The dynamics of heterochromatin markers and other histone methylations are described in Chapters 3, 4 and 5. The summary of quantification of histone methylation in the present study and previous publications is shown in Figure 1.9. The data showed that demethylation of heterochromatin markers H3K9me2, H3K9me3 and H4K20me3 occurs at the two-cell embryo stage and then *de novo* methylation takes place at the four-cell stage. H4K20me3 however, was absent during most of mouse development. The mark disappears from the maternal genome before fertilisation and does not

reappear in most tissues until well after mid-gestation. Intriguingly, demethylation of heterochromatin and zygotic gene activation occurred concurrently at the two-cell stage. Therefore I propose that the advantage of histone demethylation at the two-cell stage might be to remove heterochromatin marks leading to the transcription machinery activating ZGA satellite transcripts. These are derived mainly from repetitive elements in the pericentric/centromeric area and retrotransposons that are commonly silenced in somatic differentiated cells by heterochromatin marks (Martens et al., 2005, Peaston et al., 2007). This model does not exclude the contribution of CpG demethylation in ZGA, as demonstrate in early *Xenopus laevis* embryos (Dunican et al., 2008, Stancheva and Meehan, 2000)

According to my evidence the mechanism of heterochromatin clearance of H3K9me2 and H3K9me3 marks was related to either an imbalance of epigenetic modifiers or the presence of histone demethylases. Histone methyltransferase transcripts of early preimplantation embryos tend to be maternally inherited in oocytes and their mRNAs are degraded rapidly postfertilisation, whereas demethylase transcripts tend to be newly expressed postfertilisation. Hence the new expression of demethylases and degradation of methyltransferases has the potential for causing an imbalance of epigenetic modifiers. Additionally, the appearance of demethylase *Jmjd2a* and *Jmjd1a* in the embryo might be responsible for the removal of H3K9me2 and H3K9me3. I found that depletion of these gene products causes a failure of zygotic pronuclear histone asymmetry and very early embryo development. Many *Jmjd2a*- and *Jmjd1a*-deficient embryos arrested at zygote and the two-cell stage suggesting that these two enzymes were essential for early preimplantation development and might be involved in epigenetic reprogramming after fertilisation. Unlike H3K9, the mechanism of H4K20me3 depletion might be a consequence of the lack *HP1 α* and *Suv4-20h* methyltransferase to maintain and catalyse H4K20me3. A model for heterochromatin marker depletion is shown in Figure 6.1.

6.1.2 Heterochromatin maturation in postimplantation development and in ES cells

Heterochromatin maturation in mouse development was described in Chapters 3, 4 and

5. Heterochromatin in the preimplantation embryo was immature due to a lack of several chromatin modifications such as H4K20me2, H4K20me3 and HP1 α . The immature heterochromatin in the preimplantation embryo probably lead to chromatin adopting a globally more open organisation, allowing transcriptional machinery access to chromatin and to reactivating embryonic genes that are generally repressed before fertilisation (Svoboda et al., 2004, Evsikov et al., 2004, Peaston et al., 2004, Ma et al., 2001, Puschendorf et al., 2006).

Initially, heterochromatin acquired mature epigenetic marks during early implantation and was limited to the trophectoderm lineage (about day 4.5) whereas maturation of the embryonic lineage began at a much later stage. Surprisingly, I demonstrated that the process of creating ES cells from ICM could induce heterochromatin maturation *in vitro*. H4K20me3 and HP1 α appeared during the early passage of ES cells. Although undifferentiated ES cells have all heterochromatin markers, our results and others (Kobayakawa et al., 2007, Meshorer et al., 2006, Efroni et al., 2008, Martens et al., 2005) have revealed that ES cell-heterochromatin is still highly dynamic, which may make ES cell chromatin more accessible for transcriptional complexes than differentiated cells.

