Development and application of sensitive genome-wide platforms to study the genetic and epigenetic (DNA methylation) makeup of gametes and early bovine embryos

Thèse

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Résumé

Pour ce projet, nous avons développé une plateforme pour l'analyse pangénomique de la méthylation de l'ADN chez le bovin qui est compatible avec des échantillons de petites tailles. Cet outil est utilisé pour étudier les caractéristiques génétiques et épigénétiques (méthylation de l'ADN) des gamètes soumis aux procédures de procréation médicalement assisitée et des embryons précoces.

Dans un premier temps, une plateforme d'analyse de biopuces spécifiques pour l'étude de la méthylation de l'ADN chez l'espèce bovine a été développée. Cette plateforme a ensuite été optimisée pour produire des analyses pangénomiques de méthylation de l'ADN fiables et reproductibles à partir d'échantillons de très petites tailles telle que les embryons précoces (≥ 10 ng d'ADN a été utilisé, ce qui correspond à 10 blastocystes en expansion). En outre, cet outil a permis d'évaluer de façon simultanée la méthylation de l'ADN et le transcriptome dans le même échantillon, fournissant ainsi une image complète des profils génétiques et épigénétiques (méthylation de l'ADN). Comme preuve de concept, les profils comparatifs de méthylation de l'ADN spermatique et de blastocystes bovins ont été analysés au niveau de l'ensemble du génome.

Dans un deuxième temps, grâce à cette plateforme, les profils globaux de méthylation de l'ADN de taureaux jumeaux monozygotes (MZ) ont été analysés. Malgré qu'ils sont génétiquement identiques, les taureaux jumeaux MZ ont des descendants avec des performances différentes. Par conséquent, l'hypothèse que le profil de méthylation de l'ADN spermatique de taureaux jumeaux MZ est différent a été émise. Dans notre

étude, des différences significatives entre les jumeaux MZ au niveau des caractéristiques de la semence ainsi que de la méthylation de l'ADN ont été trouvées, chacune pouvant contribuer à l'obtention de performances divergentes incongrues des filles engendrées par ces jumeaux MZ.

Dans la troisième partie de ce projet, la même plateforme a été utilisée pour découvrir les impacts d'une supplémentation à forte concentration en donneur de méthyle universel sur les embryons précoces bovins. La supplémentation avec de grandes quantités d'acide folique (AF) a été largement utilisée et recommandée chez les femmes enceintes pour sa capacité bien établie à prévenir les malformations du tube neural chez les enfants. Cependant, plus récemment, plusieurs études ont rapporté des effets indésirables de l'AF utilisé à des concentrations élevées, non seulement sur le développement de l'embryon, mais aussi chez les adultes. Au niveau cellulaire, l'AF entre dans le métabolisme monocarboné, la seule voie de production de S-adénosyl méthionine (SAM), un donneur universel de groupements méthyles pour une grande variété de biomolécules, y compris l'ADN. Par conséquent, pour résoudre cette controverse, une forte dose de SAM a été utilisée pour traiter des embryons produits *in vitro chez* le bovin. Ceci a non seulement permis d'influencer le phénotype des embryons précoces, mais aussi d'avoir un impact sur le transcriptome et le méthylome de l'ADN.

En somme, le projet en cours a permis le développement d'une plateforme d'analyse de la méthylation de l'ADN à l'échelle du génome entier chez le bovin à coût

raisonnable et facile à utiliser qui est compatible avec les embryons précoces. De plus, puisque c'est l'une des premières études de ce genre en biologie de la reproduction bovine, ce projet avait trois objectifs qui a donné plusieurs nouveaux résultats, incluant les profils comparatifs de méthylation de l'ADN au niveau : i) blastocystes versus spermatozoïdes ; ii) semence de taureaux jumeaux MZ et iii) embryons précoces traités à de fortes doses de SAM versus des embryons précoces non traités.

Summary

In this project, we developed a bovine genome-wide DNA methylation platform compatible with small sample size to study genetic and epigenetic (DNA methylation) makeup of ART-treated bovine gametes and early embryos.

Initially, a bovine-specific array-based DNA methylation analysis platform was developed. This platform was subsequently optimized to produce reliable and reproducible genome-wide DNA methylation analysis from very small sample sizes, *e.g.* bovine early embryos (≥ 10 ng gDNA input, corresponding to 10 expanded blastocysts). In addition, this platform permitted concurrent assessment of both DNA methylation and transcription in the same sample, thereby providing a very complete picture of genetic and epigenetic (DNA methylation) profiles. As proof of concept, for the first time, comparative DNA methylation profiles of bovine sperm and blastocysts were analysed at a genome-wide level.

Using this platform, global DNA methylation profiles of monozygotic (MZ) twin bulls were analysed. Despite being geneticially identical, MZ twin bulls consistently have different progeny performance. Therefore, it was hypothesised that the DNA methylation profile of sperm from MZ twin bulls is different. In our study, there were significant differences between MZ twin for semen end points, as well as for the sperm epigenome (DNA methylation), all of which would be expected to contribute to incongruous divergent performances of daughters sired by MZ twins.

In the next part of this project, using the developed platform, impacts of supplementation of a high-concentration global methyl donor on bovine early embryos was investigated. Supplementation with large amounts of folic acid (FA) has been extensively used and recommended in pregnant women for its well-established ability to prevent neural tube defects in children. However, more recently, several studies reported adverse effects of high FA concentrations, not only on embryo development, but also in adults. At the cellular level, FA enters one-carbon metabolism, the only pathway to produce S-adenosyl methionine (SAM) as the global methyl donor for a wide variety of biomolecules, including DNA. Therefore, to address this controversy, a high dose of SAM was used to treat *in vitro* -produced bovine embryos. This not only affected early embryo phenotypes, but also the transcritome and genome-wide DNA methylome.

Overall, the current project resulted in development of a user-friendly and cost-effective bovine genome-wide DNA methylation analysis platform, which is compatible with small cell number such as early embryos. In addition, as one of the first studies of its kind in bovine reproductive biology, this project had three objectives which yielded several novel results, including comparative genome-wide DNA methylation profiles of: i) bovine blastocysts versus sperm; ii) sperm from monozygotic twin bulls and iii) high dose SAM-treated versus non-treated bovine early embryos.

Table of Contents

List of tables

Chapter 1

Chapter 3

Chapter 5

List of figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

List of abbreviations

%	Percent
°C	Celsius degree
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
5-methyl-THF	5-methyl-tetrahydrofolate
AI	Artificial insemination
AID	Activation-induced cytidine deaminase
AOT	Acridine orange test
APEX	APEX nuclease (multifunctional DNA repair enzyme)
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like,
AR reaction	Acrosome reaction
aRNA	Anti-sense RNA
ART	Assisted reproductive technologies
ART2A	ADP-ribosyltransferase 2a
ASMA	Automated sperm head morphology analysis
ATF	Artificial transcription factors
BER	Base excision repair pathway
Bisulf	Sodium bisulfite treatment
Blst	Blastocysts
BMP	Bone morphogenetic proteins
BODIPY	4-bora-3a,4a-diaza-s-indacene
BovA2	Short interspersed nuclear element (SINE) sequences of the *Bovidae*

BOvB	*Bov-B* consensus sequence
BovtA	Short interspersed nuclear element (SINE) sequences of the *Bovidae*
bp	Base pair
BRD9	Bromodomain Containing 9
BS	Bisulfite sequencing
BSA	Bovine Serum Albumin
BSE	Breeding soundness evaluation
bST	Bovine somatotropin
BWS	Beckwith Wiedemann syndrome
C	Non-methylated cytosine
CAM	Calcein acetomethyl ester
CARS	Cysteinyl- tRNA Synthetase
CASA	Computer Assisted Sperm Analysis
CChIP	Carrier Chromatin Immunoprecipitation
cDNA	Complementary DNA
CE	Capillary electrophoresis
CFDA	6-carboxyfluorescein diacetate
CG	Cytosine phosphate Guanine
CGIs	CpG islands
CHARM	Comprehensive high-throughput arrays for relative methylation
CHG	Cytosin H Guanine (where H corresponds to A, T, or C)
ChIP-chip	Chromatin immunoprecipitation (ChIP) followed by microarray hybridization
CHOTHF	10-Formyltetrahydrofolate
CIDR	Controlled intravaginal drug release
CMA3	Chromomycin A3
CMFDA	5-chloromethylfluorescein diacetate

Cnt.-EB	Non-treated expanded blastocysts
CNV	Copy number variants
CO2	Carbon dioxide
COBRA	Combined bisulfite restriction analysis
COCs	Cumulus oocyte complexes
CpG	Cytosine phosphate guanine
Ct	Threshold cycle
ΔCT	Delta cycle threshold
ΔΔCT	Delta Delta cycle threshold
CTC	Chlortetracycline
d	Day
DAVID	Database for Annotation, Visualization and Integrated Discovery
DE	Differentially expressed
DEG	Differentially expressed gene
DIC	Differential interference contrast
Dig	Restriction endonuclaese digestion
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferase enzymes
dNTPs	Deoxynucleotides
DOHaD	Developmental Origin of Health and Diseases
DTT	Dithiothreitol
dUMP	Deoxyuridylate
E2	Oestradiol
EB	Expanded blastocysts
EBVs	Estimated breeding values
ECP	Oestradiol cypionate
EDMA	EmbryoGENE DNA methylation analysis

EDTA	Ethylenediaminetetraacetic acid,
EGA	embryonic genome activation
ELMA	EmbryoGENE LIMS and microarray analysis
EmbryoGENE	A Network was created to address important issues concerning embryo development in livestock, mainly cattle and swine.
EMBV3	EmbryoGENE bovine transcriptome version 3
EpiTYPER	A bisulfite-treatment-based method for detection and quantitation of DNA methylation
ERCC	External RNA Controls Consortium
ERCR	Estimated relative conception rate
ERVs	Endogenous retroviruses
ESCs	Embryonic stem cells
ET	Embryo transfer
EtBr	Ethidium bromide
FA	Folic acid
FACS	Fluorescence-activated cell sorting
FAD	Folate acid deficiency
FC	Flowcytometry
FC	Fold change
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FQRNT	Fonds Québécois de la Recherche sur la Nature et les Technologies
FR	Folate receptor
FSH	Follicle-stimulating hormone
FTAI	Fixed-timed artificial insemination
FTET	Fixed-timed embryo transfer
Gb	Giga base

GC-MS	Gas chromatography-MS
gDNA	Genomic DNA
GEO	Gene Expression Omnibus
GnRH	Gonadotrophin-releasing hormone
GO	Gene ontology
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GWAS	Genome wide association studies
h, hr	Hour
H2AK119	Histone 2A Lys4 119
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H3K27me3	Histone 3 lys27 trimethylation
H3K4	Histone 3 lys4
H3K4me3	Histone 3 lys4 trimethylation
H3K9me3	Histone 3 lys9 trimethylation
hCG	Human chorionic gonadotropin
HCP	High CpG density promoters
HDL	High-density lipoproteins
HE	Hydroethidine
HELP	*HpaII* tiny fragment enrichment by ligation-mediated PCR
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOST	Hypo-osmotic swelling test
HPCE-ESI/MS	High-performance capillary electrophoresis–electron spray Ionization/mass spectrometry
hpf	Hour post-fertilization
HPLC	High Performance Liquid Chromatography
IAPs	Intracisternal A particle genes
ICM	Inner cell mass
ICP	Intermediate CpG promoters

ICRs	Imprinting control regions
IGF2	Insulin-like growth factor 2
IGFBP-4 and -5	Insulin-like growth factor binding proteins 4 and 5
IP	Immunoprecipitation
IPA	Ingenuity Pathway Analysis
iPSs	Induced pluripotent stem cells
ITC	Fluorescein isothiocyanate
IVC	*In vitro* cultured embryo
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
K	Kilo
Kb	Kilo base
Kbp	Kilo base pair
LAMP	Loop-mediated isothermal amplification
LC	Liquid chromatography
LC-MS	LC-mass spectrometry
LCP	low density CpG promoters
LH	Luteinizing hormone
LIG	Ligase I, DNA, ATP-Dependent
LIMMA	Linear Models for Microarray and RNA-Seq Data
LINE	Long Interspersed Nuclear Elements
LM-PCR	Ligation-mediated PCR
lncRNAs	Long noncoding RNA
LOS	Large offspring syndrome
LTR	Long-terminal-repeat retrotransposons
LUMA	Luminometric methylation assay

M	Use for millions, and molar
M540	Merocyanine 540
MACS	Magnet-activated (or magnetic-bead activated) cell sorting
MALDI-TOF	Matrix-assisted laser desorption ionization–time of flight
Mass spec.	Mass spectrophotometry
MAT	Methionine adenosyltransferase
Mb	Mega base
MBD2	Methyl-CpG binding domain protein 2
MBD4	Methyl-CpG-binding domain protein 4
MCA	Methylated CpG island amplification
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
MET	Maternal embryonic transition
mg	Milligrams
Mg2+	Magnesium
MgCl2	Magnesium chloride
MI	Metaphase I
MII	Metaphase II
miRNA	Micro RNAs
mL	Milliliter
mm	Millimeter
mM	Millimolre
MOET	Multi-Ovulations Embryo Transfers
mRRBS	Multiplexed RRBS
MS	Mass spectrometry
MS-HRM	Methylation-sensitive high-resolution melting
MS-MCA	Methylation-sensitive melting curve analysis
MSP	Methylation specific PCR
MSRE	Methyl-sensitive restriction endonucleases

MS-SnuPE	Methylation-sensitive single nucleotide primer extension
MTHF	Methyltetrahydrofolate
MW	Molecular-weight
MZ	Monozygotic
Nacl	Sodium chloride
natRNA	Natural antisense
ncRNA	Non-coding RNA
ng	Nanogram
NGS	Next generation sequencing
nmol	Nanomolar
NNAT	Neuronatin gene
NRC	National Research Council
NRR	Non-return rate
NSERC	Natural Sciences and Engineering Research Council of Canada
nt	Nucleotide
NTDs	Neural tube defects
Oligo dT	A short sequence of deoxy-thymine nucleotides
OPU	Ovum pick-up
OvF	Ovulatory follicle
oxBS-Seq	Oxidative bisulfite sequencing
p and P	Stands for probability
P4	Progesterone
PARP1	Poly (ADP-Ribose) Polymerase 1
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCFT	Proton-coupled folate transporter
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PE	Phycoerythrin

PEG10	Paternally expressed 10 gene
pg	Picogram
PGCs	Primordial germ cell
PGF2α	Prostaglandin F2α
PGF	Placental Growth Factor
phi29 DNAP	Wild-type phi29 DNA polymerase
PI	Propidium iodide
piRNA	Piwi-interacting RNA
PNA	Peanut agglutinin
PNKP	Polynucleotide Kinase 3'-Phosphatase
PR	Progesterone receptor
PRC2	Polycomb Repressive Complex 2
Pr.Ext	Primer extension
PSA	Pisum sativum agglutinin
pY	Phosphotyrosine
Pyro	Pyrosequencing
QC	Quality control
q-RT-PCR	Quantitative real-time reverse-transcriptase polymerase chain reaction
QTL	Quantitative trait loci
R123	Rodamine 123
rasiRNAs	Repeat-associated siRNA
RdDM	RNA-directed DNA methylation
RdDM	RNA-directed DNA methylation
RE	Restriction endonuclease
REDIH	CIHR Training Program in Early Development, and the Impact on Health
RFC	Reduced folate carrier
RIP-seq	RNA Immunoprecipitation coupled with sequencing

RLGS	Restricted landmark genome scanning
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	ROS reactive oxygen species
RPM	Revolutions per minute
RQR	Réseau Québécois en Reproduction
RRBS	Reduced-representation bisulfite sequencing
rRNA	Ribosomal RNA
RTE	A non-long-terminal-repeat (non-LTR) retrotransposable element
s	second
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SAM-EB	SAM-treated expanded blastocysts
SCNT	Somatic cell nuclear transfer cloning
SCR	Sire conception rate
SCSA	Sperm chromatin structure assay
SD	Standard Deviation
shRNA	Short hairpin RNA
SINEs	Short Interspersed Nuclear Elements
siRNA	Small interfering RNA
SMRT	Single-molecule real-time
SMUG1	Single strand-selective monofunctional uracil DNA glycosylase 1
SNARF-1	Seminaphtorhodafluor-1
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
SOF	Synthetic oviduct fluid
SRY	Sex-determining Region on the Y chromosome

SSRs	Simple sequence repeats
SYBR14	Membrane-permeant fluorescent nucleic acid stain
T	Thymine
TAI	Timed artificial insemination
TALE	Transcription-activator-like effector
TALEM	Transcription-activator-like effector methyltransferase
TBARS	Thiobarbituric acid reactive substances
TDG	Thymine DNA glycosylase
TE	Trophectoderm
TE	Transposable elements
TEP	Trophectodermal projections
TEs	Transposable elements
TETs	Ten eleven translocase enzymes including
TFO	triplex-forming oligomers
THF	Tetrahydrofolate
TLC	Time-lapse cinematography
TLH	HEPES-buffered Tyrode's medium
Tsix	X-Inactivation-Specific Transcript-Antisense
TSS	Transcription start sites
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling.
TZP	Transzonal projections
U	Unit
UCSC	University of California, Santa Cruz
UHRF1	Ubiquitin-Like With PHD And Ring Finger Domains 1
ULI-NChIP	ultra-low-input micrococcal nuclease-based native ChIP
ULS	Universal Linkage System
USDA	United States Department of Agriculture
V	Volt
WGSBS	Whole-genome shotgun bisulfite sequencing

XCI	X chromosome Inactivation
xg	Times gravity
Xi	Inactive X chromosome
Xic	X inactivation center
Xist	X-inactive specific transcript
Xm	Maternal X
Xp	Paternal X
XRCC	X-Ray repair complementing defective repair in chinese hamster cells
YOPRO1	A commercial green-fluorescent stain
ZFP	Zinc finger proteins
ZGA	Zygotic genome activation
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolare

Dedication

I dedicate this thesis to my wife, Somayeh Golzari Movafagh, who has been beside me in every moment of my life since our marriage to support, encourage and help me. It was not possible to accomplish my PhD and achieved all of my successes during the life since I have been with her without her patients, care and love.

I also dedicate this thesis to my mother, Maryam Abdollahi and my father Hamid Shojaei for being such a great parents supporting me with their infinite love. Nothing can explain their dedication and self-devotion for their children especially me to succeed and be happy in my life. Thank you mom and dad!

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Publications

The chapter 1(introduction) comprises a published book chapter and a review article in addition to some updated information which incorporated appropriately in the text. Chapter 3 & 4 are published articles and chapter 5 will be submitted shortly.

Chapter 1

Book chapter: Shojaei, H. and Robert, C. (2014). Modern reproductive technologies and breeds improvement. The Genetics of Cattle. 2nd Edition. Chapter 13. CABI. UK.

Authors' contributions

HASS and CR wrote the manuscript; designed the figure and tables. All authors read and approved the final manuscript.

Review article : **Shojaei, H*.**, McGraw*****, S., Robert, C. Meeting the methodological challenges in the molecular mapping of the embryonic epigenome. Molecular Human Reproduction. 2013; 12:809-827. ***** These authors contributed equallly to this work.

Authors' contributions

HASS and SM provided equal contribution to the conception and writing of the manuscript; C.R. wrote parts of the manuscript; S.M. and C.R. revised the manuscript. All authors approved the final version.

Chapter 3

Shojaei, H., O'Doherty, A., Gagné, D., Fournier, E., Grant, J. R., Sirard, MA., Robert, C. (2014) An integrative platform for efficient survey of the bovine DNA methylome suitable for low amount of starting material. BMC Genomic. 15:451

Authors' contributions

HASS worked on platform development, performed bovine sperm and blastocyst experiments, and designed the analysis pipeline. AOD performed pyrosequencing for candidates. DG leaded platform development. EF performed bioinformatic analyses, array design and programmed the plot generation pipeline. JG performed the *in silico* survey of CpG islands. MAS and CR conceived the general strategy for the platform's development. CR designed and supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

Chapter 4

Shojaei, H., Fournier, E., Vigneault, C., Bailey, J., Robert, C. (2016) Genome-wide analysis of sperm DNA methylation from monozygotic twin bulls. Reproduction Fertility and Development Journal. Published online: 12 January 2016; http://dx.doi.org/10.1071/RD15384

Authors' contributions

HASS worked on bovine sperm intact gDNA extraction development, performed semen and sperm analysis, MZ twin sperm DNA methylation experiments. EF performed

bioinformatic analyses. CV and PB provided the MZ information and samples. JB provided the CASA analysis system and reviewed the manuscript. CR designed and supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

Chapter 5

Shojaei, H., Fournier, E., Gagné, D., Baldoceda, L.M., Sirard, MA., Robert, C. Responses of bovine early embryos to S-adenosyl methionine supplementation in culture (*Accepted in Epigenomics journal to publish in July 2016*).

Authors' contributions

HASS, MAS and CR designed the study. HASS performed the IVF, phenotypic, transcriptome (RT-qPCR) and epigenetics (DNA methylation and pyrosequencing) experiments, acquired and contributed to dara analysis. DG performed the transcriptome microarray. EF performed bioinformatics analyses. LMBB and HASS performed the mitochondria staining. CR supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

1. Introduction

1.1. Reproductive systems

Reproductive systems are different for male and female and consist of a series of glands and tubes that produce and nurture gametes and transport them to the site of fertilization. Therefore, the major function of the reproductive system is to ensure survival of the species. The processes of male (sperm) and female (oocyte) haploid gamete production are known as spermatogenesis and oogenesis, respectively. Following puberty, spermatogenesis can persist throughout lifetime in males. In contrast, oogenesis initiates during fetal development, arrests at prophase I of meiosis before birth, and does not resume until prior to ovulation in adulthood.

1.1.1. Male reproductive system

The male reproductive system of mammals consists of two testes (testicles) in the scrotum, accessory organs including ducts and glands, and the penis. The organs and glands of the male reproductive tract produce the sperm and deliver it to the female reproductive tract during intercourse.

1.1.1.1. Testis

The testicles have two functions; i) production of: spermatozoa (also called sperm) and ii) synthesis of the male sex hormones, most importantly testosterone. The testicles are located outside of the body cavity in the scrotum, which is important for thermoregulation of the testicles and provides a favorable environment for the production and maturation of spermatozoa (Jung and Schuppe 2007; Kim *et al.,* 2013). This is a favorable environment as successful sperm development occurs at a

temperature several degrees below normal body temperature. Each testis consists of a mass of coiled seminiferous tubules where spermatogenesis takes place. In bovine, it has been estimated that the approximately 2×10^{9} sperm are produced in a single day (Rowen D. Frandson, 2009). Histologically, within the seminiferous tubules there are sertoli cells that surround and support developing spermatozoa and their precursors (Kaur *et al.,* 2014). Sertoli cells are involved with nourishing the developing sperm and mediate the effects of follicle-stimulating hormone (FSH) and testosterone on the germ cells (Kaur *et al.,* 2014). The connective tissue between the seminiferous tubules contains the interstitial cells (leydig cells) which secrete the main male hormone (testosterone) when stimulated by the pituitary gonadotropin luteinizing hormone (LH) (Smith and Walker, 2014).