6.1.3 The function of H4K20me3 in development

Collective results from the present and previous studies show that H4K20me3 might serve many functions. It is involved in silencing repeat sequences by heterochromatinisation at pericentric heterochromatin (Kourmouli et al., 2004, Schotta et al., 2004, Kourmouli et al., 2005, Martens et al., 2005) and at telomere and subtelomere regions (Blasco, 2007, Martens et al., 2005). It is related to progressive cancer both human and animals (Fraga et al., 2005, Pogribny et al., 2006). H4K20me3 can also modulate telomere length and sister chromatid recombination (Benetti et al., 2007), as well as combining with H3K9me3 and HP1 to regulate imprinted genes (Pannetier et al., 2008, Delaval et al., 2007, Regha et al., 2007). The present study and data presented by Schotta et al (2008) showed that H4K20me3 is important for pre/perinatal development. A reduction in H4K20me3 might lead to foetal mortality. There is evidence that H4K20me3 may be related to an ageing mechanism, as tissues

from old rats gain more H4K20me3 than those from young rats (Sarg et al., 2002). Others have revealed that some human cell lines increase the level of H4K20me3 in late passage or senescent cells (Sarg et al., 2002, Shumaker et al., 2006). I found that H4K20me3 has an additional role as it appeared to be associated with cellular differentiation *in vivo* development, consistent with other observations in mouse (Biron et al., 2004) and *Drosophila* (Karachentsev et al., 2007). I also found that the greater proportion of H4K20me3 was observed in late development rather than early embryo development suggesting a role for H4K20me3 in tissue maturation; similar results have also been reported in rat brain development (Stadler et al., 2005).

6.1.4 Global epigenetic different between ES cell and inner cell mass

The difference between chromatin modification of ES cells and the inner cell mass (ICM) of embryos is detailed in Chapters 3, 4 and 5 and summarised in Figure 6.2. Typically, ES cells are derived from the inner cell mass of the blastocyst and are commonly used as a model for developmental biology based on the assumption that ES cells are functionally equivalent to their cell origin, the ICM. However transcription profiles of either human or mouse cells reveal a substantial difference between ICM compare to ES cells (Reijo Pera et al., 2009, Zwaka and Thomson, 2005, Horie et al., 1991, Clark et al., 2004, Toyooka et al., 2003). In terms of epigenetic and chromatin modifications, our results clearly show that the ICM differs from ES cells with regards to the lack of H4K20me3 and HP1 α to form mature heterochromatin. Chromatin modifiers histone demethylases also exhibited a divergence. *Jmjd1a* was specific to ES cells whereas *Jmjd2a* was expressed only in ICM. Surprisingly, we found that the transcriptional pathway was also different between ES cells and ICM. For instance, the demethylase and transcription factor *Jmjd2c* typically regulates *Sox2* and *Cdx2* in ICM, but it switched to regulate *Nanog* after ICM *in vitro* transformation to ES cells. In contrast to *in vivo* development, in which H4K20me3 was strongly associated with cell differentiation, H4K20me3 in ES cells seems not to be involved in differentiation. We found that H4K20me3 was already established in early passage ES cells and did not undergo a significant alteration after differentiation. This evidence suggests that the epigenetic and chromatin modifications regulating early differentiation *in vivo* development might differ from *in vitro* differentiation in ES cells. Based on the

epigenetic divergence of between ICM and ES cells, I would recommend that researchers should ensure that the relevant gene expression/pathways are similar between ES cells and ICM prior to using ES cell as a model for developmental biology.

6.1.5 The importance of demethylases in preimplantation development

Although histone methylation and histone methyltransferases were discovered about 30 years ago (Honda et al., 1975, Spivak and Peck, 1979), histone demethylases have only recently been discovered (Shi et al., 2004, Chen et al., 2006, Agger et al., 2007). Apart from their demethylation function, not much is known about histone demethylases. Approximately 248 publications in several species have been found in PUBMED with approximately 200 publications related to mouse and human studies. However only one publication described the function of histone demethylase in preimplantation embryos (Wang et al., 2009) and other two reports expression of histone demethylase using microarray analysis (Hamatani et al., 2004, Zeng et al., 2004). Our present study confirms previous studies (Hamatani et al., 2004, Zeng et al., 2004, Wang et al., 2009) showing that many histone demethylases are present in preimplantation embryos and unfertilised oocytes. This could be linked to the importance of these genes for epigenetic and chromatin reprogramming postfertilisation and during preimplantation development. One recent study (Wang et al., 2009) and my data show that *Jmjd2c* are important for blastocyst formation, however, the gene expression profile of *Jmjd2c* during preimplantation appears to be variable (Hamatani et al., 2004, Zeng et al., 2004, Wang et al., 2009). This could be due to different breeds of mice and different culture systems. In the present study we show that *Jmjd2a* and *Jmjd1a* are important for early preimplantation development between zygote and 2-cell embryo stages, and that most *Jmjd2a* or *Jmjd1a* deficient embryos arrest at the two-cell stage. The failure to develop may result from the interruption of histone demethylation, the absence of parental pronuclear epigenetic asymmetry and subsequent aberrant expression of ZGA-repeat elements such as MuERV transcripts, which are normally silenced by H3K9 methylation.

6.2. Conclusions

This thesis demonstrates that epigenetic and heterochromatin markers are dynamic during development. Heterochromatin is immature postfertilisation and becomes more mature throughout development. The dynamics of epigenetic and heterochromatin may be regulated by the balance of their modifiers. Moreover we discover a novel function of histone demethylases during preimplantation development as they may be vital for development and epigenetic reprogramming postfertilisation. These results have emphasised gaps in our understanding of the basic mechanisms that allow the initiation of development from the fertilised zygote but have also contributed new clues. The new knowledge generated from this project will be beneficial to other epigenetic and cell reprogramming studies relevant to reproductive biology, stem cells, developmental biology and regenerative medicine.

6.3 Directions for future research

In addressing the research questions in this work, several interesting research topics have been observed. These open up several avenues for future work to further evaluate and expand the findings of this thesis:

6.3.1 Chromatin modifications in development embryos

In this study the dynamics of global chromatin modification have been investigated using immunofluorescence and microscopy methods. In preimplantation embryos I did not find the signal of H4K20me₃, similar to other groups (Kourmouli et al., 2004, van der Heijden et al., 2005 and Beaujean, personal communication), however; it does not mean that there is no H4K20me₃ present. It could be simply that the immunofluorescence epitope detected by anti-H4K20me₃ may be completely masked by protein bound to this histone modification. In order to prove that I would need to extract embryonic proteins and analyse with western blot. In *Drosophila* (Karachentsev et al., 2007), H4K20me₃ is also not detected in early development using

immunofluorescence, however, H4K20me3 was detected by western blot specifically in free histone not in chromatin.

Furthermore, in order to investigate this change at gene specific level, chromatin immunoprecipitation (ChIP) a common technique could be used to study chromatin modifications and protein-DNA interactions at gene level in many cell types. However, standard ChIP assays require large cell numbers which limit the capacity of ChIP to analyse small/expensive cell samples such as preimplantation embryos. Although recently modified ChIP protocols have been designed that work on very small samples (Dahl and Collas, 2008, O'Neill et al., 2006), there has been no report to assure that these protocols will work efficiently in very early mouse preimplantation embryos especially zygote and two-cell stage when chromatin is extensively reprogrammed. Further investigations on chromatin modifications at single gene level of postfertilisation embryo will be required. ZGA genes will be the first target to be examined as they express first and seem most important for very early development.

6.3.2 Heterochromatin and pluripotency

Although report of ES-like cells and inner cell mass lacking heterochromatin enzymes Suv3-9h or Suv4-20h have appeared in the literature (Benetti et al., 2007, Peters et al., 2001), these cells have not been tested for pluripotency capacity. My studies and previous studies (Peters et al., 2001, Schotta et al., 2008) suggest that these enzymes may not be essential for *in vivo* pluripotency of ICM and epiblast cells; however, it is speculated that these cells may exhibit differentiation abnormalities due to terminally differentiated cells being unable form proper mature heterochromatin. To test this hypothesis, cellular markers for pluripotency and differentiation, and differentiation functional tests such as *in vitro* ES cell differentiations and teratoma test would be required.

6.3.3 Functions of Histone demethylases in developments

In the present study some but not all histone demethylases have been investigated regarding their expression and functions. The results suggest that some demethylases are essential for preimplantation development and their gene expression is dynamic.

However the mRNA levels in preimplantation embryos are frequently not related to the protein level, and many proteins remain in the embryo although their mRNAs are degraded to contribute to zygotic gene activation and proper development (Minami et al., 2007, Schultz, 1993). Moreover the diversity of demethylases genes (Shin and Janknecht, 2007) also suggests that they could have a functional redundancy role during preimplantation development. Further studies will need to investigate the complete proteomic profile, non-demethylation function and redundancy of histone demethylases during early embryonic development.