1.1.1.2. Other male reproductive system structures

These structures include the epididymis and ductus deferens, accessory sex glands (ampullary glands, vesicular glands, prostate, and bulbourethral glands), the urethra, and the penis. Following spermatogenesis, testicular spermatozoa must undergo further maturation to enable fertilization of oocytes. Maturation occurs during the transit of immature spermatozoa through the male reproductive tract via interactions with epididymal epithelium (usually 10–15 days) and accessory gland fluid (Caballero *et al*., 2011). Male accessory sex glands include ampulla of the ductus deferens, vesicular gland, prostate gland, and bulbourethral gland which produce semen to provide favorable conditions for nutrition of sperm and acts as a buffer against the natural acidity of the female genital tract (Rowen D. Frandson, 2009).

1.1.1.3. Hormones of Male Reproduction and functions

The secretion of gonadotrophin-releasing hormone (GnRH), stimulates the release of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (adenohypophysis) (Schlatt and Ehmcke, 2014). Both FSH and LH are protein hormones which are the primary endocrine regulators of testicular function, therefore both are considered to be gonadotrophins. FSH promotes spermatogenesis by its actions on germ cells and sertoli cells in the seminiferous tubules to support the development of the spermatozoa (Schlatt and Ehmcke, 2014). However, LH acts on leydig cells to promote the secretion of androgens, primarily testosterone. Testosterone produced by the leydig cells is necessary for the completion of spermatogenesis (Smith and Walker, 2014).Therefore, both FSH and LH are required for successful spermatogenesis.

1.1.2. Female reproductive system

In mammals, the female reproductive system generally consists of two ovaries, uterine tubes (also called oviducts), uterus and vagina. Oocytes grow and mature in the ovaries, and following ovulation transit into the oviduct, the site of fertilization and early embryo development. Subsequently, embryos transit to and implant in the uterus resulting in pregnancy.

1.1.2.1. Ovary

Mature female gametes (oocyte) and some of the hormones necessary for female reproduction are produce in ovaries. Unlike the testis, ovaries contain a limited number of gametes following birth, (Van den Hurk and Zhao, 2005). They contain a variant

population of follicles including primordial, primary, secondary and tertiary follicles which the latter can be classified as small and large antral follicles (Aerts and Bols, 2010). In primates and most domesticated animal species, the stock of primordial follicles develops during fetal life. According to dogma in the field, in females, the peak number of ovarian follicles occurs in the fetus at mid-gestation and is approximately 2.7 million and seven million follicles in cow and women, respectively. This number then declines to 135,000 and 700,000 follicles in cow and women, respectively at birth meaning up to 95% loss at birth due to atresia (Van den Hurk and Zhao, 2005). Furthermore, in ovaries, folliculogenesis takes place which is the process of ovarian follicle development causing the primordial follicle to undergo several steps of maturation in order to develop into a preovulatory follicle. In fact, these changes occur within the three major cell populations: oocytes, granulosa cells, and theca cells under control of several hormones and growth factors. In order to reach the ovulatory stage, an ovarian follicle will pass through the following stages: primordial (resting), primary, secondary (pre-antral), tertiary (antral), and, finally, the pre-ovulatory (Graafian) follicle stage. Folliculogenesis can be divided into two phases: i) preantral growth which is gonadotropin-independent phase and initiates from recruitment of a primordial follicle to the end of the secondary stage and ii) antral growth which is gonadotropin-dependent phase when a fluid-filled cavity or antrum develops within the follicle which is tertiary (Graafian follicle) and ultimately fully developed into a preovulatory follicle (Conti and Chang, 2016).

1.1.2.2. Other female reproductive system structures

Other female reproductive system structures consist the Fallopian tubes (also called oviducts), uterus and vagina. The oviducts comprise infundibulum, ampulla, isthmus, uterotubal junction and are the site of fertilization and function in the transfer of the oocyte to the uterus. Furthermore, oviducts support sperm survival and have functions to prevent infection (Sheldon *et al*., 2014). The uterus of the domestic mammal consists of a body, a cervix (neck), and two horns and is the site for embryo implantation and fetus development during pregnancy.

1.1.2.3. Hormones of Female Reproduction and functions

Similar to male, in female the hypothalamic secretion of GnRH promotes the release of both FSH and LH from the adenohypophysis (Christensen *et al*., 2012). However, ovarian steroids (estradiol and progesterone) and peptide hormones (inhibin) can be modulated by the release of GnRH. Cellular receptors for FSH and LH are expressed in the granulosa and theca of secondary follicles, respectively and they become responsive to these hormones (Xu *et al*., 1995; Aerts and Bols 2010a). From this point, the coordinated effects of FSH and LH are both required for normal follicular development. During the gonadotrophin-dependent stage of follicular development, FSH- and LH-signaling pathways play essential role in follicular differentiation, selection and survival (Palermo, 2007; Gervásio *et al*., 2014). Under the effect of LH, theca cells produce androgen which diffuses into the mural granulosa cell (MGC) layer (those granulosa cells surround the antrum), where it is the substrate for FSH-induced aromatase for follicular estradiol synthesis. This is the landmark of ‘two cells-two

gonadotrophins' concept for follicular estradiol biosynthesis and the physiology of ovarian function in mammals (Palermo, 2007; Gervásio *et al*., 2014; Conti and Chang, 2016). Estrogen produced by the granulosa cells acts as a paracrine agent with positive feedback that promotes the development of the follicle, however systematic circulation of estrogen has a negative feedback effect on FSH secretion from the adenohypophysis (Aerts and Bols 2010a; Forde *et al*., 2011). In fact, follicles are involved in the positive and negative feedback mechanisms of the hypothalamic–pituitary–gonadal (HPG) axis which also has a governing role in the regulation of the oestrous cycle of cattle (Forde *et al*., 2011). Together these feedback mechanisms can select which follicle ultimately produces the ovum and ovulates (Aerts and Bols, 2010b). In addition, estrogens from developing follicles are also essential for modulating of the hypothalamic–adenohypophyseal axis for ovulation and increasing the LH receptors in thecal cells to response to short-term large LH release (LH surge) necessary for ovulation (Aerts and Bols, 2010b).

1.2. Introduction to Assisted Reproductive Technologies (ARTs)

Assisted reproductive technologies (ARTs) encompass a wide range of techniques designed primarily to aid couples unable to conceive without medical assistance (E. Oluwole Akande, 2008). One in six couples worldwide experience some form of infertility problem at least once during their reproductive lifetime. It is now estimated that more than 5 million babies have been born worldwide since the first IVF baby was born in 1978 (ESHRE, July 2014).

The largest part of the agriculture economy in developing countries relies on cattle and small ruminants (Rodriguez-Martinez, 2012). In many countries of the southern hemisphere, such as Australia, New Zealand and South American countries, cattle and small ruminants represent the major economical asset in terms of milk, meat and wool production (Rodriguez-Martinez, 2012). In Canada, there are more than 11,600 dairy farms, with approximately 1,000,000 dairy cow and 500,000 dairy heifers accounting for an income over six billion dollars in 2014 (http://www.dairyinfo.gc.ca/) (Canadian Dairy Information Center, 2015).

In cattle, ARTs are routinely carried out to shorten generational intervals and to propagate genetic material among breeding animal populations (Rodriguez-Martinez 2013). ARTs are important tools for improving the production of dairy and beef cattle. Hence, ARTs have been developed over the years for manipulation of both male and female gametes and embryos. In a way that today ARTs face a strong wave of increasing commercialization and profits for the cattle industry through a positive economical impact on beef and milk production and genetics exports (Rodriguez-

Martinez, 2012). For instance, in Canada up to 80 % of the Canadian Dairy Genetics Exports in 2014 were accounted for by embryo and semen exportation, whereas export of dairy cattle provided only 20 % of the Canadian Dairy Genetics Exports money values (Canadian Dairy Information Centre, 2015). Among different ARTs in cattle, artificial insemination (AI) is by far the most important and routinely performed of the ARTs (see page 17). Worldwide, it has been estimated that over 130 million cattle are inseminated artificially each year (Vishwanath, 2003). In addition, *in vitro* embryo production is one of the most frequently employed ARTs in the cattle industry. However, *in vitro* production of bovine embryos is not without consequence, as they have been shown to differ greatly from their *in vivo* produced counterparts in many facets, including developmental competence; which has been suggested to be related to disruption of the epigenetic profile of the gametes and/or embryos (Urrego *et al*., 2014). Similar observations have been reported for other species such as human and mouse (van Montfoort *et al*., 2012; El Hajj and Haaf, 2013; de Waal *et al*., 2015; Song *et al*., 2015).

ARTs represent an exciting prospect for conservation biologists for the preservation of both the ecologically valuable, wild, rare, and indigenous species and the genetic variability within such species (Long, 2008). Considering the intensive agriculture, extinction of natural habitats, environmental pollutions and several other factors, biodiversity has been sharply threatened through extinction of several mammalian species. Hence, due to the cultural, historical and genetics importance attempts are being made to conserve genomes by employing ART and biotechnology for the

endanger species (Cseh and Solti, 2000; Long, 2008). However, in practice there are few examples of success, of course not as the result of a failure on the part of the technologies per se, but rather is due to lack of knowledge about the fundamental biology of the species in question (Pukazhenthi *et al*., 2005; Long, 2008).

1.2.1. ART applications in cattle

Well-defined objectives are essential for success in livestock breeding and will usually evolve over time in relation to societal concerns as well as technological and economic developments. In case of cattle, higher output per unit input have been the main breeding objective but other issues including well-being, longevity and reproduction have been raised (Flint and Woolliams, 2008). Dairy cattle breeding is undergoing a paradigm shift to genomic selection of sires and dams (Amann and DeJarnette, 2012). Genetic improvement relies on two different physiological applications namely genetic selection and selective mating. The first requires estimation of genetic potential, the second pertains to the selection of mating pairs and reproductive technologies used to disseminate high merit genetics and is the focus of this chapter. Reproductive technologies have a crucial role in improvement of livestock reproduction to meet the rising challenges to the food supply of the 21st century (Murphy, 2012). Despite major advances associated with reproduction in dairy cattle (Moore and Thatcher, 2006), reproductive decline in elite dairy cattle is a serious concern of farmers and the dairy industry worldwide (Lucy, 2007; Dobson *et al*., 2007; Lucy, 2001; Pryce *et al*., 2004; Walsh *et al*., 2011). Fertility has emerged as a growing and biggest genetic and

management challenge in high-producing dairy herds (Funk, 2006). Fertility requires both paternal and maternal contributions to produce an embryo which eventually leads to live birth. The following aims at portraying the current and emerging reproductive technologies that are destined to increase the rate of genetic improvement with a focus on dairy herds.

1.2.1.1. Gametes

1.2.1.1.1. Sperm

Male fertility is an important factor in cattle breeding as bulls in service are used to breed numerous cows and defective semen quality can have a profound contribution on reproductive failure (Kathiravan *et al*., 2011; DeJarnette *et al*., 2004). It was estimated that sperm from majority of sires are not able to fertilize 100% of the oocytes (>90% oocytes in heifers or non-lactating cows) and male-associated deficiencies account for approximately 5 to 20% of embryos dying by Day 8 of development (Amann and DeJarnette, 2012). In cattle, primarily, post-thaw semen evaluation and analysis of several sperm characteristics are routinely performed in breeding centers to assess potential bull fertility.

Sperm is the ultimate example of a structural and functionally differentiated cell in which its genetic component located in the highly condensed nucleus results in gene expression silencing (Eddy, 2006; Johnson *et al*., 2011). This high level of compaction is achieved through chromatin remodeling during spermatogenesis by removing and replacing most somatic histone cores with transition proteins and then protamines (arginine-rich, nuclear proteins which have higher DNA affinity), leading to extreme

chromatin compaction in the late haploid phase of spermatogenesis (Ward, 2010). The extent of histone replacement differs between species and varies between 85-98% in mammals (Balhorn, 2007; Ward, 2010). Interestingly, recent studies imply that retained histones in mature sperm genome are believed to be gene-specific and non-randomly distributed and have unique histone modifications, indicating their potential epigenetic regulatory mechanisms in early embryo development (Hammoud *et al*., 2009; Arpanahi *et al*., 2009; Miller *et al*., 2010). In the sperm nucleus, interaction of the protamines and DNA make a unique structure of sperm DNA coiling called toroidal subunits, or doughnut loops, that contain roughly 50 kb of DNA (D'Occhio *et al*., 2007).

Mammalian spermatozoa are endowed with a unique cytoskeleton (Fouquet and Kann, 1994) that varies in morphology, biochemical and physiological characteristics among the species (Pesch and Bergmann, 2006). Substantial differences in sperm head shape and size were found within and between breeds in livestock (Phetudomsinsuk *et al*., 2008). Bull sperm has a very flat and highly condensed sperm head due to the extent and/or efficiency of disulfide bonding in its nucleus (Perreault *et al*., 1988). Sperm DNA is enclosed in a vestigial nuclear envelope that is protected by several outer membranes formed when the typical elongated spermatozoa take shape during spermiogenesis (Hess and Franca, 2009). These membranes are known to delineate subcellular compartments and different regions that are essential for the complex physiological transformation which sperm must undergo to achieve successful fertilization (Brewis and Gadella, 2010). Acrosome is a unique membranous secretory organelle located in the sperm head (Mayorga *et al*., 2007; Berruti and Paiardi, 2011;

Yanagimachi, 1994) which contains enzymes to digest and interact with zona pellucida of the oocyte (Buffone *et al*., 2008). During the early stages of sperm-egg interaction acrosome undergoes an exocytotic process known as the acrosome reaction (AR) which is an irreversible step and functions as a behavioral switch, converting sperm into a state in which they are competent to interact with oocytes (Florman *et al*., 2008). However, the dogma of the zona pellucida-induced acrosome reaction was challenged in 2011. Prior to the AR in the female reproductive tract, mammalian sperm must undergo capacitation which is a complex series of biochemical and physiological modifications followed by changes in sperm motility pattern (hyperactivation) which altogether are crucial changes in sperm for successful fertilization (Ickowicz *et al*., 2012). Depending on the species, about 100 mitochondria are localized in the sperm mid-piece, having several key roles in sperm pathology and physiology such as providing energy (ATP) for sperm metabolism, membrane function and motility (Pena *et al*., 2009).

Some of these structural, physiological and biochemical characteristics of sperm have been routinely examined through techniques to evaluate and determine the quality of the semen and characterizing the most elite bulls in terms of fertility potential. These sperm attributes can be evaluated *in vitro* at molecular and cellular levels. Semen evaluation using light microscopy provides useful information at cellular level (morphology and motility), but is subjective which limits its prognostic value for the reproductive performance of males or the outcome of assisted fertilization. However, at molecular level sperm characteristics (mainly abnormalities) can be detected by an

array of biomarkers including fluorescent markers for sperm plasmalema integrity, permeability and stability, acrosomal status, sperm mitochondrial integrity and activity, capacitation status and membrane fluidity of sperm, altered sperm chromatin or DNA integrity, apoptotic, oxidative stress and lipid peroxidation events in sperm, and antibodies detecting proteins that are either up- or down-regulated in defective spermatozoa (Figure 1-1) (Sutovsky and Lovercamp, 2010). Using flow cytometry (FC), the majority of these biomarkers can be efficiently evaluated (Martínez-Pastor *et al*., 2010). Flow cytometry enables objective measurement of these biomarkers (individually or simultaneously) in semen through automated, high throughput and rapid methods (Gillan *et al*., 2005; Hossain *et al*., 2011).

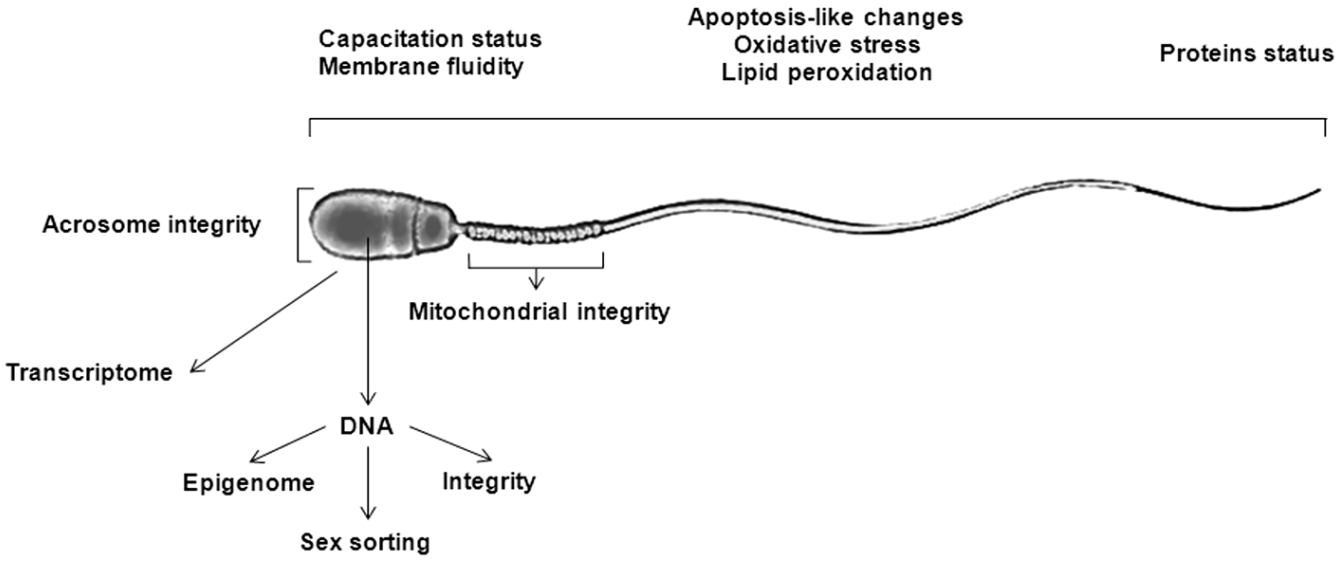

Figure 1-1 Different spermatic characteristics that can be evaluated at the molecular level to either assess sperm quality or gender selection.

1.2.1.1.1.1. Current applied ARTs for cattle sperm

1.2.1.1.1.1.1. Breeding soundness evaluation (BSE) and estimation of sire fertility

Recently, bull fertility has received increasing attention as the results of artificial insemination (AI) are declining in highly selected dairy cattle populations (Karoui *et al*., 2011). One of the most important challenge in AI industry is to identify bulls producing large numbers of fertile sperm and accurately predicting fertility of dairy bulls with apparently normal semen (Foote, 2003). Breeding soundness which refers to a bull's ability to get cows pregnant can be classically evaluated to identify bulls with substantial deficits in fertility, but does not consistently identify sub-fertile bulls (Kastelic and Thundathil, 2008). There is urgent need for accurate biomarkers of fertility to complement traditional breeding soundness evaluation, identifying and eliminating bulls with inferior fertility/semen quality (Shojaei *et al*., 2012; Sutovsky and Kennedy, 2013). Currently, relative sire fertility can be retrospectively estimated based on AI records for the calculation of the very general parameter of non-return rate (NRR) which is based on whether or not the female had a second insemination (indicative of a failed gestation causing a return to estrus) within a certain period (either 28, 56 or 128 days post-insemination). Usually 56 days after the first insemination (day of AI = Day 0) is currently the most commonly used time point for recording fertility in Canada and Europe (Rodríguez-Martínez, 2003). While, NRR provides reliable results over longer periods of time, it is not completely accurate and is highly prone to bias by several factors including unexplained variation. (Rodríguez-Martínez, 2003; Foote, 2003; Amann and DeJarnette, 2012). The NRR value represents the combined

contribution of sire and dam to successfully sustain gestation. Female factors such as age and lactation status are known to impact fertility as heifers are more fertile than cows. In USA, the estimated relative conception rate (ERCR) was used from 1986 to 2008. It was based on 70-day NRR of an AI service sire relative to service sires of herd-mates and provided more reliable fertility estimates for bulls through exclusion of services to cows that had left the herd (Cornwell *et al*., 2006; Amann and DeJarnette, 2012). Since 2008, sire conception rate (SCR), phenotypic predictor of bull fertility, has been made available to dairy producers from USDA as a new and more accurate evaluation AI bull (service-sire) fertility in USA compared to ERCR. The SCR is based on 70 d post-AI confirmed pregnancy (or not pregnant) after ≥ 300 total inseminations (≥ 100 in last 12 mo) and reported as a sire's fertility deviation from the average fertility of a population (> 10 herds). (Kuhn and Hutchison, 2008; Kuhn *et al*., 2008; Norman *et al*., 2010).

1.2.1.1.1.1.2. Sperm attributes

1.2.1.1.1.1.2.1. Sperm count

The most traditional and “gold standard” method to count sperm is microscopic manual chamber counting using a hemocytometer which is a time-consuming and has several limitations such as variation between operators and hemocytometer designs. Several methods have been developed to precisely count the sperm, amongst which the flow cytometer approach was found to be the most precise, however, the need for appropriate semen dilution, flow rate adjustment, high cost and overestimation are its

drawbacks (Petrunkina and Harrison, 2010). Overall, frequency of use, size of sample required, the number of samples routinely assessed, species and cost are important factors to consider for standardizing a laboratory procedure for sperm counts (Prathalingam *et al*., 2006).

1.2.1.1.1.1.2.2. Sperm morphometry

Computer automated sperm head morphology analysis (ASMA) was introduced to objectively evaluate several sperm morphometric parameters (Gravance *et al*., 1996) and identify sperm subpopulations. Due to some technical challenges, inconsistency of the results and low predictive value for bull fertility assessment, so far it has received little attention specially in dairy bulls (Gravance *et al*., 2009; Hoflack *et al*., 2005; Jenkins and Carrell, 2012). However, recent attempts have been done to improve its performance (Vicente-Fiel *et al*., 2013).

1.2.1.1.1.1.2.3. Sperm motility

Motility is considered to be one of the most important characteristics of sperm indicating their viability and structural integrity as well as a good indication of their fertilizing ability (Gaffney *et al*., 2011). Traditionally, assessment of sperm motility has been based on subjective optical microscopic evaluation of parameters , such as population and individual motility which resulted in 30 to 60% variations of motility parameters of the same ejaculates (Verstegen *et al*., 2002). Computer-assisted sperm analysis (CASA) is a precise, automated and objective method to evaluate sperm head motion characteristics which provides several sperm kinematic parameters. A combination of some of these parameters have been found to be correlated with

fertility as well as classification of different sperm sub-populations with specific patterns of movement in a given semen based on path velocity or hyperactivated motility in bulls (Shojaei *et al*., 2012; Muiño *et al*., 2008; Kathiravan *et al*., 2011).

1.2.1.1.1.1.3. Adding “new” parameters to sperm quality evaluation

Although the aforementioned standard seminal parameters like motility, morphology as well as sperm concentration measurements are the most currently implemented methods to evaluate the quality of a bull’s ejaculate, they are insufficient as some service bulls with good semen quality parameters still provide low NRR values (Kastelic and Thundathil, 2008; Petrunkina *et al*., 2007). It is not known if these are genetically-based incompatibility or are associated with other spermatic characteristics. As such, several groups are currently looking into integrating different assays to complement the current set of parameters. Most of these additional semen quality parameters are based on older assays developed to study spermatic physiology (Table 1-1). They mainly target the assessment of the integrity of membranes as they play crucial roles in the spermatic functions for capacitation and AR or of the mitochondrial potential needed to sustain motility and also of the integrity of genetic material itself as DNA fragmentation could lead to embryonic failure (Figure 1-1).

1.2.1.1.1.1.4. Sperm Sorting

Using flowcytometric approaches, sperm can be sorted based on the sex or several parameters and markers (Martínez-Pastor *et al*., 2010).

1.2.1.1.1.1.4.1. Sex sorting

The availability of sexed semen in dairy cattle has been eagerly anticipated for many years and is growing in adoption (Weigel, 2004) such that sex selected sperm straws have become a new product offered by many AI Centers (Garner and Seidel Jr, 2008). Sex sorting separates semen into fractions enriched with either X- or Y-bearing sperm. One of the most efficient and widely used methods for sex sorting sperm is based on fluorescence-activated cell sorting (FACS) and staining sperm with a DNA-binding fluorescent dye to separate sperm based on the quantity of the DNA content. The bovine X-chromosome bearing sperm contain 3.8% higher DNA than Y-chromosome bearing sperm which allows their separation by FACS (Weigel, 2004; Garner, 2006; Seidel, 2007). The demand is for sperm containing an X chromosome for the production of replacement heifers. Despite current advances (Sharpe and Evans, 2009), sex sorting is not completely accurate (with 85 to 95% of the sperm containing the desired chromosome), and the slow procedure, compromises viability, motility, lifespan, and fertility potential of the sex-sorted sperm (Vazquez *et al*., 2008; Seidel, 2012; Wheeler *et al*., 2006). However, this technology is continuously being improved and is expected to be more widely available and used in the near future (De Vries *et al*., 2008; Garner and Seidel Jr, 2008; Sharpe and Evans, 2009).

1.2.1.1.1.1.4.2. Marker-based sorting of fertile spermatozoa

Many techniques were developed to isolate sperm capable of fertilizing oocytes especially for the context of medically assisted reproduction (Ickowicz *et al*., 2012). Recent studies on human sperm proved that using different fluorochromes and FACS,

sperm can be sorted based on apoptosis (Annexin-V) (Hoogendijk *et al*., 2009) as well as apoptotic and dead sperm (YO-PRO)(Ribeiro *et al*., 2013). Magnet-activated (or magnetic-bead activated) cell sorting (MACS) technique is another sorting method (immunomagnetic) which eliminates apoptotic sperm from a sperm suspension through Annexin V-conjugated paramagnetic microbeads (Grunewald *et al*., 2001; Said *et al*., 2008; Dirican, 2012). Overall, these testify the possibility that sperm can be physically sorted depending on one or several parameters providing new possibilities for research and for future practical use in breeding (Martínez-Pastor *et al*., 2010).

Table 1-1 Summaries of current possible methods of sperm quality assessments.

Male		classic		modern		References
		Non-FC	**FC-based**	**Non-FC**	**FC-based**	
Sperm characteristics						
Enumerating		hemocytometry, spectrophotometry, and Microcells	-	fluorescent plate reading, and image analysis	flow cytometry	(Prathalingam *et al.*, 2006)
Morphometry		eosin-nigrosin staining	-	ASMA	-	(Gravance *et al.*, 1996)
Motility		Microscopic	-	CASA	-	
Sperm intactness (plasma membrane)	Integrity	eosin-nigrosin staining, HOST	CFDA, CFDA/PI, CMFDA, CAM	SYBR-14/PI,	SYBR-14/PI	(Jeyendran *et al.*, 1984; Resli *et al.*, 1983) ; (Garner *et al.*, 1986; Garner *et al.*, 1994)
	permeability and stability	-	-	-	Annexin V/PI, Hoechst 33342, YO-PRO-1, Merocyanine 540, SNARF-1, ethidium homodimer	(Hallap *et al.*, 2006; PeÑA *et al.*, 2005)
Acrosome integrity		Phase-contrast & DIC microscopy, Dyes for bright-field microscopy, Fluorescent labels	-	FITC-PSA/PNA	SYBR-14/PE-PNA/PI, FITC-PNA/PI, FITC-PSA/PI, LysoTracker™	(Cross and Meizel, 1989); (Thomas *et al.*, 1997a; Hinsch *et al.*, 1997; Thomas *et al.*, 1997b)
Mitochondrial status			R123-EtBr	-	JC-1, Mitotracker Green, Mitotracker Deep Red	(Evenson *et al.*, 1982) ;(Garner and Thomas, 1999; Garner *et al.*, 1997; Hallap *et al.*, 2005)
Chromatin intactness		AOT	-	Comet assay, CMA3	SCSA, TUNEL, CMA3	(Boe-Hansen *et al.*, 2005; Evenson, 2013; Evenson and Wixon, 2006; Rahman *et al.*, 2011; Simoes *et al.*, 2009; Tavalaee *et al.*, 2010)

Table 1-1. Summaries of current possible methods of sperm quality assessments.

	classic		modern		
	Non-FC	**FC-based**	**Non-FC**	**FC-based**	
Male					
Sperm characteristics					
Changes induced during capacitation	-	-	CTC	CTC, M540/Yo-Pro 1/Hoechst 33342, Fluo, Indo-1	(Pons-Rejraji *et al.*, 2009; Piehler *et al.*, 2006; Hallap *et al.*, 2006; Rathi *et al.*, 2001; Thundathil *et al.*, 1999)
Apoptotic-like changes	-	-	-	Annexin V/PI, YO-PRO-1	(Anzar *et al.*, 2002; Martin *et al.*, 2004)
Detection of oxidative stress and lipid peroxidation	TBARS assay	-	-	H2DCFDA, HE, MitoSOX, BODIPY probes	(Hossain *et al.*, 2011; Bansal and Bilaspuri, 2011)
sperm surface targets	-	-	-	PR, ubiquitin-PNA, pY	(Odhiambo *et al.*, 2011; Gadkar *et al.*, 2002; Piehler *et al.*, 2006)

Abbreviations: **AOT**: Acridine orange test , **BODIPY**: 4-bora-3a,4a-diaza-s-indacene , **CAM**: calcein acetomethyl ester , **CASA**: Computer Assisted Sperm Analysis, **CFDA**: 6-carboxyfluorescein diacetate, **CMA3**: chromomycin A3, **CTC**: chlortetracycline, **EtBr**: ethidium bromide, **FC**- : Flowcytometry , **FITC**: Fluorescein isothiocyanate , **H2DCFDA**: 2',7'-dichlorodihydrofluorescein diacetate , **HE**: hydroethidine, **HOST**: hypo-osmotic swelling test, **JC-1**: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide , **M540**: Merocyanine 540, **PE**: phycoerythrin, **PI**: propidium iodide, **PNA**: peanut agglutinin, **PR**: progesterone receptor, **PSA**: pisum sativum agglutinin, **pY**: phosphotyrosine, **R123**: rodamine 123, **SCSA**: sperm chromatin structure assay , **SNARF-1**: seminaphtorhodafluor-1, **SYBR14**: Membrane-permeant fluorescent nucleic acid stain , **TBARS**: Thiobarbituric acid reactive substances , **TUNEL**: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

1.2.1.1.1.1.5. Artificial insemination (AI)

Due to the ease of access and rate of production of male compared to female gametes, reproductive technologies have initially focused on maximizing benefits from artificial insemination (AI). More than two centuries ago (1784) the first successful insemination was performed in dog, however, 100 years later it had been used for studies in rabbits, dogs and horses (Funk, 2006). Early 20th century Ivanow established AI as a practical procedure in farm animals and later was followed by other scientists mainly in Europe. Modern development of AI in dairy cattle was initiated over 70 years ago in USA by establishing AI cooperatives and in the 1980s, tremendous growth occurred because of strong exports markets, especially for Holstein semen (Funk, 2006). Artificial insemination has been considered as the first remarkable and most widely used biotechnology applied to improve reproduction and genetics of farm animals, and because of its worldwide acceptance it opened the doors for other reproductive biotechnologies such as semen evaluation techniques, semen freezing and sexing, bull sexual behavior and sire power, genetic selection of bulls for milk and detection of estrus, synchronization, and timing of insemination (Foote, 2002; Cardellino, 2003). Currently based on all of the collected and analyzed data the trend in AI industries would be on the following: breeding for long-lasting, durable, and profitable dairy cattle through shifting emphasis from production (*i.e.,* yield) to nonproduction traits; selection based on fertility potential; inbreeding for purebred dairy breeds and crossbreeding to overcome inbreeding depression within the pure breeds (Funk, 2006).

1.2.1.1.1.1.6. Sperm cryopreservation

Cryopreservation in general is a procedure by which cells (gametes, embryos and somatic cells) are suspended in a solution of salts and a low-molecular-weight (low-MW) organic compound, cooled to very low subzero temperatures (usually −196°C in liquid nitrogen), stored for some theoretically unlimited period of time, then warmed and recovered to resume their normal function (Leibo and Pool, 2011). Semen cryopreservation is extensively used by the dairy cattle AI industry for conservation and distribution of animal genetic resources and was the main reason for rapid growing of bovine AI industry since early 1950s (Funk, 2006). Contrary to many other species, bull sperm are characterized by superior cryoresistance, because of its physiology, biochemistry and structure which enable them to efficiently survive cryopreservation (Holt, 2000a; Słowińska *et al*., 2008). This cryotolerance and the anatomy and physiology of sperm transport in bovine female reproductive tract (Holt, 2000b) led to remarkable success with cryopreservation of bull semen enabling worldwide distribution of selected male genetics as cryopreserved semen straws. Currently several methods are available for semen cryopreservation (Barbas and Mascarenhas, 2009; Vishwanath and Shannon, 2000). Despite all of the advances, improvements in the field of cryoprotection are still ongoing due to the low survival rate (30- 50% viability) as well as approximately seven-fold reduction in fertilizing ability of post-thaw sperm using the current methods (Benson *et al*., 2012; Muiño *et al*., 2007; Hu *et al*., 2011; Sullivan, 2004; Watson, 2000). Sperm post-thaw survival depends on several factors such as physiology, biochemistry and structure of sperm, storage temperature,

cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control (Barbas and Mascarenhas, 2009). The ejaculated semen is composed of a heterogenous cohort (*e.g.* different morphology, motility and viability). This heterogeneity is believed to be an important aspect of fertility where a more heterogeneous gametic population will offer a larger window of opportunity for successful fertilization since the time of estrus, insemination and ovulation occur over a variable time window (Curry, 2000). Hence, there have been always a subpopulation of sperm which are more sensitive than others to environmental conditions and various stresses such as cryopreservation, leading to survival, cell death or functional impairment which can be detected in several assays (Watson, 1995). In addition to within-species variations, sensitivity to cryopreservation is also known to exhibit some variance within the individual bull population (Lisa and Paul, 2002). Cryo-damage in sperm has been shown to cause, premature capacitation and induced DNA damage that would compromise embryonic development (Barbas and Mascarenhas, 2009; Bailey *et al*., 2000). In AI centers, the ultimate safeguard in a Quality Control Program is the post-thaw semen evaluation program (Amann and DeJarnette, 2012).

1.2.1.1.1.1.7. Improving male fertility through integration of the “omics”

The recent development, implementation, and acceptance of genomic evaluations (based first on the BovineSNP50 Bead Chip) have great impacts on AI industry and breeding, such that currently all young bulls entering the major AI centers are preselected based on genomic evaluations resulting in extensive marketing of 730 days

old bulls (Wiggans *et al*., 2011). However, genomics advances in terms of male fertility have not been significant and currently there is limited information on genes associated with bull fertility (Peñagaricano *et al*., 2012). Several studies using genome-wide analysis, comparative genomics, SNP identification (whole-genome SNP Chip using BovineSNP50 Bead Chip or other methods plus Imputation) and candidate pathway approaches showed that bull fertility is influenced by genetic factors (Khatib *et al*., 2010; Li *et al*., 2012; Blaschek *et al*., 2011; Fortes *et al*., 2012; Peñagaricano *et al*., 2012; Peñagaricano *et al*., 2013; Feugang *et al*., 2009). Although the prominent place of AI in animal breeding where a single male has greater impact on genetic improvement than any female, study of the genetic variation behind the expression of male reproductive traits have received less attention than the cow (Li *et al*., 2012). The few studies targeting the identification of quantitative trait loci (QTL) or of molecular markers for male reproductive traits only found weak association, indicating the importance and needs for replications and further studies (Casas *et al*., 2010; Druet *et al*., 2009). The difficulty in finding genetic markers of fertility is likely attributable to the complex nature of its etiology in addition to the challenging phenotypic data collection where gestational failure alone is actually the combined result of different phenotypes. Hence, it is expected that the industry-wide data collection and analysis to evaluate genetic merit would be the most important tool for genetic progress in the future (Powell and Norman, 2006). Increasing the level of precision at which the genome is mined using high-density or whole-genome SNP chip and or even specially designed ‘fertility chip' might be future tools to better identify genomic fertility-

markers and characterize the fertility of elite sires (Amann and DeJarnette, 2012). Alternate perspectives include the study of gene products such as RNA and proteins.

Recently, sperm transcriptome studies in dairy bulls have received increasing attention but remain controversial (Lalancette *et al*., 2008). Some studies found sperm mRNA (Gilbert *et al*., 2007; Card *et al*., 2013; Feugang *et al*., 2010; Kasimanickam *et al*., 2012; Arangasamy *et al*., 2011) and microRNAs (Govindaraju *et al*., 2012) may have potential to be applied as molecular biomarkers for male gamete quality and bull fertility.

Recently, proteomics approaches specially 2D-PAGE coupled with mass spectrometry (MS) technique, have been increasingly used on fertile and sub fertile dairy bulls to identify candidate proteins as biomarkers associated with bull fertility potential which are differentially expressed in sperm membrane (Park *et al*., 2012; D'Amours *et al*., 2010; Moura *et al*., 2006a; Gaviraghi *et al*., 2010), seminal plasma (Kumar *et al*., 2012; Killian *et al*., 1993), accessory sex gland fluid (Moura *et al*., 2007; Moura *et al*., 2006b) or epididymal fluid (Moura *et al*., 2006a).

Epigenetics is the study of potential heritable changes in gene function that occur independently of alterations to primary DNA sequence (Kiefer, 2007; Bernstein *et al*., 2007b). Epigenetics may help find missing causality and missing heritability of complex traits and diseases which genomics approaches have to date been unable to address (Gonzalez-Recio, 2012). Among all of the epigenetic effectors DNA methylation is the most intensively studied and the most stable type of the epigenetic modification modulating the transcriptional plasticity of mammalian genomes (Eckhardt *et al*., 2006;

Suzuki and Bird, 2008b; McGraw *et al*., 2013b). Recent studies showed that sperm is heavily methylated compared to oocytes with approximately 85% vs. 30% global CG methylation levels respectively (Seisenberger *et al*., 2013). Sperm DNA methylation mainly occurs outside of the promoters regions (intergenic) similar to embryonic stem cells and embryonic germ cells (Schagdarsurengin *et al*., 2012). Developing embryos require appropriate epigenetic marks from sperm (Jenkins and Carrell, 2012). This emphasizes the importance and crucial role of the sperm epigenome in successful fertilization, embryo development and full term pregnancies. Recently there is an emerging interest and demand to study the sperm epigenome in livestock to improve breeding through identification of potential fertility biomarkers, environmental effects and transgenerational epigenome inheritance.

1.2.1.1.1.2. Conclusions

Accurately identifying bulls that produce large numbers of fertile sperm is critically important for the AI industry. From an AI Center standpoint, profits are associated with the lineup of bulls that are under contract which dictates the demands but also the quality of the semen that is produced to maximize the number of straws per ejaculate in order to efficiently provide the offer to meet the demands (DeJarnette *et al*., 2004). Hence, the AI Centers are trying to define characteristics on the sperm population to estimate fertility potential before putting the sire in service. Simple assessment of mating ability and physical examination of a sire cannot predict the potential fertility of a sire. Rather, based on the current available knowledge and modern technologies, there is strong consensus that a combination of laboratory methods testing a large,

heterogeneous sperm population for characteristics relevant to both fertilization and embryo development should be applied (Rodriguez-Martinez and Barth, 2007; Kastelic and Thundathil, 2008; Rodríguez-Martínez, 2003).

1.2.1.1.2. Oocyte

1.2.1.1.2.1. Current applied ARTs for cattle oocytes

A lowering of dairy herd fertility especially in high producing cows has been reported worldwide. The exact nature of this decline is still not clear but some mechanisms have been suggested (Lucy, 2007; Dobson *et al*., 2007; Lucy, 2001; Pryce *et al*., 2004; Diskin *et al*., 2006; Evans and Walsh, 2011; Walsh *et al*., 2011). Despite male whose testes and mainly sperm attributes play central roles in fertility assessments for the paternal contribution in fertility (Krawetz, 2005), in female in addition to oocyte (maternal gamete) and ovaries, female endocrine function is of great importance in physiology and management of cow reproduction and its maternal role in fertility.

Female endocrine function and communication between the hypothalamic-pituitary-ovaries axis in cows play a crucial role for normal oocyte development, ovulation, fertilization, early embryo development, implantation, fetal development and parturition. Their manipulations have been of great aspect in dairy cow reproductive managements. With current advances in AI some proven sire can produce up to 50,000 offspring in 1 yr (Funk, 2006) while the best females have much limited impacts with generally less than 100 offspring. In addition, the current important limiting factor for the efficiency of dairy production systems is failure of cows to successfully establish

pregnancy after AI (Evans and Walsh, 2011). Together, these emphasize the importance of maternal contribution in fertility and breeding.

Oocyte quality is instrumental for fertility. Oocytes are the largest cells in the body and are produced through oogenesis which is a complex process regulated by a vast number of intra- and extra-ovarian factors in the ovaries (Sánchez and Smitz, 2012). Contrary to sperm, oocytes are limited resources and cattle normally release only one oocyte at ovulation. The bovine follicular development occurs through two or three consecutive waves of follicle growth during the estrous cycle (lasting approximately 21-28 days) (Figure 1-2). Each follicular wave includes the initial recruitment of a group of follicles each containing a single oocyte arrested at the GV stage, from which one is selected to pursue its growth, resume meiosis and eventually ovulate, while the others undergo atresia (Adams *et al*., 2008; Shoubridge and Wai, 2007; Mapletoft *et al*., 2009). The bovine follicle initiates growth following recruitment from the ovarian reserve several months before the observable antral stage development that can be monitored by ultrasonography. During antral growth, the follicle increases in size and volume (300 to 400 fold in diameter from the primary (50 µm) to the preovulatory (15-20 mm) stage (Rajakoski, 1960). The bovine oocyte and follicle grow in parallel until the follicle reaches a diameter of 3 mm; which the oocyte is fully grown (diameter plateaus at about 120–130 µm) at this stage and already harbor transcriptionally

inactive, highly condensed chromatin (Macaulay *et al*., 2011). The follicle can continue to grow up to 15–20 mm in diameter before ovulation (Fair, 2003; Fair *et al*., 1995).

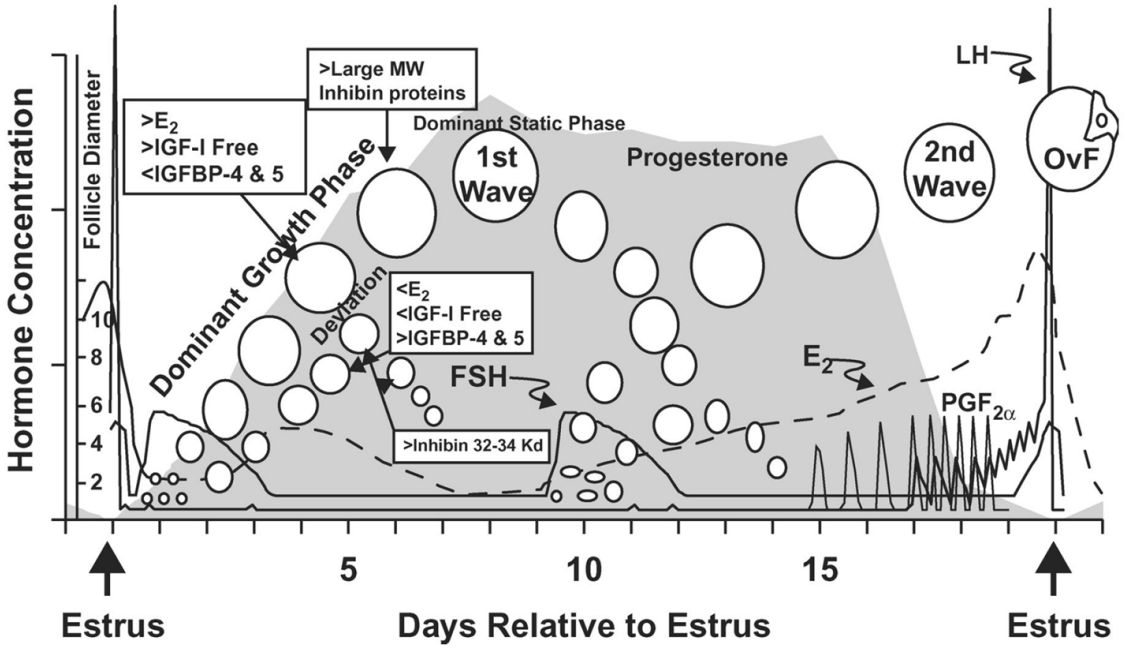

Figure 1-2 Ovarian follicular and corpus luteum development correlated with endocrine changes during the bovine oestrous cycle. E2, Oestradiol; IGFBP-4 and -5, insulin-like growth factor binding proteins 4 and 5; OvF, ovulatory follicle. (Reproduced from Moore and Thatcher, 2006, with permission.)

In addition to providing half of the nuclear genetic material, oocytes endow the embryo with almost all membrane and cytoplasmic determinants such as maternal RNAs, mitochondria and other organelles required for successful fertilization and embryo development and quality (Ferreira *et al*., 2009; Sirard *et al*., 2006). The prime example of such asymmetric contribution to embryogenesis is represented by mitochondrial inheritance. While one sperm has nearly 100 mitochondria, an oocyte has up to 100,000 mitochondria. The existence of a mitochondrial bottleneck has been established but still remains to be explained where as few as 0.01% of these

mitochondria in the oocyte actually contribute to the offspring of the next generation while selective destruction of paternal mitochondria leads to the maternally exclusive lineage of mitochondria (Shoubridge and Wai, 2007; Krawetz, 2005). Hence oocyte has a crucial role in embryogenesis compared to sperm and pregnancy establishment and maintenance through follicular determinants (Pohler *et al*., 2012; Geary *et al*., 2013).

Bovine oocytes similar to other mammals, initiate meiotic maturation during fetal life then arrest at diplotene stage of the first meiotic prophase, also called the germinal vesicle stage (GV) oocyte. Only fully grown female gametes individually enclosed in a large antral follicle will resume meiosis *in vivo* following the luteinizing hormone (LH) surges that initiates at puberty (Mehlmann, 2005). Oocyte maturation, involves complex and distinct, although linked, events of nuclear (chromosomal segregation) and cytoplasmic maturation (organelle reorganization and storage of mRNAs and proteins) (Ferreira *et al*., 2009). Over the years, nuclear maturation has been the most studied. During follicular growth, the oocyte remains at the GV stage but its chromatin undergoes remodeling which is concomitant with transcriptional silencing. The bovine oocyte exhibits unique patterns of chromatin configurations (from GV0 to GV3) distinct of the mouse (Liu *et al*., 2006; Lodde *et al*., 2008). Following the endogenous LH surge or following oocyte extraction from the follicle, meiotic resumption is morphologically characterized by germinal vesicle breakdown (GVBD) which is followed by progression to Metaphase-I (MI) manifested by extrusion of the first polar body, and then meiotic cell cycle gets arrested a second time at metaphase-II (MII) until fertilization. Cytoplasmic maturation is more discreet and less understood. It consists of three main

events: (1) redistribution of cytoplasmic organelles, (2) dynamics of the cytoskeletal filaments, and (3) molecular maturation (Ferreira *et al*., 2009).