6.3.4 Function of histone demethylases in cell reprogramming

Cell reprogramming methodologies such as somatic cell nuclear transfer (SCNT) and induced pluripotent stem cells (iPSC) have been shown to reprogram somatic histone methylation into a totipotency- or pluripotency-like state (Wang et al., 2007, Mikkelsen et al., 2008). Recently, histone demethylase Jmjd3 has been suggested to be involved in the early step of iPSC (Banito et al., 2009) and a dynamic change of histone demethylases has been observed using microarray technology (Mikkelsen et al., 2008). Future studies will need to investigate the importance and the function of histone demethylases during cell reprogramming, as it may help to support reprogramming and may improve efficiency of reprogramming technology.

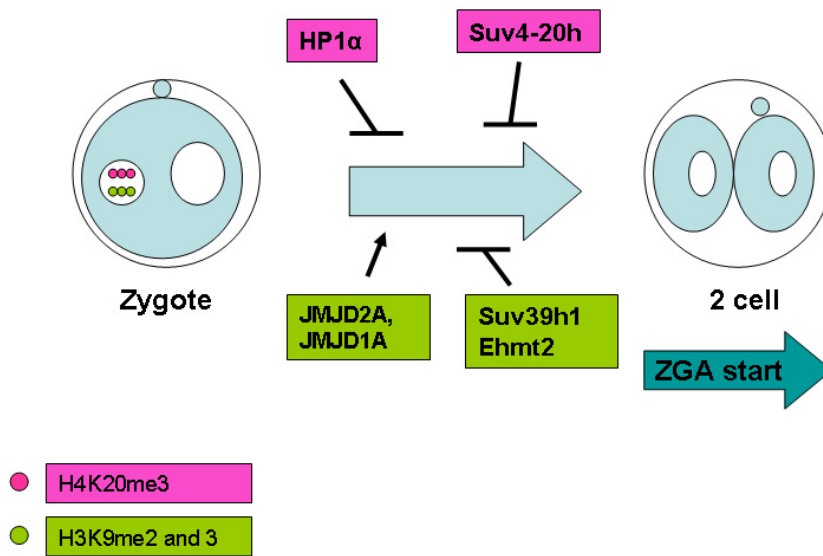
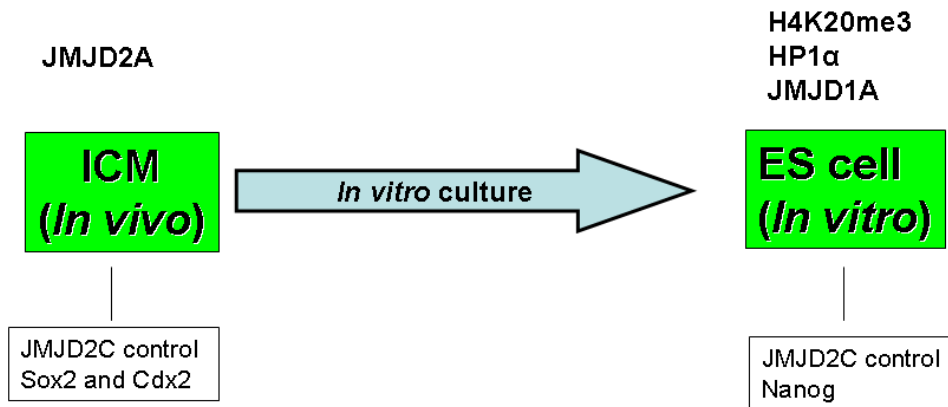


Figure 6.1 Model for removal of heterochromatin markers (H3K9me2, H3K9me3, and H4K20me3). H3K9me2 and H3K9me3 is removed by Jmjd2a and Jmjd1a together with lack of Suv39h and Ehmt2. H4K20me3 was depleted due to deficiency of HP1 α and Suv4-20h.