It is the sequence of all of these cellular and molecular events that leads to the proper preparation of the oocyte to successfully sustain early development. The acquisition of the intrinsic developmental potential by the oocyte is referred to as its developmental competence. The nature and underlying mechanisms of oocyte developmental competence have yet to be elucidated (Duranthon and Renard, 2001). It is known that during oogenesis the growing antral follicle oocyte's cytoplasm enriches with stabilized transcripts (Gilbert *et al*., 2009b) which can be stored for several days before they are used to support early embryonic development at least until embryonic genome activation (EGA) (8-16 cell stage embryo in bovine) after 3-4 cell cycles (Macaulay *et al*., 2011). Considering that within an *in vitro* context where hundreds of collected cumulus-oocytes complexes can be submitted to the same developmental opportunity and that most embryonic losses occur before the developmental stage at which the embryonic genome is known to activate, many studies are looking at the oocyte's transcriptome to identify the potentially lacking transcripts that fails to support protein synthesis before the embryo take-over (Nivet *et al*., 2012; Nivet *et al*., 2013; O'Shea *et al*., 2012; Bunel *et al*., 2013; Assidi *et al*., 2010; Assidi *et al*., 2008)

As mentioned, the mechanisms by which an oocyte acquire its potential for embryonic development is not known but it is clear that the oocyte and its surrounding somatic cells have a series of paracrine and junctional interactions (through transzonal

projections) which allows for the exchange of many regulatory signals that control oocyte metabolism, cytoskeletal remodeling, cell cycle progression and fertilization, all of which are key events for initiating and sustaining early embryogenesis (Li and Albertini, 2013; Albertini *et al*., 2003). In spite of remarkable advancements in reproductive biology and the array of techniques for dairy cattle reproduction (Moore and Thatcher, 2006) oocyte quality in cattle is poorly defined and the effects of metabolic disorders and disease in the post partum period on oocyte quality are not well understood (Walsh *et al*., 2011).

Over several decades, a number of therapies have been developed that manipulate ovarian follicle growth to improve oocyte quality and conception rates in cattle (Baruselli *et al*., 2012a). Many of the current reproductive technologies in application or being developed are aiming at increasing and maximizing the maternal contribution to genetic improvement efforts. The following aims at portraying the current and emerging technologies that might increase the rate of genetic improvement in dairy herds.

1.2.1.1.2.1.1. Synchronization

Ovarian follicular growth and development is of great importance as there are a number of intriguing aspects of reproductive physiology that differ somewhat in lactating dairy cows and may be related to the control of follicular wave dynamics. Most of those changes become more dramatic as milk production increases. Synchronization mainly on ovulation have been applied currently to overcome reproductive inefficiencies in dairy cows (Wiltbank *et al*., 2011). Furthermore,

synchronization programs (including synchronization of estrus and/or ovulation) have been successfully used in many dairy cows management systems to decrease intervals from calving to conception (Macmillan, 2010).

Any method that will synchronize estrus will also synchronize the time of ovulation; however, synchronization may not be sufficient to yield good success with timed AI. Synchrony programs such as Ovsynch (Figure 1-3) and its various modified protocols have been developed for dairy cattle and some showed more efficiency over others (Rabiee *et al*., 2005).

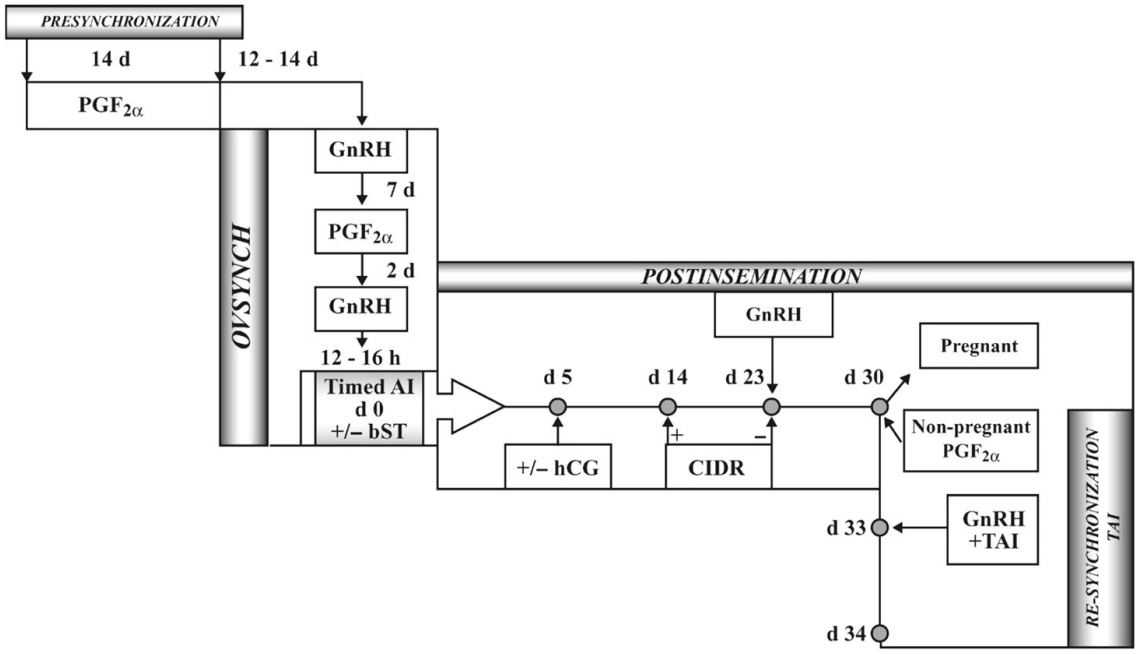

Figure 1-3 Reproductive management alternatives to improve reproductive performance of lactating dairy cows with the use of presynchronization. Ovsynch for timed artificial insemination (TAI), post-insemination endocrine treatments and resynchronization for TAI. Endocrine treatments involve injection of bovine somatotropin (bST) at TAI and injection of human chorionic gonadotropin (hCG) at day 5 after TAI. Resynchronization of non-pregnant cows involves the insertion of an intravaginal progesterone device (CIDR) between days 14 and 23 after artificial insemination and injection of GnRH at day 23 at the time of CIDR withdrawal. Cows diagnosed non-pregnant at day 30 receive an injection of PGF2α, and on day 33 are injected with GnRH and concurrently inseminated. (Reproduced from Moore and Thatcher, 2006, with permission.)

1.2.1.1.2.1.1.1. Estrus/ovulation synchronization

In using AI, estrus synchronization becomes an important tool, mainly because of its application to improve the efficiency and accuracy of estrus detection (heat) or by reducing the labor requirement for estrus detection (Xu, 2011a). However, using estrus synchronization, estrus detection is problematic in modern herds of high producing cows as they may display less obvious behavioral (symptoms of estrus) and shortened estrus period (Evans and Walsh, 2011; Walsh *et al*., 2011; Roelofs *et al*., 2010; Macmillan, 2010; Wiltbank *et al*., 2011). Therefore, due to all these challenges associated with efficient estrous detection, the current trend is toward ovulation synchronization or induction which allows timed breeding without the need for estrus detection (Xu, 2011a; Macmillan, 2010). Ovulation synchronization (synchronization of follicle wave emergence and growth which is deliberately terminated by induction of ovulation through administration of luteinizing hormone) is the key component of recent protocols for AI and embryo transfer, namely fixed-time artificial insemination (FTAI) or fixed-time embryo transfer (FTET) (Mapletoft *et al*., 2009). These technologies provide organized approaches to enhance the use of AI or ET.

1.2.1.1.2.1.1.2. Fixed-timed artificial insemination (FTAI)

Currently, FTAI programs are widely used as an integrated part of reproductive management strategies with satisfactory pregnancy rate in many parts of the world with various protocols (Baruselli *et al*., 2012b). The key point is synchronization of the follicular wave at initiation of the program. GnRH-based (Ovsynch program) (Figure 1-3) and Estradiol/Progesterone-based programs for ovulation synchronization

are the most common programs currently used (Figure 1-4) (Wiltbank *et al*., 2011). The GnRH-based program is the original FTAI protocol introduced in 1995 (Pursley *et al*., 1995). It starts with GnRH administration (to synchronize a new follicular wave and to assure the presence of a corpus luteum (CL) during the program), followed 7 d later by Prostaglandin F2α (PGF) treatment (to regress the CL and allow the dominant follicle to proceed toward ovulation), 56 h later by a second GnRH injection to synchronize ovulation, and finally timed artificial insemination (TAI) at 16 h after the second GnRH injection (Wiltbank *et al*., 2011). Presynch–Ovsynch, Co-synch, Heatsynch and Selectsynch are recent variations to Ovsynch which keep the interval of 7 days between the first GnRH injection and the one following PGF used in Ovsynch program (Macmillan, 2010). Recently, there has been some advancements in reproductive management strategies for timed insemination (Xu, 2011b). A representative typical Estradiol (E2)/Progesterone(P4)-based program begins with a P4-releasing device inserted into the vagina (mainly controlled intravaginal drug release (CIDR) inserts) and treatment with E2 (to regress follicles that are present on the ovaries and approximately 3 to 5 d later, there is initiation of a new follicular wave) followed 7 d later by PGF treatment (to regress any CL). One day later, cows are given E2 cypionate and the CIDR is removed (to synchronize ovulation), with FTAI 48 h later (Wiltbank *et al*., 2011).

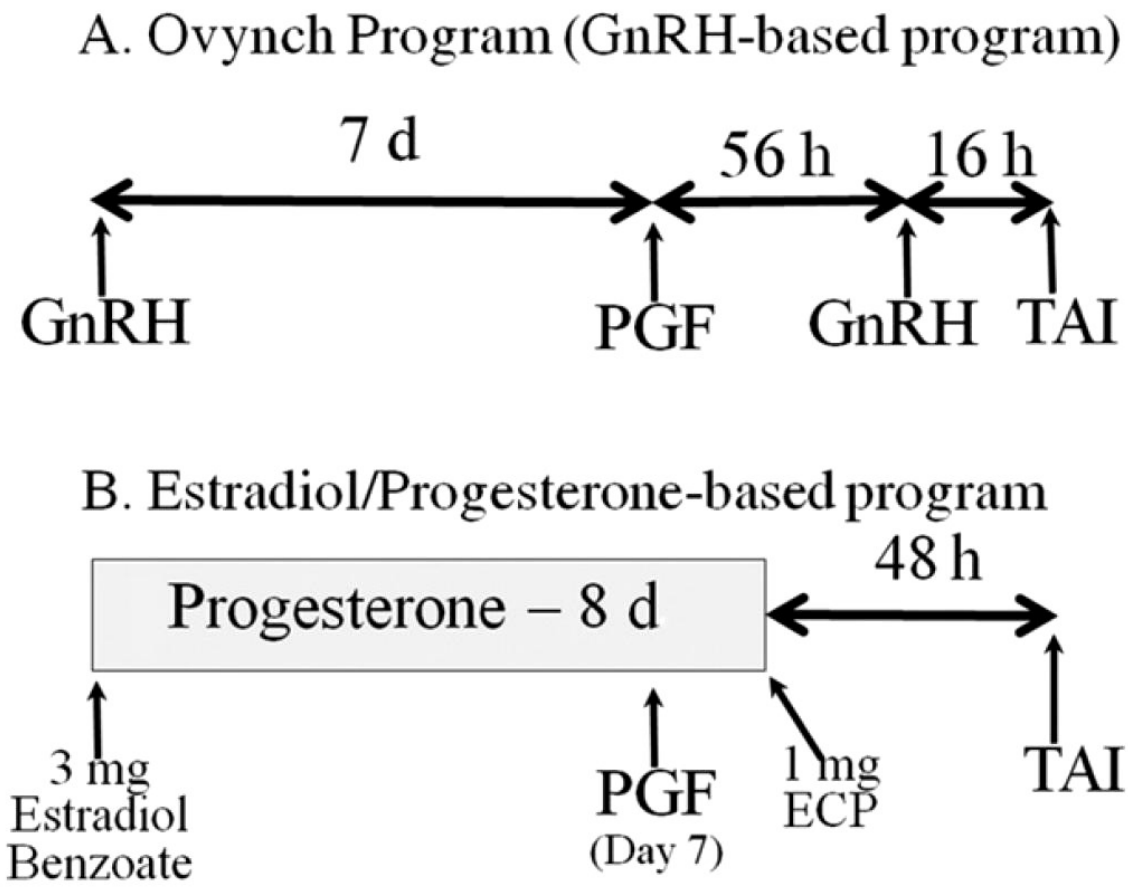

Figure 1-4 Typical programs designed to synchronize ovulation of the dominant follicle and facilitate success with a timed AI (TAI) protocol. (A) Representation of the typical Ovsynch programme that begins with GnRH treatment, followed 7 days later by PGF treatment, 56 h later by a second GnRH treatment to synchronize ovulation, and finally TAI at 16 h after the second GnRH. (B) Representation of a typical E2/P4-based programme which begins with insertion of a P4-releasing device and treatment with E2 (in this case 3 mg E2-benzoate), followed 7 days later by PGF treatment. One day later, cows are given 1 mg of oestradiol cypionate (ECP), with TAI 48 h later. (Reproduced from Wiltbank *et al.*, 2011 with permission).

1.2.1.1.2.1.2. *In vitro oocyte* maturation (IVM)

In general, oocyte maturation refers to the final stages of oogenesis when the oocyte acquires the properties required to initiate and sustain embryonic development. These processes require oocyte undergoes significant series of changes at both ooplasm and nuclear and molecular levels, namely “cytoplasmic” and “nuclear” and “molecular” maturation, respectively (Albertini, 2012; Coticchio *et al*., 2015). Very recently, it has been shown that the cumulus cell (somatic cells encompass oocyte) also contribute transcripts to the oocyte through transzonal projections (TZPs) (Macaulay *et al*., 2014) before the initiation of meiosis resumption suggested for the acquisition of developmental competence (Macaulay *et al*., 2015). As an essential requirement for *in vitro* production of embryo, *in vitro oocyte* maturation (IVM) is necessary. IVM is an assisted reproductive technique in which the collected oocyte (either by ovum pickup or from slaughterhouse in case of cattle) placed in culture maturation medium and incubated *in vitro* for a certain period of time (up to 24 hr). During IVM oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation (resume the first meiotic division) (Sirard and Coenen, 2006). However, studies have been shown that *in vitro* vs. *in vivo* mature oocytes have less oocyte competency due to variation in several factors such as transcription (Labrecque and Sirard, 2014) and epigenetics (Franciosi *et al*., 2012; Urrego *et al*., 2014).

1.2.1.2. Early embryo

1.2.1.2.1. Current applied ARTs for early embryos

1.2.1.2.1.1. Fixed-Time Embryo Transfer (FTET)

Widespread application of embryo transfer has been limited due to inefficiency of oestrus detection in dairy herds, especially in recipients, although it has been used commercially for many years. Fixed-time embryo transfer (FTET) allows ET independent of oestrus detection and has been applied as the most useful alternative to increase the sufficient number of well-synchronized recipient cattle utilized in an ET program (Rodrigues *et al*., 2010). Such an increase in proportion of recipients reported to cause higher pregnancy rate without compromising the conception rate in case of a single FTET (Baruselli *et al*., 2010). The basic protocol for FTET is similar to that of FTAI (Ovsynch), with exception to perform embryo transfer at 8 d after the 2nd GnRH (d 17) instead of timed-AI at 16-20 h after the 2nd GnRH injection. Improvements in protocols are ongoing (Bó *et al*., 2011; Baruselli *et al*., 2011b).

1.2.1.2.1.2. Multiple Ovulation and Embryo Transfer (MOET)

Being a mono-ovulant, a cow generally produces one offspring in each nine months gestation. To increase the dissemination of high genetic merit females, it is common to manipulate the endogenous hormonal response and supplement it with exogenous hormones to bypass the natural negative hormonal feedback that limits ovulation to exactly one follicle. Through hormonal manipulation of cow reproductive cycles, a number of oocytes can be simultaneously ovulated and fertilized by AI. Embryos may then be collected and graded according to their quality to either transfer into synchronized recipients of lesser genetic merit (which allows dairy producers to obtain

multiple offspring from genetically superior females) or be frozen for later transfer or sale (Moore and Thatcher, 2006). Generally, using superovulation the mean ovulation rate will be about 15 (ovulated oocytes) of which about 10 unfertilized ova/embryos will be recovered. From such cohort, 60–70% of the embryos will be of good quality and can be stored or transferred to recipients (Lonergan and Boland, 2011). Multi-Ovulations Embryo Transfers (MOET) breeding schemes have been widely established (~ 80% of commercial ET) in cattle (Rodriguez-Martinez, 2012). This technology has not reached optimality due to some limitations (mainly inefficiency of superovulation protocols to generate large homogenous cohorts of high developmental potential oocytes), however, currently several approaches to improve and simplify superovulation protocols in cattle have been developed (Mapletoft and Bó, 2011; Bo *et al*., 2010; Blondin *et al*., 2002; Nivet *et al*., 2012).

Compared to natural breeding, the use of MOET substantially increases the number of descent per animal thus increases genetic improvement rates when applied to elite animals by producing more embryos from genetically superior cows in shorter time periods. The standard approach involves the aforementioned hormonal regimen to induce and support follicular growth that will lead to multiple ovulations. The eggs are fertilized *in vivo* by artificial insemination and the embryos are allowed to develop for 6-7 Days until their exit from the oviducts to the uterine environment where they are collected by flushing the uterine lumen. The current most efficient embryo production strategy involves superovulation and collecting the immature oocytes by ultrasound guided transvaginal follicular puncture and aspiration (termed ovum pick-up (OPU)).

The oocytes are then matured and fertilized *in vitro* and the embryos are kept in *in vitro* culture until they reach the early blastocyst stage (about 7 days post fertilization). The combination of OPU and *in vitro* embryo production (IVP) can produce 3.4 more embryos per cow per year than conventional ET (Bousquet *et al*., 1999). Using OPU up to 1,000 oocytes may be collected and up to 300 *in vitro* produced embryos can be obtained annually per cow (Rodriguez-Martinez, 2012; Wu and Zan, 2012; Baruselli *et al*., 2011a). Future improvements of standard MOET or of the OPU-IVP based MOET aim at reducing the number animal handlings without compromising embryo production and pregnancy rates but most importantly to better control the inter-animal variance in ovarian response to the hormonal regimen. The extent of ovarian response being intrinsic to any animal and repeatable through time where low yielding animals are consistently providing low numbers of oocytes in response to a specific hormonal regimen, means to adapt hormonal stimulation protocols are needed (Keller and Teepker, 1990; Yaakub *et al*., 1999).

1.2.1.2.1.3. *In vitro* Production of Embryos

Since production of the first live calf by *in vitro* fertilization (IVF) in 1981 (Brackett *et al*., 1982) and from an *in vitro* matured (IVM) and IVF oocyte in 1986 (Hanada A *et al*., 1986) and IVM/IVF/*in vitro* culture (IVC) in 1998 oocyte/embryo (Sirard *et al*., 1988), significant advancements have been achieved in nonsurgical techniques of ovum pick-up (OPU), protocols, materials, culture media, etc. Nonetheless, the efficacy of *in vitro* embryo production is still suboptimal. Typically, under normal IVP conditions, >90% of retrieved oocytes are able to complete nuclear maturation (transiting from an

immature oocyte to a fertilizable egg), and of which 80% can be fertilized and reach the first cell division (cleavage stage). However, about a third of the collected oocytes will reach the late developmental stages (morula and blastocyst) *in vitro* (Mermillod, 2011). In cattle, it is at these later developmental stages, preferably at compacted morula or early blastocyst, that embryos are transferred into synchronized recipients. For breeders who want to generate as many offspring as possible from a single elite cow, IVF is currently the most efficient approach. Hence, a combination of MOET and IVF is applied by breeders for the genetically superior females such as those identified via genomic testing (Schefers and Weigel, 2012).

To maximize embryo production, oocyte developmental competence plays a critical role (Geary *et al*., 2013; Pohler *et al*., 2012; Sirard *et al*., 2006). This developmental potential intrinsic to the oocyte differs between species where ovulated mouse oocytes express about 80% developmental potential to reach the blastocyst stage *in vitro* whereas, in bovine, the developmental competence of immature oocytes collected from medium size follicles seldom display blastocyst rates higher than 33% (Geary *et al*., 2013; Pohler *et al*., 2012). So far, improving embryonic yield through a better control of oocyte maturation have proven to be complex since most of the compounds tested so far have yielded marginal results or are affected by a lack of consistency where blastocyst rates greatly fluctuate from one run to the other without modification to the procedures. Strategies to modulate oocyte *in vivo* quality prior to follicular aspiration have been developed. One involves withdrawal of growth stimulation about two days prior to oocyte aspiration. The rationale is that this

"coasting" period permits follicular differentiation where growth signal alone cannot. It has been successfully used in commercial embryo production settings (Blondin *et al*., 2002). Using this stimulation regimen, blastocyst yields have been increased from 50 up to 80% (Blondin *et al*., 2002; Nivet *et al*., 2012). In addition to the quality of the gamete, culture conditions have been shown to affect embryonic development and quality expressed as post-cryopreservation survival and/or post-transfer survival rates (Hansen *et al*., 2010).

On the down side, this higher rate of embryonic production through IVP is currently plagued by concerns regarding the quality of the produced embryos. Reports are indicating that IVP embryos lead to lower conception rates (30 to 40%) compared to AI or embryos recovered non-surgically from donor animals (50 to 70%); lead to higher rate of early embryonic mortality (mainly during the first 30 days); and are more prone to developmental aberrations such as the large offspring syndrome (LOS), abortions and dystocia (Moore and Thatcher, 2006).

Similar afflictions have been reported for human embryos produced *in vitro* and recently, there have been attempts and promising advances in reproductive medicine on embryo culture platforms to improve developmental rates and embryonic quality through modifications of the microenvironment (Swain and Smith, 2011; Smith *et al*., 2012a). There are also great incentives to select the best gametes and embryos to increase pregnancy success rates which will lower costs. Some of the promising approach are based on metabolomics by assessing the excreted metabolites in the

culture medium or through oxygen respiration measurement (Montag *et al*., 2013). Timing of the first zygotic cleavage is a valuable, non-invasive marker of embryonic competence in cattle (Lechniak *et al*., 2008). Recently, development in time-lapse cinematography (TLC) system has been considered to be a high promising, simple and non-invasive technology to objectively classify IVP embryos according to developmental competence. The TLC system enables simultaneous monitoring of embryo morphology and measurement of the length of each developmental stage, together with morphokinetics of embryo (Somfai *et al*., 2010; Kirkegaard *et al*., 2012; Sugimura *et al*., 2010).

1.2.1.2.1.4. Embryo sexing

Sex determination of embryos has been performed on site for more than a decade. It is routinely performed by veterinarians following collection of embryos. Embryonic biopsies are performed by taking away several cells from the trophectoderm (extra-embryonic tissue) that are destroyed to detect the presence of a specific sequence found on the Y chromosome by DNA amplification (Figure 1-5). Biopsied embryos can be kept alive in a portable incubator or frozen for later transfer. The most common hurdles are false negatives as many protocols do not include a positive control of amplification and when the reaction fails, it is by default determined to be a female embryo. The recurrent amplification of a single target can also lead to amplification carry over where template from previous amplifications contaminates stock solutions

or equipments which lead to false positives where all embryos are detected as males (Machaty *et al*., 2012).