I



Chromatin modification	ICM (BL)	ES cell
H4K20me3 (Methylation)	Absent	High
HP1 α (Protein)	Absent	High
Jmjd2a (mRNA)	High	Very low
Jmjd1a (mRNA)	Very low	High

Figure 6.2 Summary of the differences between inner cell mass (ICM) and ES cells. Inner cell mass (ICM) of blastocyst expresses Jmjd2a and Jmjd2c but not Jmjd1a, HP1 α or H4K20me3. After *in vitro* culture ICM transforms into ES cells when the makers H4K20me3, HP1 α and Jmjd1a start to be expressed while Jmjd2a decreases. The gene regulation pathway is also different, Jmjd2c (which activates Sox2 and Cdx2 *in vivo*), changes to control Nanog after culture *in vitro*.

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Appendix 1

Detail of Chemicals and Reagents

Name	Companies	Cat. no.
Primary Antibodies		
Mouse anti-HP1 α monoclonal antibody (Immunocytochemistry)	Millipore (Chemicon), UK	MAB3584
Mouse anti-HP1 α monoclonal antibody (Western blot)	Millipore (Chemicon), UK	MAB3446
Rabbit anti-nanog mouse affinity purified polyclonal antibody	Millipore (Upstate), UK	AB5731
Mouse anti-Oct-3/4(POU5F1) monoclonal antibody	Santa Cruz Biotechnology, USA	sc-5279
Mouse monoclonal anti-beta actin	Abcam, UK	ab8226
Rabbit anti-trimethyl-histone H3 lysine 9 polyclonal antibody	Millipore (Upstate), UK	07-442
Rabbit anti-dimethyl-histone H3 lysine 9 polyclonal antibody	Millipore (Upstate), UK	07-212
Rabbit anti-monomethyl-histone H3 lysine 9 polyclonal antibody	Millipore (Upstate), UK	07-450
Rabbit anti-trimethyl-histone H4 lysine 20 polyclonal antibody	Millipore (Upstate), UK	07-463
Rabbit anti-dimethyl-histone H4 lysine 20 polyclonal antibody	Millipore (Upstate), UK	07-367
Rabbit anti-monomethyl-histone H4 lysine 20 polyclonal antibody	Millipore (Upstate), UK	07-440
Rabbit anti-trimethyl-histone H3 lysine 27 polyclonal antibody	Millipore (Upstate), UK	07-449
Rabbit anti-trimethyl-histone H3 lysine 4 polyclonal antibody	Millipore (Upstate), UK	07-473
Secondary antibodies		
Donkey anti-rabbit IgG phycoerythrin conjugate (PE)	Santa Cruz Biotechnology, USA	sc-3745
Donkey anti-mouse FITC	Jackson Lab	715095151
Donkey anti-rabbit FITC	Jackson Lab	711095152
Donkey anti-rabbit TRITC	Jackson Lab	711075152
Donkey anti-mouse Alexa Fluor 488	Molecular probe, Invitrogen, UK	A21202
Donkey anti-rabbit Alexa Fluor 488	Molecular probe, Invitrogen, UK	A21206
Donkey anti-mouse Alexa Fluor 546	Molecular probe, Invitrogen, UK	A10036
Donkey anti-mouse HRP	Santa Cruz Biotechnology, USA	Sc2005
Donkey anti-rabbit HRP	Amersham, UK	NA934V
Serum & BSA		
Donkey serum	Sigma, UK.	D9663
Chicken serum	Gibco, Invitrogen, UK	16110-082