It is common in laboratories sexing embryos to have only female technicians perform genotyping to avoid exogenous contamination from human source. The choice of candidate locus located on the Y chromosome can have an impact on PCR efficiency.

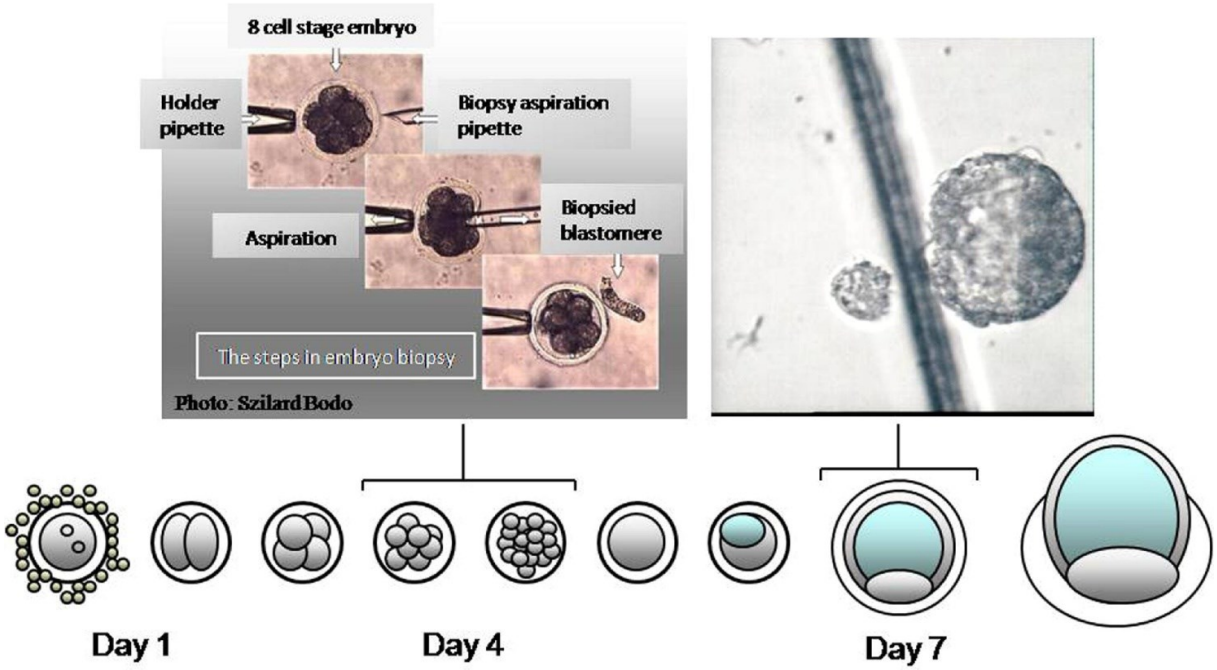

Figure 1-5 Biopsy of bovine embryo for sex diagnosis. (Reproduced from Machatya *et al*., 2012, with permission).

Traditionally, the Sex-determining Region on the Y chromosome (SRY) locus has been targeted for sexing. A multiplexed approach targeting an X chromosome sequence as positive control and a Y specific sequence has been shown to lead to more precise results. Sensitivity can be improved by targeting a repeated sequence found on the Y chromosome. Different means of DNA amplification and detection have been proposed. The Loop-mediated isothermal amplification (LAMP) reaction does not

require any expensive equipment and generates results in under an hour. The amplified product can be run on a gel for detection but the amplification of the target being very efficient, it leads to solution turbidity which can be used to detect the presence of amplification using a turbidity meter (Hirayama *et al*., 2004). In our hands, this method is efficient but has sometime given ambiguous results when turbidity is low. However, recent improvement permits detection with the naked eye without electrophoresis or a turbidity meter (Zoheir and Allam, 2010).

1.2.1.2.1.5. Splitting Embryos

Naturally, the frequency of monozygotic identical twins and, rarely, triplets are low in most mammalian species. Due to the commercial and financial interests the idea evolved of producing identical multiplets in farm animals. Through many attempts on different species, eventually in 1981 , Willadsen, S.M. *et al*. (Willadsen *et al*., 1981) developed an approach for cattle and horses, which was to recover post-compaction embryos nonsurgically and simply divide the embryos in two with a microblade (killing some cells in the process), and then transfer the resulting two demi-embryos back to uteri of recipients nonsurgically. Since then, the results showed this approach worked well, with pregnancy rates of approximately 50% per demi-embryo in cattle (Williams *et al*., 1984). Therefore, the main application of the embryo splitting has been to obtain approximately 50% more calves than by transferring them whole. However, the chance for producing lived identical twins is approximately 25% most of the time. It should be noted that through embryo splitting (cut into thirds) it is possible to occasionally

produce identical triplets resulting, but pregnancy rates per 1/3 embryo were too low to be practical, approximately 30% in skilled hands (Seidel, 2015).

Currently, except the very few highly valuable cases at commercial level, embryo splitting is not widely used despite its continued efficacy. One reason could be on the farm recovery and transfer the bovine embryo with minimal personnel (rarely more than two), making it practically difficult to perform this technique. Another very important reason and limitation for extensive application of embryo splitting is the lack of sufficient suitable recipients available at the time embryos are collected. Although, an alternative it would be possible to cryopreserve the hemi-section embryos for transfer at another time and place, but the double insult of splitting and freezing or freezing and splitting results in quite low pregnancy rates per demi-embryo making it impractical (Seidel, 2015) .

Furthermore, although splitting embryos postcompaction in most cases resulted in formation of two inner cell masses and is similar to how identical twins form naturally but recent data collected from cattle industry showed the generated identical twins does not necessarily have identical performances. The overall efficiency of cow embryo splitting (number of calves born per embryos bisected and transferred) can reach almost 60% (Rodriguez-Martinez, 2012).

1.2.1.2.1.6. Oocyte /Embryo Cryopreservation

As breeders wish to take the full benefit of elite cows it drives the development and increase the usage of assisted reproductive methodologies. The exports of

cryopreserved semen represent a significant market and to take the full advantage of both genders, the trade of cryopreserved embryos is expanding. Freezing bovine embryos is now common and with improved methodologies and cryoprotectant to limit embryonic damage, pregnancy rates can only be slightly less than those achieved with fresh embryos (Mapletoft and Hasler, 2005).

Controlled-rate freezing and vitrification are two basic cryopreservation techniques. Slow freezing commonly leads to intracellular ice crystallization and cell damage. Vitrification is the alternative method of cryopreservation (a non-equilibrium method) which uses an ultra-rapid cooling rate, using high concentrations of cryoprotectants which avoids water precipitation, preventing intracellular ice crystal formation. Vitrification is getting more popular, but has yet to achieve convincing results capable of widespread application. Oocyte and embryo cryopreservation can be performed for animal genetics conservation (Youngs, 2011; Saragusty and Arav, 2011; Prentice and Anzar, 2011).

1.2.1.2.2. Occurrence of Embryonic Death and Strategies to Improve Embryo Survival

While fertilization rates (> 80%) are not considered to be a main contributor to the poor fertility seen in dairy cows, embryo and fetal mortality are (Evans and Walsh, 2011; Sartori *et al*., 2009; Xu, 2011b). It is estimated that based on fertilization rates of 90%, embryonic and fetal loss (from fertilization to birth) may be up to 60% (Figure 1-6) while calving rates may only be 30%-40% in high yielding dairy cows (Moore and Thatcher, 2006; Diskin and Morris, 2008). Furthermore, fertility depreciation in dairy cows is believed to be caused by abnormal early embryo development rather than

fertilization failure (Lucy, 2007; Bamber *et al*., 2009) or failure of the cow to maintain the pregnancy (Bamber *et al*., 2009). In high-producing dairy cows, embryo mortality is the single biggest factor reducing calving rates. Poor oocyte quality (probably caused by the adverse metabolic environment) and by poor maternal uterine environment (probably caused by carry-over effects of uterine infection and low circulating progesterone concentrations) are possible reasons for embryo mortality (Evans and Walsh, 2011). Hence, embryonic mortality is a big challenge and can be distinguished as being early embryo or late embryo/foetal loss (Diskin *et al*., 2006; Santos *et al*., 2004).

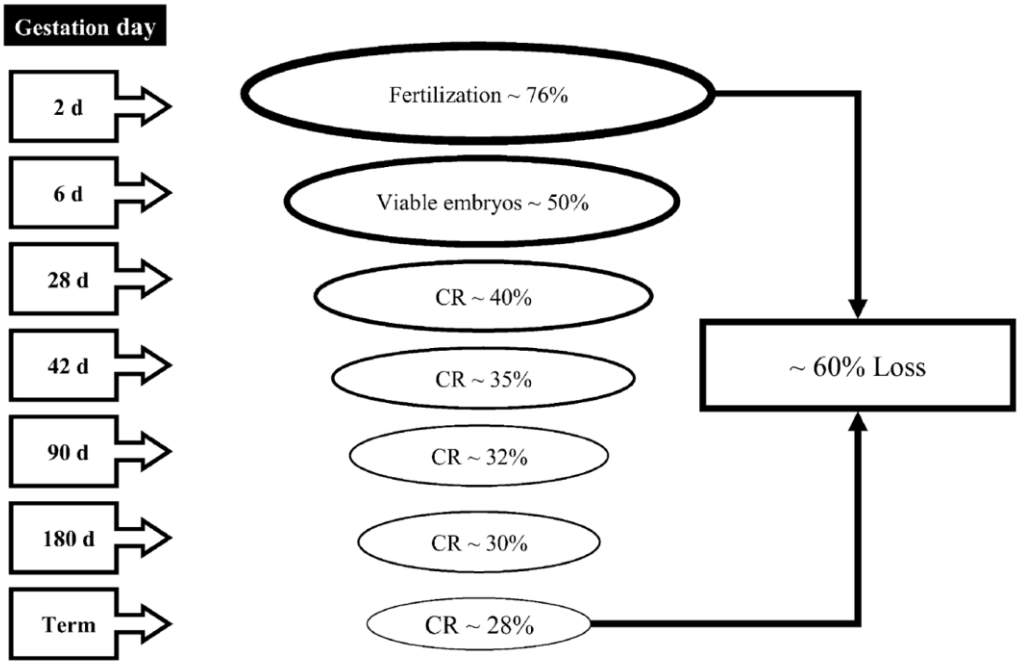

Figure 1-6 Timing and extent of pregnancy losses in the high producing lactating dairy cow. CR, conception rate. (Reproduced from Santos *et al*., 2004, with permission).

Early embryo mortality occurs between fertilization (day 0) and Day 24 of gestation which includes the developmental stages of cleavage, compaction, blastulation, expansion, hatching, and elongation or termed together as the pre-attachment period (Figure 1-7) (Peippo *et al*., 2011). Late embryo mortality occurs between Day 25 and 45 at which time embryonic differentiation is mostly completed, while fetal mortality occurs after this and up to parturition (Nomenclature, 1972). In high-producing dairy cows (very) early embryo loss occurs mainly within the first eight days postfertilization (accounts for ∼35% of embryonic mortality in the first week post fertilization) (Courot *et al*., 1985; Santos *et al*., 2004; Sartori *et al*., 2009).

The causes of early embryonic failure are not well understood mainly due to the fact that it is generally detected as a return into estrus on the following cycle. It has been proposed to have a paternal origin from inadequacies in the fertilizing sperm. However, maternal failure to recognize pregnancy (estimated to be up to 25% of failures of conception in dairy cows) and gross chromosomal abnormalities (approximately 5% of embryonic mortalities) are other important factors leading to the same phenotype of early embryo loss (Walsh *et al*., 2011). The extent of late embryo and early fetal mortality is relatively low (~ 7% on Day 24 and 80 for lactating cows) and similarly to early embryo losses the causes a numerous ranging from genetic, physiological, endocrinological or environmental factors (Diskin *et al*., 2011; Van Soom *et al*., 2007; Evans and Walsh, 2011; Silke *et al*., 2002; Diskin and Morris, 2008). Overall, embryo viability is currently a big challenge and is the topic of many research programs aiming at developing means to define and increase embryonic quality.

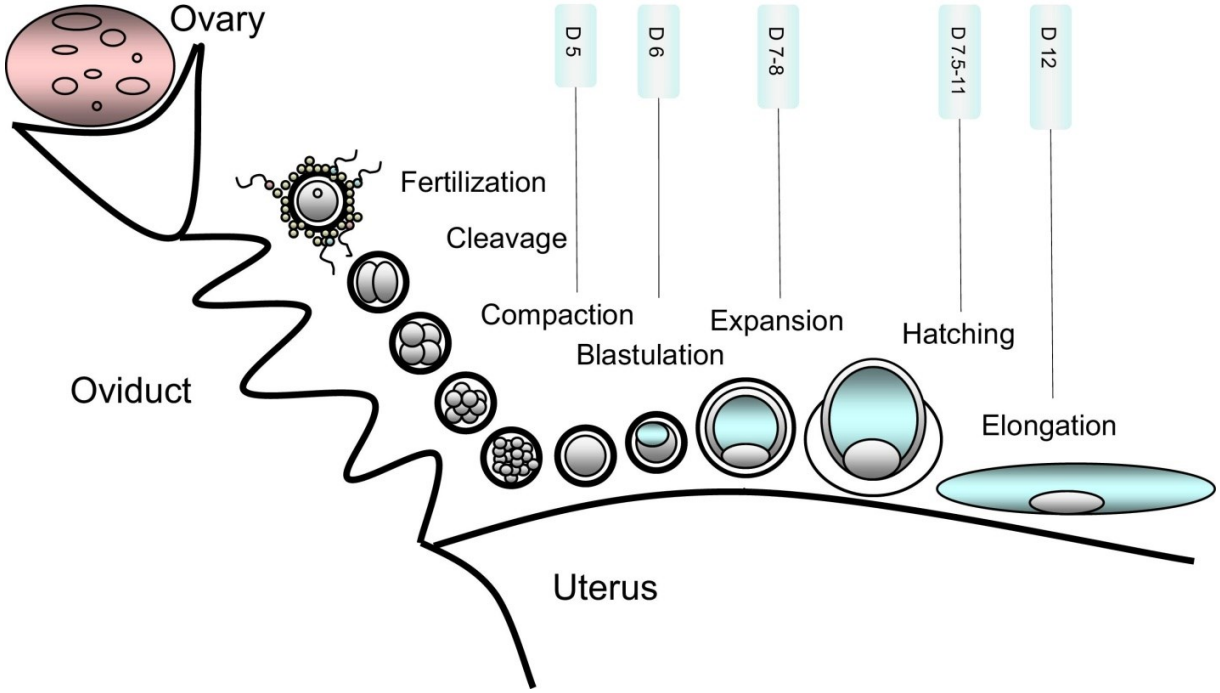

Figure 1-7 Terms and concepts in the pre-attachment period in a bovine embryo. (Reproduced from Machatya *et al.,* 2012, with permission).

1.2.2. Other applications of ARTs in cattle

1.2.2.1. Cloning

One of the main incentives driving the need to generate genetically identical copies of dairy cattle is to increase the potential semen offer from the next top of the breed bulls. Producing one or more copies of an individual either naturally, artificially (embryo splitting), or by nuclear transfer is called “cloning” (Moore and Thatcher, 2006). Two general methods can be used to obtain two and or multiple identical embryos from one embryo in cattle; i) cleavage-stage (2–4 and 8-cell embryos) blastomere separation technique to produce multiple (twins, triplets, quadruplet monozygotic) calves; and ii) embryo bisection technique on post compacted embryos (morula or blastocysts) to produce mainly identical twins (hemi-embryos) using

microsurgery (Tagawa *et al*., 2008; Rho *et al*., 1998). Both techniques (separation and bisection) have proven efficient to yield monozygotic twins after laparoscopic transfer to recipient cows with satisfactory pregnancy rate and up to 60% overall efficiency of cow embryo splitting (number of calves born per embryos bisected and transferred). Somatic (adult or foetal cells) nuclear transfer (SCNT) has been successfully used, however it is quite inefficient (due to aberrant DNA reprogramming) and very costly. Such drawbacks prohibit widespread application at commercial level. In addition to generating genetic copies of genetically superior animals, SCNT can be used for production of transgenic animals (Rodriguez-Martinez, 2012).

1.2.2.2. Transgenesis

Several methods are available to produce transgenic animals Transgenesis is used to genetically modify cattle for production improvements (altered production characteristics and quality), better health and welfare (increased disease resistance) (Lewis *et al*., 2004) or for use as bioreactors (Yang *et al*., 2008a; van Berkel *et al*., 2002; Rudolph, 1999) for biopharmaceutical applications. Due to extremely low efficiencies (< 1%) and ethical considerations, transgenesis remains far from acceptance for widespread commercial use (Lewis *et al*., 2004; Moore and Thatcher, 2006; Murphy, 2012).

1.2.2.3. Integrating genomic selection and reproductive technologies

Reproductive technologies have traditionally been applied to cattle for two main objectives: (1) as palliative measures to lower fertility; (2) as means to increase the dissemination rate of elite genetic merit. Nowadays, breeding companies are using the

latest genetic selection tools to improve all traits of economical importance including fertility (Veerkamp and Beerda, 2007; Berglund, 2008) It is a mean to address the problematic of reduced fertility from a genetic perspective with long term consequence while the application of reproductive technologies is destined to help producers in managing the problem on a daily basis. Genetic selection is used through a number of approaches and technologies such as conventional breeding (*e.g.* EBVs, PT), marker-gene assisted selection (*e.g.* microsatellites, DNA polymorphisms including single nucleotides), more recently genomic selection (*e.g.* GEBV) and functional genomics. The main hurdles facing genetic improvement of fertility traits are the low accuracy of fertility breeding values, its low heritability and the fact that animals must be selected as parents in order to achieve individual observations on their fertility. The fact fertility traits are difficult to grasp is partly explained by the numerous etiologies that may result in lowering fertility. As any other biological systems, the reproductive system is complex but it is not a vital system where individuals can live without procreating. Furthermore, it is an accessory system that is highly responsive to environmental conditions such as nutrition, stress, temperature or presence of pathogens. As such, phenotypic recording of infertility is therefore very complex and confounding. Current fertility evaluations are based on recording unsuccessful induction and maintenance of gestation which can all have numerous causes. Nonetheless, genome wide association studies (GWAS) and the candidate gene approach have been successfully used in identification of Quantitative Trait Loci (QTLs) associated with traits related to dairy cow fertility (*e.g.* ovulation rate, multiple

ovulations, twinning) (Huang *et al*., 2010a; Pryce *et al*., 2010; Sahana *et al*., 2010; Veerkamp and Beerda, 2007).

Research in the functional genomics are also conducted to identify biomarkers to better understand the physiology of lowering fertility but also as means to improve reproductive technologies and also to better phenotype and classify fertility (Veerkamp and Beerda, 2007; Robert, 2008). More precise phenotyping would translate into more precise genetic evaluations and increase genetic improvement. Functional genomics includes transcriptomics and proteomics studies aim at identifying biomarkers that are expressed in relation to the studied physiology. It involves every aspects of the reproductive system from oocyte developmental competence and its associated follicular environment (Misirlioglu *et al*., 2006; Christenson *et al*., 2013; Mondou *et al*., 2012; Gilbert *et al*., 2011; Gilbert *et al*., 2012; Nivet *et al*., 2013; Walsh *et al*., 2012; Berendt *et al*., 2009) ; the mechanisms supporting early embryo development (Vigneault *et al*., 2009; Kues *et al*., 2008; Robert *et al*., 2011; Clemente *et al*., 2011; Driver *et al*., 2012; Huang and Khatib, 2010; Chitwood *et al*., 2013; Massicotte *et al*., 2006; Memili *et al*., 2007; Bhojwani *et al*., 2006; Chaze *et al*., 2008) and conceptus-maternal interactions that will lead to a successful gestation (Forde *et al*., 2012a; Mamo *et al*., 2012; Mamo *et al*., 2011; Berendt *et al*., 2005; Forde *et al*., 2009; Forde *et al*., 2012b; Walker *et al*., 2012; Ledgard *et al*., 2009).

Recently, an extra layer of biological complexity has been added that can affect reproductive performances where environmental conditions can modulate gene

expression by modifying the epigenome which represents the sum of the alterations to the chromatin structure unaffecting the DNA sequence itself but modulating how genes are expressed. Simplified, the epigenome represents a layer of chemical factors (simple elements such as phosphate to more complex ones such as ubiquitin and even binding of RNA molecules) that is added to the chromatin structure (the DNA and the proteins that package it) and can influence how a gene is expressed to the extent of even long term silencing it (For review see: (Faulk and Dolinoy, 2011; Zaidi *et al*., 2011; Inbar-Feigenberg *et al*., 2013; Kohda and Ishino, 2013; van Montfoort *et al*., 2012)). It is believed that the epigenome is a mean through which biological systems adapt to environmental conditions. The reproductive system is of particular interest since it has been shown that some stress applied early on during development can carry long term consequences. Examples of the impact of the epigenome are actually numerous. Some of the most striking ones are phenotypes derived from the application of reproductive technologies where a stress applied in the first days of development could carry post-birth consequencesvan (Montfoort *et al*., 2012). For example, although SCNT derived clones are genetically identical copies (aside from the mitochondrial background) some clones exhibit distinct phenotypic performances although raised in the same environment. In cattle, *in vitro* derived calf can suffer from fetal overgrowth (LOS) where *in vitro* culture conditions such as the presence of serum in the medium has been proposed to be a causing agent (Young *et al*., 1998; Hiroyuki *et al*., 2004).

An important focus is now directed at better understanding how early events can shape later performances. Several studies are focusing on the first embryonic cell

divisions since it is known to be a window of sensitivity to epigenetic perturbations as the paternal and maternal genomes must reprogram to erase their ultra-specialized gametic program to become embryonic stem cells that will give rise to all cell types to form a complete individual. Another point of focus is directed during gestation where maternal cues can lead to fetal programming. It has been recently proposed that lowering fertility in dairy cattle is attributable to the metabolic stress provided by the negative energy balance during early lactation which leads to the birth of heifers with lower ovarian reserves thus lower fertility (Walsh *et al*., 2011). These reports are now raising numerous questions pertaining to the long term impacts of the uterine environment which would translate into a better selection of recipients for embryo transfer. Other impacts of the epigenome could also be modulated from the rearing conditions provided to heifers and young bulls especially in later; around the time of puberty.

1.2.2.4. Studying the (epi)-genome

Whole-genome sequencing allows complete discovery of SNPs without any ascertainment bias but also permit identification of structural variants like copy number variants (CNVs). Current sequencing platforms have not yet reach a low cost per genome that would permit routine sequencing of large cohorts of animals within the context of academic research funding. It is anticipated that sequencing the genome of sires or dams will soon become available at the bench in every laboratory and will eventually revolutionized livestock breeding (Pérez-Enciso and Ferretti, 2010; Murphy, 2012). The same impact can be expected in term of epigenetics study especially for

DNA methylation and the study of RNA populations (McGraw *et al*., 2013b; de Montera *et al*., 2013b). In this regard, while third generation platforms for genome-sequencing (use single-molecule templates, limiting sample amplification, using less starting material and being less error prone) are being used and improved, fourth generation platforms such as Oxford Nanopore (Oxford, UK) technology offer future promise (Ku and Roukos, 2013; Pennisi, 2012).

Until then, all high throughput platforms performing genomic profiling are array based and are thus limited to survey the information represented on the arrays. This information can range from several thousand up to >1.2 M loci (Zhang *et al*., 2011; Mardis, 2008; Metzker, 2010). Overall, the wealth of data generated from all of these high throughput platforms is currently generating a bottleneck where data processing and analysis is becoming challenging and requires dedicated expertise in bioinformatics.

1.2.2.5. Conclusions

Modern genetic improvement is becoming increasingly intertwined with the application of reproductive technologies. Traditionally, it has been used for the dissemination of semen of elite bulls with a clear focus on improving AI and semen cryopreservation. Although research is still ongoing to improve sire conception, the application and development of reproductive technologies are now considering the maternal contribution with a clear aim at increasing the number of descendant per cow and reducing the generation intervals. From the improvements in the reliability and efficacy of reproductive technologies, breeding companies are now looking into

combining the latest genetic selection tools (genomic selection) with the most advanced reproductive technologies (ovarian stimulation, OPU, IVP) to increase the rate at which the next generations of high genetic merit animals reach market. Genomic selection bypasses the need for progeny testing and thus offer the potential to look for the next top elite bull readily at birth and even earlier when determining the genetic value of the embryos pre-transfer through an embryonic biopsy. Furthermore, improvements in the control of ovarian and testicular functions combined with optimized *in vitro* embryo production could foresee the production of offspring from pre-pubertal parents.

The awareness that pre- and post-conception as well as gestational and pre- and post-puberty conditions can modulate fertility of the animals on the long term (and even of the next generation) is opening an entire new field of research opportunities to improve and maximize cattle fertility.

1.3. Introduction to epigenome

The term epigenome refers to all potentially heritable chemical modifications to DNA and histone proteins throughout the genome (Bernstein *et al*., 2007a). Epigenomic effectors now include the actions of non-coding RNA (Goldberg *et al*., 2007; Geiman and Robertson, 2002; Baker, 2011), although these remain less known than other types of epigenetic modulators such as DNA methylation, histone modification, histone variants and chromatin-remodeling protein complexes (Mattick *et al*., 2009; Zhou *et al*., 2010; Costa, 2008; Narlikar *et al*., 2013). Through a complex regulatory network that acts on chromatin structure, the epigenome determines how genes are expressed and thus modulates cellular function. In addition, the epigenome regulates the specific gene expression programs that are unique to each cell type and is thereby responsive to physiological states as well as developmental and environmental stimuli (Szyf, 2007; Bernstein *et al*., 2007a). Unlike the genome (the DNA nucleotide sequence), the epigenome undergoes short-term modification and is believed to be at the core of adaptive measures that allow cells to cope with environmental dynamics. Epigenetics is the term we now use to refer to the decades-old concept of the influence of the environment on gene expression (Jaenisch and Bird, 2003), which has been the focus of studies attempting to explain the apparent triggering of diseases such as cancer by environmental factors (Herceg, 2007) or complex behavioral pathologies such as schizophrenia, autism, obesity and so on (Petronis, 2010). The creation of the international society DOHaD (Developmental

Origin of Health and Diseases) is a prime example of the increasing interest in epigenetics.

1.3.1. The RNA component of the epigenome

Non-coding RNAs (*i.e.* RNAs that do not encode a protein) are involved in a wide array of intracellular signals (depending on their structure and types) that control gene expression. They are believed to be key regulators of cell physiology through roles in chromatin architecture/epigenetic memory, transcription, RNA splicing, editing, translation and turnover (Mattick and Makunin, 2006; Lai *et al*., 2013), and to act directly and/or indirectly on the epigenome (Costa, 2008). This category of RNA controls aspects of embryogenesis such as maternal–zygotic transition, maintenance of pluripotency, and organ morphogenesis (Pauli *et al*., 2011). There are two main types of non-coding RNA (Costa, 2010): small(< 300 nt) and long (≥ 300 nt). The small non-coding RNAs include molecules of known cellular function such as transfer RNA, snRNA, snoRN and small ribosomal RNA (5S and 5.8S). Epigenomic effects are mediated by another subtype of small RNA molecules, namely miRNA, siRNA, piRNA and repeat-associated siRNA (rasiRNA) (Chu and Rana, 2007; Malone and Hannon, 2009). So far, the involvement of small non-coding RNA in modulating gene expression at the transcript level has been determined by either destroying it or impairing its translation (Liu and Paroo, 2010) but the mechanism by which it influences chromatin remodeling remains unclear.

Long non-coding RNAs also include functionally characterized molecules such as large ribosomal RNA (18S, 28S). For the most part, the functions of the large contingent of long non-coding RNAs remain to be elucidated. These molecules are classified according to their genomic position: intergenic (lincRNA) and opposite strand of transcripts related to those known to be protein coding, natural antisense (natRNA). Unlike most natRNAs, lincRNAs exert their regulatory roles in trans to alter chromatin structure and gene expression at distant loci (Magistri *et al*., 2012; Werner *et al*., 2009; Knowling and Morris, 2011; Moran *et al*., 2012; Baker, 2011; Taylor *et al*., 2015).

Long non-coding RNAs can regulate gene expression through chromatin modification, or at the transcriptional and post-transcriptional processing stages (Mercer *et al*., 2009; Rinn and Chang, 2012; Pauli *et al*., 2011; Baker, 2011). As is the case for small RNAs, their role in modulating chromatin structure is currently under investigation. Some key examples include H19, which regulates the expression of the neighboring Igf2 gene, and the long non-coding pair of Xist and its antisense Tsix, which play a pivotal role in X chromosome inactivation (Andersen and Panning, 2003; Moran *et al*., 2012; Gendrel and Heard, 2014). It has been hypothesized that long non-coding RNAs may act as adaptor molecules to scaffold protein complexes that are involved in chromatin remodeling (Tsai *et al*., 2010; Rinn and Chang, 2012). The knock-down of specific long non-coding RNAs candidates drives stem cells to differentiate in a manner similar to that obtained by depletion of markers OCT-4 and NANOG (Guttman *et al*., 2011).

1.3.1.1. Studying non-coding RNA molecules

The study of non-coding RNAs is now quite straightforward thanks to the development of reliable whole transcriptome amplification procedures. The study of short RNA molecules requires adapted sample processing, whereas standard methods are employed for long non-coding transcripts. Many kits for the extraction and amplification of small RNA molecules have been commercialized. Most are based either on size fractionation or on enrichment through pull-down of partner proteins (Huang *et al*., 2011; Hu *et al*., 2006; Hüttenhofer and Vogel, 2006; Sharma and Vogel, 2009; Kong *et al*., 2009). A ligation step is generally used to lengthen the small RNA molecules and to introduce a known sequence that is then used for overall sample amplification. It is noteworthy that long non-coding RNA molecules are most often spliced and that some but not all bear a poly(A) tail of unknown length. RNA isolation based on the presence of a poly(A) tail will fractionate the population of long non-coding RNA, as will downstream reverse transcription primed using an oligo-dT. Recent interest in profiling the transcriptome of single cells has fueled the development of very high performance RNA extraction and whole transcriptome amplification platforms, which either target poly(A)-bearing molecules or use a non-discriminating random priming approach.

Although the identification of non-coding RNAs is fairly straightforward, identifying their biological functions and performing functional analyses is not (Baker, 2011; Pang *et al*., 2006). Among the several assays that have been used widely to study the function of non-coding RNAs are si/shRNA knockdown, mouse gene knockout, directed

mutagenesis, ectopic expression, over-expression and in situ hybridization (Hu *et al*., 2012). In addition, several new technologies and methods have been developed to facilitate identification, such as miRNA polymerase chain reaction array (Maserati *et al*., 2011), RIP-seq (capturing of the polycomb repressive complex 2 (PRC2) transcriptome to identify a genome-wide PRC2-interacting RNA pool) (Zhao *et al*., 2010; Zambelli and Pavesi, 2015) combined with dedicated computational tools (Jung *et al*., 2010; Da Sacco *et al*., 2012; Glazko *et al*., 2012; Gorodkin and Hofacker, 2011).

The presence of short and long non-coding RNAs has been described in mammalian early embryos (Castro *et al*., 2010; Yang *et al*., 2012; Yang *et al*., 2008b; Abd El Naby *et al*., 2013; Lee *et al*., 2012) and in germ cells (Tesfaye *et al*., 2009). Several studies have focused on highlighting the functional roles of small non-coding RNAs (Pang *et al*., 2011; Maserati *et al*., 2011) as well as lncRNAs (Caballero *et al*., 2014) in pre-implantation embryo development using knockout and microinjection techniques. In several species such as zebrafish, the transition from the maternal program to the embryonic program requires the destruction of maternal RNAs by miRNA (Ramachandra *et al*., 2008; Giraldez *et al*., 2006; Schier, 2007; Graf *et al*., 2014). Such a role of specific miRNAs in maternal-to-embryonic transition (MET) was also suggested for bovine embryos (Mondou *et al*., 2012) .

1.3.2. The protein component of the epigenome

1.3.2.1. Histone modification and variants

Histone modification involves several types of chemical modification of histone tails, including acetylation, phosphorylation, methylation, deimination, β-N-acetylglucosamine, ADP-ribosylation, sumoy-lation, ubiquitination, histone tail clipping, histone proline isomerization. Each of these modifications can occur to varying degrees and on various residues (Strahl and Allis, 2000; Bannister and Kouzarides, 2011). Understanding of the histone code (Jenuwein and Allis, 2001; Margueron *et al*., 2005) is proving to be a daunting task, since chromatin contains a large contingent of histones (the total histone content represents about 0.4 mg of protein per 107 cells) that are more or less independently regulated, in addition to its remodeling being very dynamic (Mellor, 2005).

Histone variants make up a non-canonical histone fraction that has specific expression, localization and species-distribution patterns, as well as having roles in a range of processes including DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, sex chromosome condensation, sperm chromatin packaging, regulating nucleosome structure and dynamics and targeting and/or activating chromatin-remodeling complexes (Talbert and Henikoff, 2010; Kamakaka and Biggins, 2005; Tessarz and Kouzarides, 2014; Suganuma and Workman, 2011). Furthermore, histone modifications and DNA methylation have a very strong cross talks, link and association for several cellular and molecular functions such as

transcriptions and DNA methylation establishment (both *de novo* DNA methylation and DNA maintenance) (Du *et al*., 2015).

1.3.2.2. Chromatin remodeling

Chromatin remodeling is described as the modification of chromatin structure by repositioning or removal of nucleosomes. It can be caused either by thermal fluctuations or by introducing a variety of chaperones and ATP-dependent enzymes known as chromatin re-modelers, which perform repositioning, restructuring, or removal of nucleosomes by exertion of ATP-generated mechanical forces (Killian *et al*., 2012; Blossey and Schiessel, 2011; Vignali *et al*., 2000; Narlikar *et al*., 2013). It has been proposed that the re-modelers act through recognition of histone-tail states and/or linker length between nucleosomes (Florescu *et al*., 2012; Clapier and Cairns, 2009).

1.3.2.3. Studying histone modifications and chromatin-related proteins

Immunofluorescence staining in association with immunoblotting and other standard techniques can be used to localize candidate proteins and to study chromatin state and remodeling (Wongtawan *et al*., 2011). However, this provides only a very general perspective, since fluctuations in protein content do not inform about potential effects on the associated genes. Modern mass spectrometry assays are now providing in-depth characterization of the proteome of oocytes and embryos (Pfeiffer *et al*., 2011). The valuable information revealed on the main actors responsible for the epigenetic marks present at specific stages of early development will be particular useful in our understanding of events occurring during reprogramming phases. Like all other

proteomic methods, lack of amplification ability renders this option very challenging, since thousands of oocytes are required (Zhang *et al*., 2009; Wang *et al*., 2010; Pfeiffer *et al*., 2011).

For most, the method of choice to study histone modifications at the genomic scale is conducted using chromatin immuno-precipitation (ChIP). This technique involves immobilizing (cross linking) DNA-binding proteins on the DNA, fragmenting the DNA strands and using a targeted approach to pull down specific candidate proteins. The genomic localization of the proteins is deduced from the identification of the accompanying DNA molecule, which can be achieved using microarray hybridization (ChIP-chip) or next-generation sequencing (ChIP-seq) (Barski *et al*., 2007; Mikkelsen *et al*., 2007; Guenther *et al*., 2007; Schones and Zhao, 2008; Park, 2009). Both of these genome-wide approaches have been used to study the presence and localization of histone variants (Zhang *et al*., 2005; Barski *et al*., 2007). Nucleosome maps have also been generated using ChIP-chip (Park, 2009). In these techniques, the potential for introducing methodological biases is not negligible, since specificity is dependent on the quality/specificity of the antibody, while histone/chromatin preparation methods such as fixation and sample fragmentation may be influenced by neighboring proteins that can mask the epitope (Schones and Zhao, 2008; Bernstein *et al*., 2007a).

Conventional ChIP-based methods require a large number of cells (ideally > 107 cells), making them unsuitable for studying mammalian oocytes and early embryos. Even the ChIP assays developed for small numbers of cells, such as the Quick and Quantitative ChIP assay or Q2ChIP (100,000 cells), and the MicroChIP (10,000 cells, Collas, 2010) still

require far too many cells for early development studies. Optimized protocols have been proposed in which the protein content is increased by adding a foreign “carrier” protein. The carrier ChIP (CChIP), proven suitable for the analysis of samples containing 100 cells (O'Neill *et al*., 2006), and the µChIP (Dahl and Collas, 2008), reportedly efficient with a minimum of 1,000 cells, offer promising alternatives. When methods are optimized and scaled down, they typically become finicky, and both CChIP and µChIP have been criticized because of cross-detection of the carrier (for CChIP), tediousness and poor sensitivity (Collas, 2010; Bernstein *et al*., 2007a).

Both the CChIP (Daujat *et al*., 2009; Rugg-Gunn *et al*., 2010; Alder *et al*., 2010; Lindeman *et al*., 2011) and the µChIP (Dahl *et al*., 2010; Smallwood *et al*., 2011a) methods have nevertheless been used successfully as small-input assays to study histone modification in germ cells and early embryos. Recently, which an ultra-low-input micrococcal nuclease-based native ChIP (ULI-NChIP) and sequencing method was introduced (Brind’Amour *et al*., 2015) to generate genome-wide histone mark profiles with high resolution and high quality results from as few as 1,000 cells which making it compatible with early embryo histone modifications studies (Biechele *et al*., 2015).

1.3.3. The DNA component of the epigenome

1.3.3.1. DNA methylation and hydroxymethylation

DNA methylation involves the addition of methyl group to cytosine and presents in three different DNA sequence contexts: CG and CHG (where H corresponds to A, T, or C), which are symmetrical sequences, and CHH, which is an asymmetrical sequence

(Law and Jacobsen, 2010). DNA methylation is often viewed as a gene-silencing mark that is established during cell differentiation (Raynal *et al*., 2012; Bird, 2002). Non-genic regions such as repeat elements and transposons are well known to be highly methylated (Hackett and Surani, 2013; Goldberg *et al*., 2007; Monk, 2015). Although in mammals, DNA methylation occurs primarily at CG residues; non-CG methylation is observed in stem cells within the body of actively transcribed genes (Varley *et al*., 2013; Du *et al*., 2015). Many researchers now refer to 5-methyl cytosine (5mC) as the fifth nucleotide base (Lister and Ecker, 2009). This epigenetic mark may occur at 20–30 million sites throughout the mammalian genome, predominantly in association with CpG dinucleotides (Trasler, 2009). Interestingly, the frequency of CpG dinucleotides is lower than what would be expected throughout most mammalian genomes (accounting for approximately 1 % of DNA bases of mammalian genome (Illingworth and Bird, 2009), and it is widely accepted that this low frequency is related to the spontaneous deamination of methylated cytosine in CpG site (Fryxell and Zuckerkandl, 2000). In fact, deamination of non-methylated cytosines leads to the product of cytosine deamination, Uracil, which is readily recognized as an aberrant nucleotide and it is repaired. However, the deamination product of methylated cytosine, Thymine, leads to transition mutations in the next round of DNA replication (Cooper *et al*., 2010). Consequently, methylated CpGs in the germ line are likely to be lost over time and result in a low frequency of CpG dinucleotides in mammals (Saxonov *et al*., 2006). However, certain regions of the genome, namely CpG islands (CGIs), have up to 10-fold higher frequency of the CpG dinucleotide than the rest of the genome (Crider *et al*.,

2012). CGIs compose < 1 % of the genome and are typically unmethylated and associated often with promoter and other regulatory regions of > 50 % of all mammalian genes (Crider *et al*., 2012). It is believed that there are two main reasons for hypomethylation at CGIs in promoters, i) Promoter CGIs transcription factors binding which avoid the DNA methylation machinery to target these regions (Stadler *et al*., 2011) and ii) Promoter CGIs specialized histone mark (histone 3 Lys4 trimethylation (H3K4me3) which inhibit *de novo* DNA methylation transferases enzymes (DNMT3) enzymes to recognize and methylate these regions (Smith and Meissner, 2013). Nevertheless, a sizable fraction of CGIs might be fully methylated in normal cells (Weber *et al*., 2007). The bovine genome has a size of approximately 2.29 Gb with more than 27,000,000 CpG sites, and 36,729 CGIs - with an average length of 1,023 nucleotides (Han *et al*., 2008). In addition to the CGIs in promoters, intergenic and intragenic CGIs, can serve as functionally important regions for DNA methylation, most importantly those CGIs located in "shore" regions, which are defined as CpG-containing regions that are within 2 kb of a CpG island, contain low densities of CpG sites (Han *et al*., 2008). These CGIs in shore regions have been shown to be genomic loci for tissue-specific differential methylation in normal tissues with altered methylation patterns in cancer cells (Irizarry *et al*., 2009).

In a somatic cell, it is estimated that 60 – 80 % of the potential CpG sites are methylated (Hermann *et al*., 2004; Neil and Robert, 2011). Pioneering works using various combinations of methylation sensitive restriction enzymes (Kafri *et al*., 1992; Monk *et al*., 1987) and 5mC immunofluorescence staining (Santos *et al.*, 2002; Beaujean *et al.*)

established that the DNA methylation landscape was dynamic in gametes and preimplantation embryos. Although these methods provide only global or partial insight of the DNA methylation setting that is taking place, they are accountable for a substantial amount of our knowledge about the epigenome of gametes and embryos. Whereas these methods are still valuable in many situations, the emergence of innovative genome-wide molecular approaches have not only confirmed most of the earlier findings, but propelled epigenomics to new heights by providing a clearer picture of when and where DNA methylation is acquired and lost during development (Smith *et al.*, 2012b; Borgel *et al.*, 2010b). Since DNA methylation patterns are stable and are retained in purified gDNA, DNA methylation studies are amenable to a wide variety of cell-free assays and technologies including DNA methylation analysis at single nucleotide resolution, next-generation sequencing, and genome-wide methylation profiling (Crider *et al*., 2012). Recent studies have uncovered that the hallmark of epigenetic modifications, 5mC, can produce less stable oxidative chemical derivatives or intermediates through the action of the Ten-eleven translocation (TET) family proteins (Kriaucionis and Heintz, 2009; Ito *et al*., 2011; Tahiliani *et al*., 2009; Hill *et al*., 2014; Xu and Wong, 2015).

One DNA modification variant which in particular has been grabbing increasing attention is 5-hydroxymethylcytosine (5hmC), which is currently referred to as the 6th base of the genome (Münzel *et al*., 2011) and has been linked with epigenetic reprogramming (Yamaguchi *et al*., 2013; Wossidlo *et al*., 2011; Hill *et al*., 2014). Although it is still not perfectly clear how these bases influence development, there is

clear evidence of differing 5mC and 5hmC contents in male and female pro-nuclei (Iqbal *et al*., 2011a) as well as increased levels and a probable role in the regulation of transcription in embryonic stem cells (Pastor *et al*., 2011).

TET enzymes and their products, different oxidation formats of 5mC, appear to have several functions including i) elimination of hypermethylation from a regulatory region commonly found in cells undergoing fate transition; ii) counteracting the ongoing *de novo* DNA methylation to keep CpG islands, transcription start sites (TSS), and enhancers free of 5mC accumulation in ESCs, adult stem cells, zygotes and neuron; iii) transcriptional regulation either through repression or promotion of transcription through different mechanisms; iv) catalyzing the oxidation of substrates other than 5mC in DNA; and v) protecting DNA methylation against hypermethylation in canyons which are regions with locally reduced DNA methylation levels that often contain CGIs and are frequently associated with promoter regions (Xu and Wong, 2015; Wiehle *et al*., 2015).

1.3.3.2. DNA methylation establishment and removal

1.3.3.2.1. DNA methyltransferases (DNMTs)

The *de novo* establishment and maintenance of DNA methylation are carried out through DNA methyltransferase enzymes called DNMTs (Jin and Robertson, 2013). In mammals, DNMTs have a central role in mediating the transfer of a methyl group from S-adenosyl methionine (SAM) to cytosine (Niculescu and Zeisel, 2002). There are three enzymatically active DNA methyltransferases in mammals: DNMT1, DNMT3A and DNMT3B and one related regulatory protein, DNMT3L, which lacks catalytic activity

(Denis *et al*., 2011). DNMT3A and DNMT3B are involved primarily in *de novo* establishment of DNA methylation (Okano *et al*., 1999). Following DNA methylation establishment, DNA methylation is perpetuated through both mitotic and meiotic divisions by DNMT1 which primarily acts as maintenance DNA methyltransferases (Du *et al*., 2015). However, the mechanisms that maintain these different types of methylation vary widely between different eukaryotes and these functional DNMTs distinctions are not absolute. For instance, DNMT1 can exhibit *de novo* DNA methylation activity depending on the type of repetitive elements and DNMT3A or DNMT3B may also be required for DNA methylation maintenance in human cancer cells (Arand *et al*., 2012; Jair *et al*., 2006). Dysregulated expression of DNMTs has been reported for various human cancers and defects and in some cases reported to cause embryonic lethality (Denis *et al*., 2011).

DNMT1 localizes to DNA replication foci during S phase which its recruitment is mediated by the proliferating cell nuclear antigen (PCNA) and UHRF1 (recognizes hemimethylated DNA substrates) to preferentially methylates hemimethylated CpG dinucleotides and maintain DNA methylation after replication (Qin *et al*., 2015). In addition, DNMT1 has two forms, namely DNMT1s (somatic form) and DNMT1o (oocyte-specific form) which maintain DNA methylation until the blastocyst stage (Branco *et al*., 2008; Cirio *et al*., 2008). Whereas DNMT1s is expressed at all cleavage stages of preimplantation development and appears to maintain DNA methylation at all stages except the 8-cell stage, DNMT1o which is oocyte-specific version of DNMT1, is expressed in oocytes and preimplantation embryos and is present only in the

cytoplasm throughout early embryo development with the exception of the 8-cell stage where it is found also in all eight nuclei (Ratnam *et al*., 2002). Recently, it has been shown that loss of DNMT1o disrupts Imprinted X Chromosome Inactivation in female blastocysts (McGraw *et al*., 2013a).

De novo methylation through DNMT3a and DNTM3B enzymes can occur globally and/or in a locus-specific fashion (Denis *et al*., 2011). It has been suggested that the intrinsic sequence preferences of the DNMT3 enzymes is important for global *de novo* DNA methylation, whereas crosstalk with other factors seems to be more important for locus-specific DNA methylation (Denis *et al*., 2011).

DNMT3L, is the cofactor of DNMT3 enzymes, shows no methyltransferase activity, but it is essential for the *de novo* methylation of most imprinted loci in germ cells and establishment of DNA methylation early embryo. DNMT3L interact directly with DNMT3A through i) stabilization the conformation of the active-site loop of DNMT3A and ii) enhancement of *de novo* methylation and increases the binding of SAM (Jia *et al*., 2007).