Foetal Bovine Serum	Gibco, Invitrogen, UK	
Foetal Bovine Serum ES cell qualify	Gibco, Invitrogen, UK	10439-016
Bovine Serum Albumin (immunodetection)	Sigma, UK.	A6003
Bovine Serum Albumin (embryo culture)	Sigma, UK.	A331-10G
DNA stain and mounting reagents	Companies	Cat. no.
Hoechst33342	Sigma, UK.	B2261
Vectashield® mounting medium with DAPI	Vector Lab	H-1200
Vectashield® mounting medium	Vector Lab	H-1000
Prolong gold antifade with DAPI	Invitrogen, UK	P36931
Western Blot		
4-12% gradient Bis-tris-Gel 12 lane	Invitrogen, UK	NP0322BOX
4-12% gradient Bis-tris-Gel 15 lane	Invitrogen, UK	NP0323BOX
4-12% gradient Bis-tris-Gel 17 lane	Invitrogen, UK	NP0329BOX
NuPage® LDS sample buffer	Invitrogen, UK	NP0007
NuPage® Reducing agent	Invitrogen, UK	NP0009
Xcell Surelock™ Mini-Cell	Invitrogen, UK	EI0001
NuPage® MES SDS running buffer	Invitrogen, UK	NP0002
NuPage® antioxidant	Invitrogen, UK	NP0005
Xcell II™ Blot module	Invitrogen, UK	EI9051
PVDF membrane	Amersham, UK	RPN303F
X-ray film Fuji	Fisher Scitific, UK	AUT-300-010M
NuPage Transfer buffer	Invitrogen, UK	NP0006
Dc Protien assay reagent A	BioRad, UK	500-0113
Dc Protien assay reagent S	BioRad, UK	500-0115
Dc Protien assay reagent B	BioRad, UK	500-0114
PCR		
RNA later, Ambion, UK	Ambion, UK	7020
RNeasy mini kit	Qiagen, UK	74104
QIA shredder	Qiagen, UK	79254
DNaseI	Qiagen, UK	79254
Pellet Paint®, Co-precipitation	Novagen, UK.	70748-3
Verso™ cDNA Kit	ABgene,UK	AB1453
Cloned AMV first strand synthesis kit	Invitrogen, UK	12328-032
0.5 ml PCR tube	ABgene,UK	AB0350
0.2 ml QPCR tube	Corbett life science, UK	P/N3001-001
ABsolute™ Blue QPCR low ROX Mix	ABgene,UK	AB4323/A
Rotor-Gene™ 6000	Corbett life science, UK	
Hybaid system, Thermo	Thermo Fisher, UK	
DNA ladder	New England Biolab, UK	N04675
SYBR gold	Molecular probe, Invitrogen, UK	S11494
Hormones, growth factors and enzymes	Companies	Cat. no.
Pregnant mare serum gonadotrophin	Intervet, UK	
Human chorionic ganadotrophin	Intervet, UK	
Follicular stimulating factor	Sigma, UK	F8174

2.5% Trypsin (10x)	Lonza, UK	BE17-160E
Epithelium growth factor	Sigma, UK	
Hyaluronidase	Sigma, UK	H4272
Leukemia inhibiting factor (ESGRO [®])	Chemicon, Millipore,	ESG1106
Trypsin tablet (antigen retrieval)	Sigma, UK	T7168-20TAB
Fetuin	Sigma, UK	F6131
Media		
KSOM culture medium	Chemicon, Millipore	MR-020P-5F
M2 medium	Sigma, UK	M7167
α MEM	Invitrogen, UK	M8042
DMEM high glucose with L-glutamine	Gibco, Invitrogen, UK	41966-029
Knockout [™] DMEM	Gibco, Invitrogen, UK	10829-018
Opti MEM	Gibco, Invitrogen, UK	31985
MEM Non essential amino acids	Gibco, Invitrogen, UK	11140
L-glutamine	Invitrogen, UK	25030-024
Penicillin/streptomycin	Sigma, UK	P4333
PBS (liquid)	Lonza, UK	BE17-512F
PBS tablet	Gibco, Invitrogen, UK	18912-014
2-mercaptoethanol	Invitrogen, UK	31350010
Ultrapure water for embryo culture	Chemicon, Millipore	TMS-006-B
Cell culture water	Lonza, UK	BE17-724Q
Chemicals		
Paraformaldehyde	Sigma, UK	P6148
Tween 20	Fisher Scientific	BP337-100
Triton X 100	Sigma, UK	X-100
Acid tyrode	Sigma, UK	T1788
HEPES (pH7.9)	Sigma, UK	H3375
MgCl ₂	BDH Lab, UK	101494V
KCl	BDH Lab, UK	1019874L
DDT	443853B	
PMSF	Sigma, UK	P7626
Tris HCl pH8	Promega, UKH5125	H5125
Tris base	Fisher Scitific	BPE152-1
Boric acid	Fisher Scitific	BPE168-1
EDTA	Sigma, UK	EDS-500G
Proteinase cocktail inhibitor	Roeach, UK	11836170001
Agarose	Fisher Scitific	BPE1356-500
Lipofectamine 2000	Invitrogen, UK	11668-019
Mitomycin C	Sigma, UK	M4287-2MG
Mitomycin C	Calbiochem, UK	47589
HCl	BDH Lab, UK	101256J
siRNA	Companies	Cat. no.
Jmjd2c	Invitrogen, UK	10620318 ID124473A12 ID124473B01
Oct4	Ambion, UK	AM16704 ID151960
Suv39h1	Invitrogen, UK	10620318 ID119928E09 ID119928E10