1.3.3.2.2. One-carbon metabolism

One-carbon metabolism consists of an integrated series of metabolic pathways including folate cycle, methionine remethylation and trans-sulfuration pathways (Ikeda *et al*., 2012). Generating and transferring of one-carbon units for the *de novo* synthesis of purines, thymidylate and remethylation of methionine are the central function of one-carbon metabolism (Xu and Sinclair, 2015). Several components,

including folate, vitamin B12, choline, betaine, methionine, cysteine, vitamin B6 and vitamin B2 which are directly influenced by dietary intake are involved in one-carbon metabolism (Figure1-8) (Johansson *et al*., 2009). Liver is considered as the major site of one-carbon methabolism, since the activities of the participating enzymes are generally (with some exception) higher in liver than other somatic tissues. Nevertheless, relatively higher one carbon methabolism activity can be seen in pancreas and kidney as well (Ikeda *et al*., 2012a).

A major substrate of one-carbon metabolism is S-adenosyl methionine (SAM), which is generated through adenosylation of methionine by methionine adenosyltransferase (MAT) enzyme. SAM is a primary methyl group donor for almost all methylation reactions, including the methylation of DNA (Smith *et al* 2012; Guo *et al*., 2012; Smith and Meissner, 2013), RNA (Wang *et al*., 2014; Lin and Gregory 2014; Batista Pedro *et al*., 2014; Yue *et al*., 2015; Geula *et al*., 2015), proteins (such as histones) (Vastenhouw and Schier, 2012; Serrano *et al*., 2013; Zhou and Dean, 2015; Bošković and Torres-Padilla, 2015) and lipids (Loenen, 2006; Locasale, 2013). Hence, SAM is considered as a global methyl donor. This highlights the crucial role of one-carbon metabolism in epigenetic regulation of gene expression (Figure1-8).

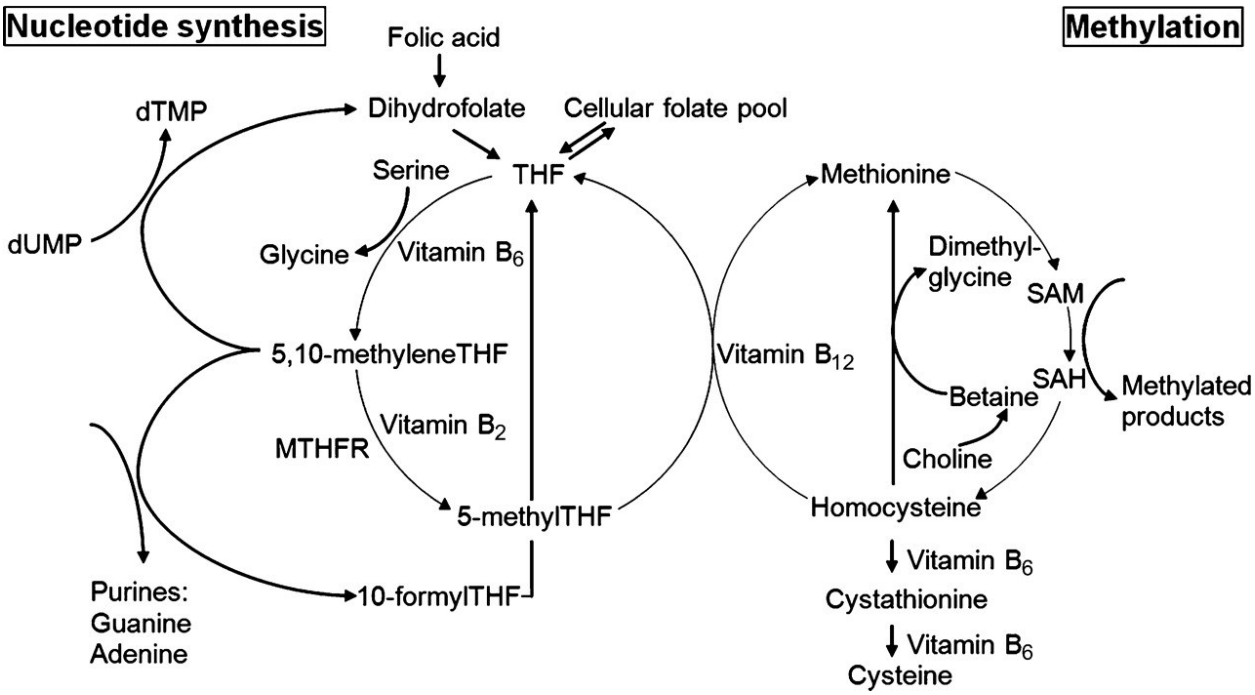

Figure 1-8 Diagram of the one-carbon metabolism pathway. Labile methyl groups are supplied by dietary serine, choline (via betaine), and methionine. One-carbon units are derived from serine through the activity of the vitamin B6–dependent serine hydroxymethyltransferase, which generates 5, 10-methylenetetrahydrofolate (5, 10-methylene THF). 5, 10-methylene THF, in turn, is then reduced to 5-methyltetrahydrofolate (5-methyl THF), the predominant form of folate in the circulation, in an irreversible reaction catalyzed by vitamin B2–dependent methylenetetrahydrofolate reductase (MTHFR). 5-methyl THF serves as a methyl donor in a reaction converting homocysteine to methionine, in which vitamin B12 serves a cofactor. Homocysteine can also be metabolized to cysteine through the sequential action of two vitamin B6–dependent enzymes. The methionine derivative, S-adenosyl methionine (SAM), is the universal methyl donor for the methylation of a vast variety of biomolecules, including DNA, RNA, protein (such as histones) and lipids. (Reproduced from Johansson *et al.*, 2009, with permission).

The majority of one-carbon metabolic enzymes are expressed in somatic cells of the ovary, mammalian oocytes and in preimplantation embryos (Xu and Sinclair, 2015). Methyl dietary supplements, as well as folate intake and availability which involve in one-carbon metabolism, can alter methylation genome-wide and/or locus-specific such as promoters of disease-related genes or specific imprinted genes in animals (van Engeland *et al*., 2003; Cooney *et al*., 2002; Andersen *et al*., 2012). One-carbon metabolism is critical for gametogenesis and embryo development for several physiological and molecular processes such as i) DNA methylation and histone modifications through SAM production; ii) conversion of deoxyuridylate (dUMP) into dTMP for DNA synthesis, repair and replication; and iii) production of the intermediate 10-Formyltetrahydrofolate (10-CHOTHF) for *de novo* synthesis of purines (Xu and Sinclair, 2015). Therefore, considering all these cellular functions, dietary deficiencies in folate, methionine, choline, vitamin B12 and vitamin B6, together with disturbances in the activity of enzymes involved with one-carbon metabolic pathways, can impair DNA synthesis and methylation (Xu and Sinclair, 2015). Furthermore, one-carbon metabolism is critical for early embryo development due to its substrate SAM which is essential for the maintenance of pluripotency (Shiraki *et al*., 2014; Shyh-Chang *et al*., 2013).

1.3.3.2.3. Folate

Folate, a water-soluble B-vitamin, is an essential vitamin for humans and is obtained from the diet, in particular from vegetables and fruits (Stover, 2004). In ruminants such as bovine, ruminal bacteria can synthetize B vitamins, including folic acid and vitamin

B12 (Duplessis *et al*., 2014). Deficiency symptoms for folate have not been observed in lactating dairy cattle and according to National Research Council (NRC) synthesis of B vitamins in the rumen is sufficient to meet requirements of dairy cows ((NRC), 2001). However, in bovine the beneficial supplementation of folic acid and vitamin B12 has been reported (Duplessis *et al*., 2014; Gagnon *et al*., 2015; Preynat *et al*., 2009). Based on these studies on non-ruminant animals, two mechanisms of folate absorption from the intestinal tract have been suggested: i) an active saturable process and ii) a non-saturable passive process which is affected by the availability of folates. However, in the case of ruminants, folate appears to degrade and are converted and synthesized in the forestomachs and even absorbed there on a small scale prior to intestinal absorption (Ragaller *et al*., 2009).

Except pig which the primary form of folate in the plasma of is tetrahydrofolate (THF), in humans and other animal species including bovine the majority of plasma folate is 5-methyl-tetrahydrofolate (5-methyl-THF)(Ref: 1992). The 5-methyl-THF in the plasma can be transported into the cell by either carrier- or receptor-mediated transport. Folate has several receptors, the α receptor (FR-α) is the major one and has a high affinity for the monoglutamate, 5-methyl-THF, and is expressed in a limited number of epithelial cells, predominantly in the proximal tubules of the placenta, kidney and the choroid plexus (Blom and Smulders, 2011).

It should be noted that based on chemical nomenclature, the difference between folate and folic acid is just one proton. However, the term folic acid is in general applied

to the synthetic form of this B-vitamin, which is also the most stable form (Blom and Smulders, 2011). In fact, natural food folates in comparison with synthetic folic acid are inherently less stable and show incomplete bioavailability (McNulty *et al*., 2012).

1.3.3.2.4. Folate metabolism

Folate metabolism is essential for cellular function, especially during periods of rapid cellular growth (McNulty *et al*., 2012). In fact, there are many critical cellular pathways dependent on folate as a one-carbon source including DNA, RNA and protein methylation as well as DNA synthesis and maintenance (Choi and Mason 2000; Steegers-Theunissen *et al*., 2013). Folic acid first needs to be converted by dihydrofolate reductase to tetrahydrofolate (THF), the naturally bioactive form (Xu and Sinclair, 2015). In the intestinal lumen, dietary folate polyglutamates are hydrolysed to their monoglutamated forms and absorbed into mucosal cells by passive diffusion and active transport via proton-coupled folate transporters. In mucosal cells, folate monoglutamates are transformed into 5-methyl-tetrahydrofolate (5-MeTHF) and transported out into circulation (Figure1-8). 5-Methyl-THF is the predominant folate form in non-hepatic tissues, which must then be polyglutamated for cellular retention and one-carbon cycle coenzyme function. From the blood, 5-MeTHF can enter the cell through specific carriers (reduced folate carrier (RFC)) or receptors (folate receptor (FR)) (Xu and Sinclair, 2015). At the intracellular level, folate functions as both a one-carbon unit acceptor and a donor. Amino acids including histidine, serine and glycine and its derivatives dimethylglycine and methylglycine (sarcosine) can serve as sources for donation of one-carbon units to THF (Locasale, 2013).

Methylation cycle of one-carbon metabolism is one of the cellular cycles that folate is involved in which occurs in all cells except red blood cells, resulting in methylation of numerous compounds. Folate-centered one-carbon metabolism is a metabolic network present in the cytoplasm, mitochondria and nucleus of cells (Xu and Sinclair, 2015). In this cycle, SAM is the key methyl group donor. Through this methylation cycle a wide variety of the biomolecules such as DNA, RNA, proteins and lipids can be methylated. During the methylation cycle, 5-MeTHF transfers its methyl group to homocysteine, via vitamin B12, to produce methionine (Locasale, 2013). Loss of a methyl group from SAM generates S-adenosyl homocysteine; SAH, which is a strong inhibitor of most methyltransferases and hence must be removed. Therefore, SAH is converted to homocysteine and adenosine by SAH hydrolase. This step produces homocysteine for methylation by 5-MeTHF, and so the methylation cycle can begin again. Insufficient amounts of 5-MeTHF or vitamin B12 leads to accumulation of homocysteine in the cell which might lead to putative inhibition methyltransferases and cause abnormal epigenetic establishment and dysregulation of gene expression, protein function and lipid (Blom *et al*., 2006).

1.3.3.2.5. DNA Demethylation

Removal of DNA methylation can take place though both active and passive ways (Kohli and Zhang, 2013). In the active DNA methylation removal, a family of proteins namely ten eleven translocation (TETs) are involved which oxidize the 5mC into different oxidation formats including 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). These oxidized 5mC bases can be actively removed by

base excision repair (BER) pathway or other unidentified pathways to regenerate cytosine. In mammals, it appears that active DNA demethylation differs between the cells, for instance in ESCs and neurons TET-catalyzed 5mC oxidation to trigger thymine DNA glycosylase (TDG)-mediated BER in ESCs and neurons (Kohli, and Zhang, 2013). In fact, TDG recognizes 5fC and 5caC, the higher oxidation products of 5mC, to initiate BER pathway for removal and substitution with unmethylated cytosine. However, in the zygote this process is unidentified and it remains to be determined whether demethylation is dependent on the BER pathway. On the other hand, activation-induced cytidine deaminase (AID) has also been implicated in active demethylation through deaminases activities, however, this suggested function is currently not fully accepted (Figure 1-9).

In the passive way of DNA methylation removal, DNA demethylation can occur either with or without oxidation of 5mC. In terms of the presence of oxidative formats of 5mC, they can decline through passive dilution during DNA replication since DNMT1 is unable to recognize these oxidized formats of 5mC. Therefore, it cannot methylate an unmodified cytosine in the newly synthesized strand that correspond with the oxidized 5mC position. However, oxidation of 5mC is, in principle, not essential for passive demethylation and 5mC by itself can undergo progressive dilution when the accessibility to DNMT1 in the newly synthesized strand in DNA replication is blocked or limited by low/lack of the DNMT1 presence (Hill *et al*., 2014; Xu and Wong, 2015; Messerschmidt *et al*., 2014) (Figure 1-9).

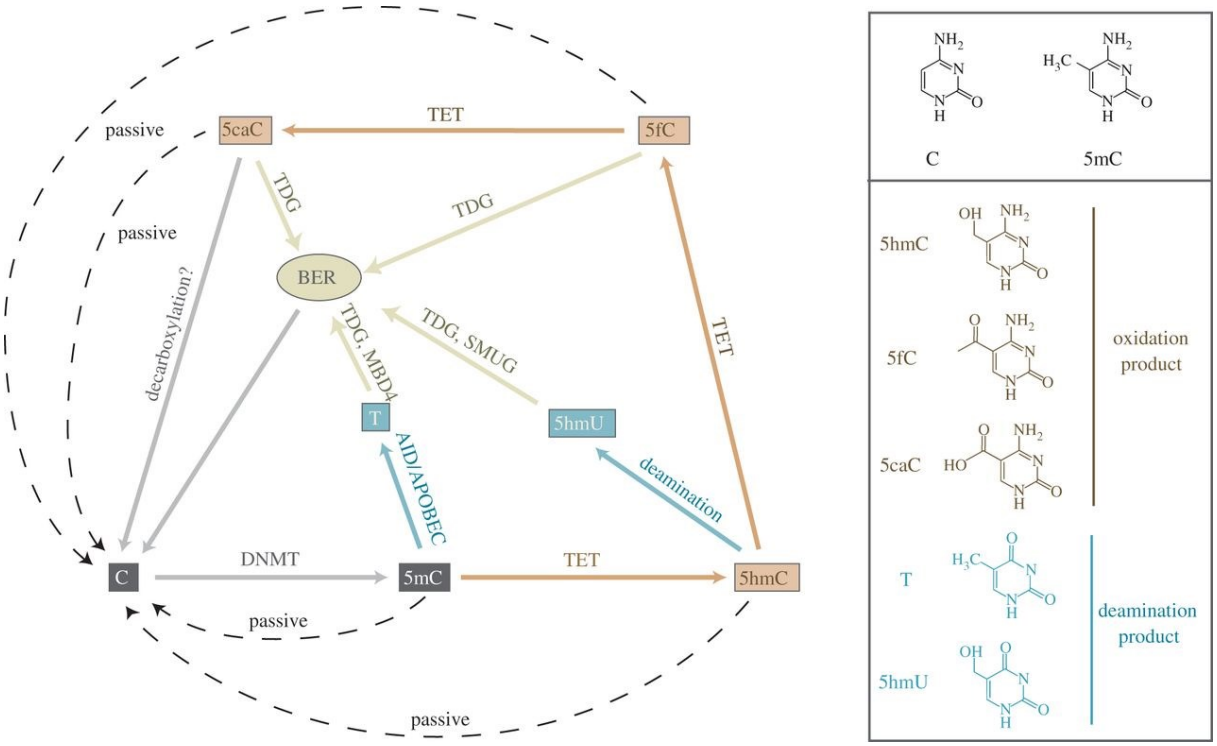

Figure 1-9 Pathways for removal of DNA methylation. Cytosine (C) is methylated at the 5' carbon position by DNMT enzymes to generate 5-methylcytosine (5mC). This can be lost passively owing to a lack of maintenance at DNA replication (dashed line), or actively processed by enzymatic activity. 5mC can be deaminated to thymine (T) by the AID/APOBEC deaminases (blue), or oxidized to 5-hydroxymethylcytosine (5hmC) by the TET enzyme family (brown). 5hmC itself may be deaminated to 5-hydroxymethyluracil (5hmC), or further oxidized by TET activity to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). The T, 5hmU, 5fC and 5caC derivatives can be excised by glycosylases (beige) such as TDG, single strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1) and methyl-CpG-binding domain protein 4 (MBD4) to initiate the BER pathway resulting in their replacement with unmodified C. Alternatively, 5fC and 5caC can be lost passively through lack of maintenance; 5caC may also be converted to C by a decarboxylation reaction. (Reproduced from Seisenberger *et al.*, 2012, with permission).

1.3.3.3. Functions of DNA methylation in transcrition and development

1.3.3.3.1. Transcription

For years, DNA methylation was directly linked to transcriptional repression and was believed to be highly correlated with gene silencing (Jones, 2012). However, recent studies have been revealing that this is not as an absolute phenomenon and is dependent on several factors and locus-specific characteristics (Messerschmidt *et al*., 2014) which are discussed as following:

1.3.3.3.1.1. Promoters

As previously mentioned (see DNA methylation and hydroxymethylation), promoters are often associated with CGIs particularly in developmental or housekeeping genes; even if the locus/gene is transcriptionally silent but still remains hypomethylated (Deaton and Bird, 2011). Nevertheless, there are functionally unknown non-promoter CGIs including those intragenic or intergenic CGIs which behave like CGIs promoter and are classed as "orphan" CGIs (Deaton and Bird, 2011).

Recently, studies using technologies which enable single nucleotide base genome-wide DNA methylation profiling as well as transcription profiling, classified CGI-associated promoters into three categories based on CpG content and sequence length including: high, intermediate and low CpG density promoters (HCPs, ICPs and LCPs, respectively). Such classification/enrichment details enable to discriminate carefully among the CGI-associated promoters in terms of their DNA methylation and impacts on transcription (Messerschmidt *et al*., 2014). It has been shown that DNA methylation of HCPs and ICPs are in agreement with traditional view that DNA methylation represses

transcription; as HCPs are rarely methylated and if it happens it does result in efficient gene silencing (Messerschmidt *et al*., 2014).

However, ICPs more frequently acquire differentiation-dependent hypermethylation than HCPs, especially at pluripotency gene promoters where DNA methylation is thought to act as a safety mechanism to reinforce silencing during differentiation, as well as at the promoters of germ cell-specific genes. Interestingly, in contrast with the dogma of CGI methylation and subsequent transcription silencing, LCPs are generally hypermethylated and remain transcriptionally active regardless of their methylation state (Meissner *et al*., 2008b; Borgel *et al*., 2010a; Messerschmidt *et al*., 2014; Weber *et al*., 2007). Furthermore, recently it has been suggested that DNA methylation may be a consequence of, not causation for, promoter silencing (Blattler *et al*., 2014).

1.3.3.3.1.2. Enhancers

The methylation status of transcriptional enhancers, that physically interact with gene promoters and support tissue-specific differentiation, impacts on transcription (Stadler *et al*., 2011) in a way that their hypomethylation is correlated with active gene expression (Sandovici *et al*., 2011). Studies showed that enhancer methylation can be found in cancer cells and is associated more closely with gene expression changes than promoter methylation itself (Aran *et al*., 2013). Recently, it has been shown that in cancer cell lines, DNA methylation not only has a regulatory role on transcription of CGI-associated promoters, but also on suppression of intragenic enhancers (Blattler *et al*., 2014).

1.3.3.3.1.3. Other genomic elements

Methylated CpGs have also been observed as variably methylated clusters in intragenic and intergenic regions and within gene silencers (Mitchell *et al*., 2015). Although the exact function of DNA methylation at these CpG sites is not clear recent data suggest that they may be even more important in controlling transcription of many genes than the CpG islands (Mitchell *et al*., 2015).

1.3.3.3.1.4. Non-coding RNAs (ncRNAs)

RNA-directed DNA methylation (RdDM) in plants has been a well-known RNA-mediated epigenetic pathway for many years (Matzke and Mosher, 2014) but in animals, recently, growing evidences have been demonstrating that non-coding RNAs through regulation of DNA methylation can alter gene expression (Mitchell *et al*., 2015; Taylor *et al*., 2015). For instance, it has been shown that DNMT3B can be recruited by DNA:RNA triplexes to silence rRNA genes (Schmitz *et al*., 2010); DNMT1-associated long non-coding RNAs regulate global gene expression as well as DNA methylation (Merry *et al*., 2015); and DNMT1-interacting lncRNAs block gene-specific DNA methylation (Di Ruscio *et al*., 2013).

1.3.3.3.2. Repetitive elements

Mammalian genomes during evolution have accumulated a vast number of retrotransposed sequences, which can be divided into: long terminal repeat (LTR) retrotransposons that include the endogenous retroviruses (ERVs), as well as long and short retrotransposons lacking LTRs, known as LINEs (Long Interspersed Nuclear

Elements) and SINEs (Short Interspersed Nuclear Elements), respectively (Mager and Stoye, 2015).

These, retrotransposed sequences or interspersed repeats are the largest class of sequences in mammalian genomes, accounting for 40 to 50 % of the total length of genomes (Adelson *et al*., 2009). In the bovine genome, interspersed repetitive elements of all classes account for approximately 46.6 % of the bovine genome comprising ~23.3 % LINEs; ~17.7 %, SINEs; ~3.2 % ERVs; ~2.0 % DNA transposons and ~0.4 % other LTRs (Adelson *et al*., 2009). Interspersed repeats are major drivers of genome evolution as they can either alter gene expression via insertions within or near promoters or insertions into exons which are often incorporated into existing protein-coding genes (Adelson *et al*., 2009).

Retrotransposons, also known as retroposons or transposable elements are the most common interspersed repeats which, similar to retroviruses, replicate and jump throughout the genome (Smit, 1996). In bovine (Bos taurus) it was found that transposable genetic elements make a large contribution to bovine gene structure, most abundantly in intronic sequences (Adelson *et al*., 2009). Retrotransposition has been considered to account for approximately 10 % of spontaneous mutations in the genome (Kazazian Jr, 1998). Bovine retroposons, elements which consist of LINE RTE type BovB and its associated SINE elements (BovA2, BOV-tA, and ART2A), account for 25-27 % of the bovine genome (Garcia-Etxebarria and Jugo, 2014).

Considering the potential impacts of repetitive DNA on transcription, from an evolutionary perspective it is essential that these elements are controlled and fully suppressed; DNA methylation plays a crucial role in this regard (Kaneko-Ishino and Ishino, 2010). Maintenance of genome integrity relies on DNA methylation to ensure chromosome stability through methylation of repetitive DNA elements which are found near centromeres and throughout the genome (Howard *et al*., 2008). Loss of methylation in these regions, which is frequently associated with tumor progression, leads to massive transcriptional activation and subsequently potential retrotransposition or production of chimeric transcripts composed of retrotransposable sequences as well as endogenous genes and ultimately genome instability (Monk, 2015). Therefore, hypermethylation of endogenous transposable elements which compose up to half of the genome significantly contributes to the overall hypermethylated state of the mammalian genome.