Suv39h2	Invitrogen, UK	10620318 ID119928E11 ID119928E12
<i>Silencer</i> ® Negative Control #1	Ambion, UK	AM4635
Stealth™ RNAi negative control duplex	Invitrogen, UK	12935300
Block-it™ alexa Fluor® Red Fluorescent Oligo	Invitrogen, UK	14750-100
Others		
Multiwell slide	Menzel GmbH, VWR	XER308B
Chamber slide	Nunc, VWR	7342050
Cell culture dish 100x20mm	Corning, MVM store	430167
Cell culture dish 60x15mm	Falcon, MVM store	353004
Cell culture dish 35x10mm	Falcon, MVM store	353001
4 well dish	Nunc, VWR	176740
50 ml centrifuge tube	Corning, MVM store	430828
15 ml centrifuge tube	Corning, MVM store	430790
1.5 ml centrifuge tube	Greiner, MVM store	616201
2 ml centrifuge tube	Eppendorf, UK	120094
1.8 ml cryotube	Nunc, MVM store	65234
1.5 ml amber tubes (light protect)	Anachem. VWR	1220-07
CUVETTES 1.6ml SEMI MICRO	Fisher Scientific, MVM store	C-CXA110025D-X

Software

1. NIH Image J (free) <http://rsbweb.nih.gov/ij/>
2. WCIF Image J (free) <http://www.uhnresearch.ca/facilities/wcif/imagej/>
3. MacBiophotonics ImageJ (free) <http://www.macbiophotonics.ca/imagej/>
4. LSM browser (free version), Zeiss.
<http://www.zeiss.de/C12567BE0045ACF1/Contents-Frame/CAA2EF638EC5F0D3C1256ADF0050E2F1>
5. Velocity (free version), Improvion, UK <http://www.improvion.com/downloads/>
6. REST software <http://www.gene-quantification.de/rest.html>

Appendix 2

Recipes of Buffer Media and Stock solutions

Mitomycin C Stock solution

Mitomycin C is a carcinogen. It may be fatal by inhalation, ingestion, or skin absorption. Do not breathe the dust. Wear appropriate gloves and safety goggles and use only in a chemical fume hood.

Add 5 ml of sterile PBS to a 2-mg vial of mitomycin C.

This stock solution should be stored at 4°C, wrapped in aluminum foil, for no more than 2 weeks. It is diluted in MEF growth medium to a final concentration of 10 µg/ml to prepare MEF feeders.

10x TBE (Tris-Boric-EDTA) stock

Makes 1 litre

- Tris 108g
 - Boric Acid 55g
 - EDTA 9.3g
 - dH₂O 800ml
-
- Stir to mix, check the pH it should be at 8.3, and adjust if necessary.
 - Make volume up to 1L with dH₂O.
 - Autoclave.

Gelatine for extracellular matrix

- Prepare 0.1% gelatine in water for by mix 1 g of gelatine (Sigma cat#G1890) in 1000 ml H₂O cell culture (Lonza cat# BE17-724Q).
- Put mixture into microwave until gelatine dissolve (Do not lid or close the bottle)

Fibroblast cell medium

- DMEM 89 ml
- FFBS 10 ml
- Pen/Strep 1 ml
- Medium was filtered with 0.2µm, medium was stored at 4 °C for a month

Mouse embryonic stem cell medium

	Reagent	company	Cat#	Storage °C	Volume	volume	Final conc
					500 ml	100 ml	
1	L-glutamine	Invitrogen	25030	-20	6 ml	1.2 ml	2mM
	OR Glutamax	Invitrogen	35050	-20			
2	2-mercaptoethanol (BME)	Invitrogen	31350-010	4	1000 ul	200 ul	0.1mM
3	MEM nonessential amino acid (NEAA)	Invitrogen	11140-035	4	6 ml	1.2 ml	0.1mM
4	Penicillin/streptomycin	Invitrogen	15140-122	-20	5 ml	1 ml	
5	Leukemia inhibiting factor (LIF)	Chemicon	ESG1106	4	500 ul	100 ul	1000 IU/ml
	OR LIF (in house)	Dundee		4 or -20	50 ul	10 ul	
6	FBS (ES qualified in house)	Invitrogen	10270-106	-20	75 ml	15 ml	15%
	OR FBS (ES qualified)	Invitrogen	10439-016	-20			
	OR Serum replacement	Invitrogen	10828-028	-20			
7	Knockout DMEM	Invitrogen	10829-018	4	406.5 ml	81.3 ml	

Trypsin solution

0.025% Trypsin

- 5 ml stock trypsin (10x) (Lonza)
- 5 ml chicken serum
- 100 ml EDTA Na salt (1.85 g/l) autoclaved
- 390 ml PBS

0.25% Trypsin

- 10 ml trypsin
- 5 ml chicken serum
- 100 ml EDTA
- 385 ml PBS
- Solution was filtered with 0.2µm and then stored at -80 °C
- After thawing, trypsin can be kept in fridge (4 °C) for a month

Phosphate Buffered Saline (PBS)

- Add 1 PBS tablet (Invitrogen) per 400 ml distilled water and allow dissolving in a glass bottle.
- Filter through 0.2 µm bottle top filter and autoclave.

Histone extraction Buffer

Components	MW	Stock	10 ml solution	Note
10mMHEPES, pH7.8	11.915	100mM	1 mL	
1.5mM MgCl ₂	4.761	100mM	150 uL	Add powder into water, do not add water onto powder
10mM KCl		1mM	100 uL	
MQ water			8.75 mL	
1.5mM PMSF		100mM in ethanol, keep dark	1.5 ul in 100 ul lysis buffer	Add only before lyses
0.5mM DTT		10mM	5 ul in 100 ul lysis buffer	Add only before lyses
Lysis buffer			93.5 ul	

Paraformaldehyde (PFA) 20 % stock solution

- Weigh 4 g PFA (Sigma) into glass bottle and add 16 ml PBS. Add 2 drops of 1M sodium hydroxide (NaOH) using P20 Gilson pipette.
- Heat to 65°C until dissolved in a water bath and allow cooling. pH, should be 7.0-7.4, add more 1M NaOH if necessary.
- Filter into universal tube and cover with foil. Store at 4°C for up to 1 month or store at -20°C in aliquots.
- Dilute to working concentration of 4 % (1:5) with PBS

Triton X-100 10 % stock solution

- Weigh 1 g Triton X-100 (Sigma) into universal tube and add 9 ml PBS.
- Place tube on a rotating platform to mix thoroughly (takes 1-2 hours)
- Store 10 % stock at 4°C for 2 weeks
- Dilute to working concentration of 0.2 % (1:50) with PBS

Tween 20 (5 % stock solution)

- Add 2.5 ml of Tween 20 to 47.5 ml PBS
- Place tube on a rotating platform to mix thoroughly
- Store 5 % stock at 4°C for up to 1 month
- Dilute to working concentration of 0.05 % (1:100) with PBS

Blocking Solutions

- **2 % Bovine Serum Albumin (BSA) in PBS:** Add 200 mg BSA (Sigma) to 10 ml PBS. Allow to dissolve and filter through 0.22 µm filter.
- **5% donkey serum.** Add 250 µl serum in 5 ml PBS and filter through 0.22 µm filter.

Cryopreservation medium (2x) (10 ml)**fibroblast cell**

- DMEM 6.9 ml
- FCS 3 ml
- DMSO 2 ml
- Pen/Strep 100 µl

ES cell

- DMEM 3.4 ml
- FCS 4.5 ml
- DMSO 2 ml
- Pen/Strep 100 µl

RIPA buffer (Radioimmunoprecipitation assay)

- 50mM Tris HCl pH 8
- 150 mM NaCl
- 1% NP-40
- 0.5% Sodium deoxycholate
- 0.1% SDS
- 2 mM EDTA pH7.4