The location of transposable elements could affect regulation of nearby genes through several mechanisms (Warnefors *et al*., 2010). Differential transposable elements (TEs) expression has been suggested to trigger sequential reprogramming of the embryonic genome during the oocyte to embryo transition as well as preimplantation embryo development (Peaston *et al*., 2004). Interestingly, recently it has been shown that de-repression and high expression of TEs in oocytes are linked with novel genes, coding or non-coding (McLysaght and Guerzoni, 2015) which suggest a regulatory mechanisms of TEs in oocyte transcription (Veselovska *et al*., 2015).

1.3.3.3.3. X-chromosome inactivation (XCI)

Mammalian females carry two X chromosomes; one of which must be transcriptionally silenced to achieve dosage compensation (Augui *et al*., 2011). This process takes place in the developing embryo, at the late blastocyst stage, and can be seen as Barr body at the nuclear periphery of interphase female cells (Dixon-McDougall and Brown, 2015).

DNA methylation is a crucial mechanism for initiation and establishment of X chromosome inactivation (XCI) which begins at the X inactivation center (Xic) with the expression of Xist which is cis acting ncRNA (Augui *et al*., 2011; Gendrel and Heard, 2014). However, the regulation of Xist expression during differentiation is regulated by the action of another ncRNA, Tsix, which is transcribed from the opposite strand to the Xist gene (Antisense) (Augui *et al*., 2011; Gendrel and Heard, 2014). The decline of Tsix expression on the future inactive X chromosome (Xi) leads to an increase level of Xist which coats the chromosome and spreading out from the Xic. The coating of Xi triggers heterochromatization which accompanies with histone hypoacetylation and several histone modifications such as reduced H3K4 methylation and high levels of H3K9me3, polycomb silencing (H3K27me3 and H2AK119 ubiquitylation) and ultimately DNA methylation (Gendrel and Heard, 2014).

In mammalian species, the strategies for achieving X inactivation appear to be rather diverse. Except the marsupials, the eutherian mammals are generally believed to display random X inactivation in somatic tissues, so that females are mosaic for cell populations with either the silenced maternal X (Xm) or paternal X (Xp) chromosome (Patrat *et al*., 2009). There are some important differences between mammalian

species in the timing and nature of XCI during early development and in the soma (Gendrel and Heard, 2014). The XCI for mouse as the best studied animal model is rather different that the other mammals except rodents. Murine imprinted inactivation of the Xp is initiated during the first cleavage stages of preimplantation development and is reversed in the inner cell mass (ICM) of the blastocyst, to give rise proper embryo for random X inactivation. Afterward, the paternal X inactivation is maintained in extra embryonic tissues such as placenta. However, human Xist expression is predicted to be biallelic initially and subsequently becomes randomly monoallelic (Okamoto and Heard, 2009). Bovine Xist expression may initiate after the time of ZGA, at 8 cell stage. The activity status of the two X chromosomes in bovine has not been examined during early embryogenesis, although it has been suggested that Xist expression is imprinted and that Xp inactivation may be found in extraembryonic tissues (placenta) (Okamoto and Heard, 2009).

1.3.3.3.4. Imprinted genes

Imprinted genes are defined by their parent-of-origin-specific monoallelic expression (MacDonald *et al*., 2014). Imprinted genes have been shown to be associated with a number of physiological processes including early embryonic development, placental function, fetal growth, energy homeostasis, brain function and behavior (Dent and Isles, 2014). Imprinted genes are a classic example of long-term epigenetic memory of parental origin since they are maintained throughout life. In fact, genomic imprinting represents the greatest complication to epigenetic reprogramming and transgenerational inheritance, since the DNA methylation patterns of the imprinted

genes must first be erased in primordial germ cell (PGCs) and then re-established in a sex-specific manner in the gamete. They must then survive another wave of DNA reprogramming which occurs during early embryonic development (Monk, 2015). Although the exact mechanisms of parent-specific DNA methylation erasure and acquisition are not completely revealed, there is evidence showing that imprinted genes are resistant to genome-wide DNA demethylation during preimplantation development (MacDonald *et al*., 2014). Recently it has been suggested that chromatin topology and nuclear architecture play roles in tissue-specific imprint domain regulation during early development and differentiation (MacDonald *et al*., 2015).

A major modification thought to be involved with regulation of allele-specific expression of imprinted genes is DNA methylation (Denomme and Mann, 2013). Establishment of parent-of-origin specific DNA methylation patterns occurs during gametogenesis, resulting in one allele being heavily methylated and the other allele being unmethylated, at regions known as differentially methylated regions or DMRs. Such an allele-specific methylation, is a hallmark characteristic of imprinting control regions (ICRs) and is thought to influence allelic expression of several neighboring genes in cis (Smallwood *et al*., 2011b; Xie *et al*., 2012; Court *et al*., 2014).

1.3.3.3.5. DNA methylation reprogramming

DNA methylation reprogramming involves several waves of global erasure of DNA methylation and global *de novo* DNA methylation, which occurs twice during the life in mammals; during PGCs and early embryo development (Hackett and Surani, 2013).

1.3.3.3.5.1. Primordial germ cells (PGCs) developemnt

DNA methylation reprograming during PGC development includes two waves of passive and active reprograming (Hackett and Surani, 2013). In the first wave, which is a passive wave, the *de novo* DNA methyltransferases are silenced and components of the active demethylation pathways such as the hydroxylase Tet1 and members of the base excision repair pathway are expressed during the time when PGCs are re-acquiring totipotency while colonizing the genital ridge; facilitating passive DNA demethylation (Seisenberger *et al*., 2012). However, the rapid expansion of PGCs in the genital ridge ensuring replication-dependent global DNA demethylation (Kagiwada *et al*., 2013) which affects genomic regions including promoters, gene bodies and intergenic regions but not the imprinted genes.

During the second wave of demethylation which is the active wave, imprinted regions undergoes active DNA demethylation (Hill *et al*., 2014) most likely through DNA deaminases of the AID/APOBEC family and oxidizing of 5mC by TET enzymes to generate 5hmC and other oxidative formats of 5mC which eventually engaged in removal pathways (Hackett *et al*., 2013).

1.3.3.3.5.2. Early embryo development

Similar to DNA methylation reprogramming in PGCs, both active and passive DNA demethylation take place during early embryo development (Hackett and Surani, 2013). However, in PGCs the extent of DNA demethylation is close to absolute (with the exception of Intracisternal A Particles (IAP) and some single copy loci; Lees-Murdock and Walsh 2008; Hackett *et al*. 2012; Seisenberger *et al*. 2012), whereas

imprinted loci and transposons are preserved from demethylation and retain their DNA methylation status during early embryogenesis (Hackett and Surani, 2013). DNA methylation reprograming is initiated post-fertilization, but with rather different mechanisms depending on the parental origin of the pronuclei (Seisenberger *et al*., 2012). Whereas the male pronucleus undergoes rapid active DNA demethylation, through oxidation of 5mC with TET3 enzymes (Iqbal *et al*., 2011b; Gu *et al*., 2011) the female pronucleus slowly undergoes a process of passive DNA demethylation following each cleavage until 8 cell satge in bovine and blastocyst stage in mouse (Dean *et al*., 2001). It was initially believed that in the female pronucleus, only passive DNA demethylation was occurring; which means the bulk of the oocyte-derived CpG methylation is reduced by replication-dependent dilution during early embryo development (Gu *et al*., 2011; Iqbal *et al*., 2011b). However, recently it has been shown that the oxidative forms of 5mC produced with TET enzymes exist in both maternal and paternal genomes, demonstrating that the paternal methylome and at least a significant proportion of maternal methylome go through active demethylation during embryonic development (Wang *et al*., 2014; Guo *et al*., 2014).

Furthermore, *de novo* DNA methylation during early embryo development is species-specific (Dean *et al*., 2001). For instance, whereas *de novo* DNA methylation initiates at blastocyst stage for mouse and human, in bovine *de novo* DNA methylation takes place at 8 cell stage of early embryo development (Dean *et al*., 2001).

1.3.3.4. Impacts of folate on gametogenesis, embryo development and pregnancy

1.3.3.4.1. Follicular development

The exact mechanisms by which folate metabolism affects ovarian function is not fully understood (Laanpere *et al*., 2010). Folate presents in follicular fluid and their levels correlate with the folate blood concentrations (Steegers-Theunissen *et al*., 1993). This finding suggests that diffusion from the ovarian circulation into follicular fluid is the main determinant of the follicular concentration of the folate (Laanpere *et al*., 2010). Furthermore, folic acid supplementation has been shown to cause higher folate concentrations in serum and follicular fluid of folic acid supplement users (Boxmeer *et al*., 2008; Twigt *et al*., 2015). This can cause changes in the follicular fluid proteome through various mechanisms, ranging from changes in DNA nucleotide, amino acid and phospholipid synthesis and oxidative stress to epigenetic changes (Twigt *et al*., 2015). In mouse cumulus-oocyte complexes (COCs), folate transport occurs predominately through an anion exchanger (SLC19A1) (Kooistra *et al*., 2013). Furthermore, ooplasm has been suggested to be an important source of one-carbon metabolites that are required for early preimplantation development. Nevertheless, currently no data are available on the content of one-carbon nutrients in oocytes (Steegers-Theunissen *et al*., 2013).

At the follicular level, it is also suggested that folate establishes its effect on the follicular microenvironment most likely through changes in intra-ovarian pathways and peripheral tissues (Twigt *et al*., 2015). Additionally, folate possibly affects follicular

metabolism and oocyte maturation through extra-ovarian mechanisms by exerting effects on apolipoproteins synthesis (HDL) in the liver (Twigt *et al*., 2015).

1.3.3.4.2. Spermatogenesis

The testis has a high rate of cell synthesis, hence, strongly relies on one-carbon metabolism (Singh and Jaiswal, 2013). Therefore, testis critically depends on normal folate metabolism to support continual cell division and attendant DNA synthesis (Singh and Jaiswal, 2013). Folic acid through one-carbon metabolism involves in DNA synthesis, repair, and replication; methylation process and polyamine synthesis which all important for proliferation of male germ cell precursors (Singh and Jaiswal, 2013). In addition, folate along with homocystein and methionine help in normal process of sperm maturation and packaging and protect it from reactive oxygen species (ROS) (Fujii and Tsunoda, 2011). Furthermore, folate is involved in maintenance of DNA integrity and protection from DNA damage during spermatogenesis. In fact, folate and its cofactor such as vitamin B2, B6, and B12 through the one-carbon metabolism maintain the steady-state level of the deoxynucleotide. Any sustained defect of this process may lead to misincorporation and retention of uracil in the DNA backbone in place of thymine which leads to futile cycles of DNA repair and ultimately resulting in chromosome breakage (Pogribny *et al*., 1995; Singh and Jaiswal, 2013). Furthermore, folate is important for process of tail structure stabilization and sperm motility through generation of homocysteine in one-carbon metabolism in which oxidation of thiols like homocysteine is a crucial process for these events (Neagu *et al*., 2011). Moreover, numerous studies showed that dietary folate deficiency impair testicular folate

metabolism (Wallock-Montelius *et al*., 2007) as well as spermatogenesis and DNA methylation (Forges *et al*., 2007; Crha *et al*., 2010; Singh and Jaiswal, 2013; Lambrot *et al*., 2013; Gong *et al*., 2015; Liew *et al*., 2015).

1.3.3.4.3. Fertilization and early embryo development

In terms of folate's role during fertilization, it has been suggested that folate through one-carbon metabolism is involved in generating cysteine in which free cysteine (in small amount) is capable of initiating a thiol disulfide exchange that is essential for decondensation of sperm nucleus after fertilization to form the male pronucleus (Singh and Jaiswal, 2013).

Nearly, all enzymes that participate in one-carbon metabolism are expressed in mammalian preimplantation embryos including bovine, supporting that they can independently metabolize all one-carbon metabolism nutrients (Ikeda *et al*., 2012b). Therefore, it is likely that preimplantation embryos have a fully functional folate cycle (Ikeda *et al*., 2012b, Steegers-Theunissen *et al*., 2013). It has been shown that antifolate, methotrexate (MTX) which can inhibit the folate cycle, causes preimplantation developmental arrest *in vitro* due aberrant DNA synthesis (depletion of thymidylate and purines) (Kwong *et al*., 2010).

Whereas the importance of endogenous folate has been shown in mammalian early embryo development (O'Neill 1998; Kwong *et al*., 2010; Kooistra *et al*., 2013), there is no evidence supporting that exogenous (*in vitro* media culture supplementation) folate can affect early embryo development (Kane, & Bavister, 1988; McKiernan & Bavister,

2000). In contrast, exogenously supplied methionine leads to impact on early embryonic development through conversion to SAM (Menezo *et al*., 1989; Ikeda *et al*., 2012a). Nevertheless, folate can be uptaken via folate receptor-mediated endocytosis (FOLR1) in mouse preimplantation embryos (Kooistra *et al*., 2013). Furthermore, folate cycle and betaine/BHMT appear to contribute to a methyl pool required for normal ICM development and establishing initial embryonic DNA methylation (Zhang *et al*., 2015).

Although the exact mechanisms of beneficial effects of folate on development are largely unknown, neural tube defects (NTDs) risk is reduced by 70 -100 % through dietary supplementation with folic acid led to mandatory folic acid fortification of grain in North America and endorsement of folic acid supplementation in women at reproductive age (Pickell *et al*., 2011; Tamura and Picciano, 2006; Blom *et al*., 2006). Therefore, as expected these studies reported elevated circulating folate concentrations in the U.S. population after folic acid fortification (Bailey *et al*., 2010; Yang *et al*., 2010) and approximately 25 % of the U.S. population had supraphysiological concentrations of serum folate (above 45 nmol/L) (Pfeiffer *et al*., 2005). Despite the observed beneficial effects of folate in reduction of NTDs, adverse effects and potential consequences of long-term high folate intake are not known (Pickell *et al*., 2011). Studies using rodent models suggest that high levels of folate during pregnancy may inhibit embryonic growth and development (Pickell *et al*., 2011).

1.3.3.4.4. Placenta

The placenta facilitates the active transport of folate from the maternal to intervillious placenta circulation (Giugliani *et al*., 1985; Henderson *et al*., 1995), mainly through folate receptors and in less extent via reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT) (Laanpere *et al*., 2010). This transport from maternal to placenta generating an intervillous blood folate concentration approximately 2-4 fold times higher than that in maternal circulation and allowing passive transport of 5-methylTHF to the fetus down the concentration gradient (Giugliani *et al*., 1985; Laanpere *et al*., 2010). This indicates a preferential fetal gradient of folate across the placenta.

1.3.3.4.5. Pregnancy

During pregnancy, a high level of folate (*i.e.* folate supplement) is required for a number of reasons, including growth and development of the fetus, rapid cell proliferation and tissue growth of the uterus and the placenta and expansion of the maternal blood volume (Rondo and Tomkins, 2000; Tamura and Picciano, 2006). Therefore, pregnant women required to have access to adequate maternal dietary intake which is 5- to 10-fold higher than those in non-pregnant women (Antony, 2007).

1.4. Current options for the study of DNA methylation

DNA handling and preparation methods have evolved tremendously over the past decade, due in large part to worldwide interest in genomics, which has fueled the improvement of sample preparation methodologies, combined with the development of very high throughput microarray and DNA-sequencing platforms. The numerous options for DNA extraction and purification, combined with a wide array of downstream methodologies, have made a large array of technological platforms available for methylation studies.

Platforms vary in terms of their different types of treatment options pretreatment and analytical approaches. The elements that dictate the choice of the methodology include the targeted epigenomic effector, the goal of the study, the amount of tissue per sample, the number of samples, the quality and quantity of the DNA, the desired coverage (several candidate loci vs. the whole genome), the required genomic resolution, budget, and to some extent adventurousness (Zilberman and Henikoff, 2007; Laird, 2010; Zhang *et al*., 2015; Yuan and Feng, 2014). The general outline of the most relevant current sample treatment options and sequence identification platforms for the study of DNA methylation is depicted in Figure 1-10. Furthermore, the developed approaches for DNA methylation analysis can also be classified as direct and indirect approaches; direct approaches mainly focus on global methylation analysis, while indirect approaches rely on specific recognition of DNA-methylation loci (Zhang *et al*., 2015). In general, according to how the developed DNA methylation analysis approaches are going to be considered, various classifications are

proposed and reviewed in details (Yuan and Feng, 2014; Zhang *et al*., 2015; Fouse *et al*., 2010; Taleat *et al*., 2015).

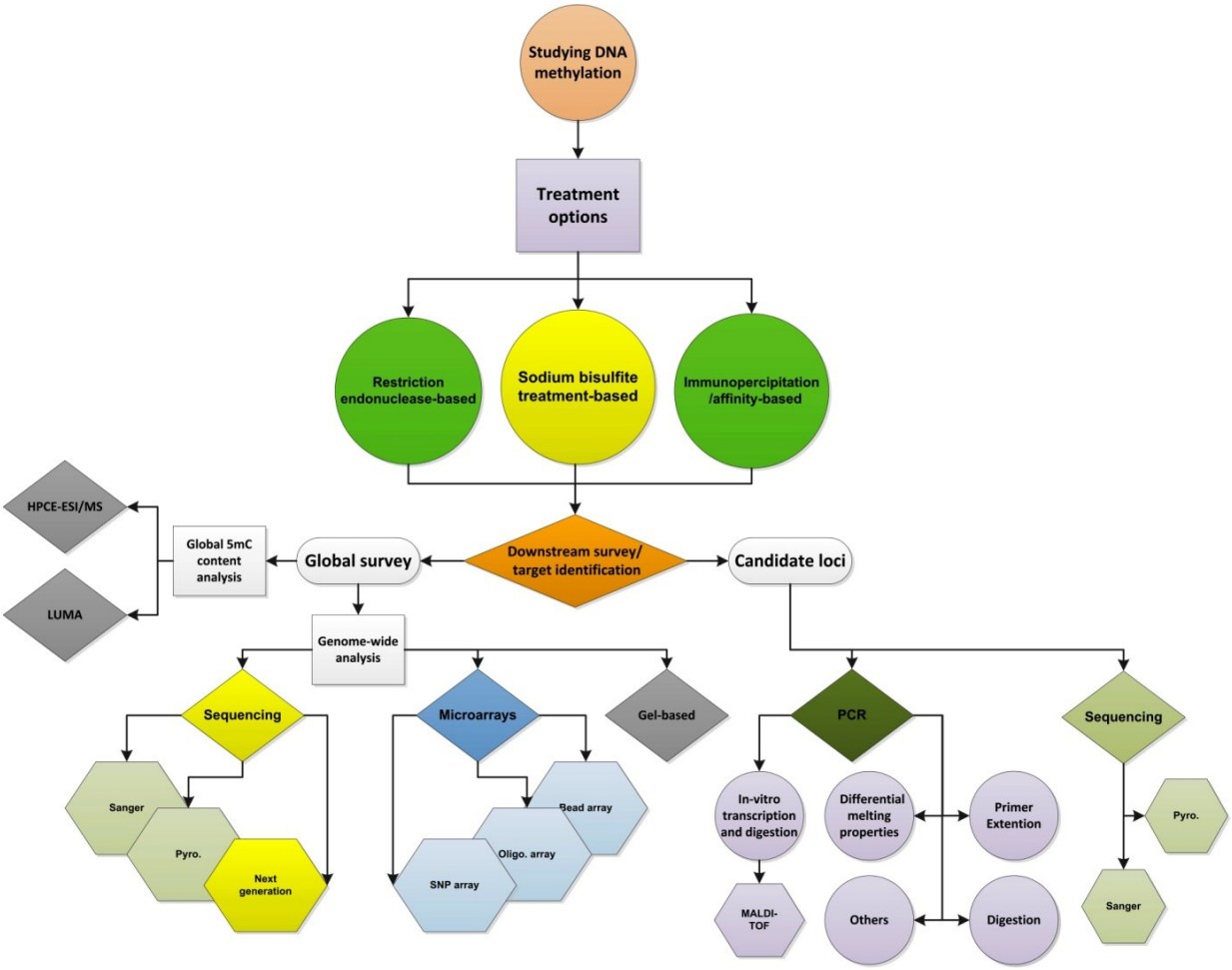

Figure 1-10 The general outline of the most relevant current sample treatment options and downstream applications and identification platforms for the study of DNA methylation. HPCE-ESI/MS: high-performance capillary electrophoresis–electron spray ionization/mass spectrometry (Berdasco *et al*., 2009); LUMA: luminometric methylation assay; MALDI-TOF: matrix-assisted laser desorption ionization–time of flight; Pyro: pyrosequencing; SNP: Singlenucleotide polymorphism.

However, for the purpose of current introduction, DNA methylation analysis approaches were classified based on the sample treatment strategies. It is noteworthy

that the first method used successfully to study DNA methylation of an entire genome was based on two-dimensional gel electrophoresis, known as restricted landmark genome scanning (RLGS) and requires 20–100 µg of DNA (Ando and Hayashizaki, 2007).

1.4.1. Sample treatment options

Sample treatment options for DNA methylation study is based on any of three principles: 1) chemical conversion using sodium bisulfite; 2) differential digestion with methyl-sensitive or methyl-specific restriction enzymes (MSRE); 3) target selection by pull-down assay using either antibodies against methyl cytosines or using methyl-binding proteins (Suzuki and Bird, 2008a; Laird, 2010; Zilberman and Henikoff, 2007).

1.4.1.1. Sodium bisulfite-based treatment

The most precise way to measure the degree of methylation of a DNA fragment remains the chemical conversion of unmethylated cytosine to uracil using sodium bisulfite. This approach is considered the gold standard for detecting changes in methylation, since it is capable of single-nucleotide-base resolution (Gupta *et al*., 2010; Yuan and Feng, 2014; Zhang *et al*., 2015). However, a positive control (spiked in) should be included in the sample in order to confirm that conversion is complete, since unconverted unmethylated cytosine will be counted as methylated sites (Zilberman and Henikoff, 2007). The bisulfite conversion approach is also very labor intensive and daunting for DNA fragments longer than 1 kb (Schumacher *et al*., 2006) and associated with incomplete conversion, false-positive results, difficult operation, and being time consuming (Zhang *et al*., 2015). Since the harsh chemical treatment can damage DNA and cause partial loss of valuable sample material (Kantlehner *et al*., 2011; Zhang *et*

al., 2015), these methods generally require micrograms of input DNA especially for whole genome DNA methylation profiling whereas input of only a few cells can be successfully used for study of targeted candidates. In addition, they do not distinguish between 5mC and 5hmC (Huang *et al*., 2010b). Bisulfite sequencing of randomly sheared genomic DNA is currently the most comprehensive whole-genome platform, but remains very expensive despite of the plummeting cost of sequencing runs and the high cost of bioinformatics (data analysis) expertise (Boyle *et al*., 2012).

Due to the nature of bisulfite treatment which converts the non-methylated cytosine to uracil (U), and will be further turned into thymine (T) after PCR amplification, while the 5-mC in the gene remains unchanged; three main analysis approaches for 5-mC after bisulfite treatment can be considered: sequencing-based (using PCR), melting temperature-based (using electrophoresis methods; flourescent signal measurement) and recognition interaction-based (using restriction enzyme; antibody; probe) approaches (Zhang *et al*., 2015).

1.4.1.2. Restriction-endonuclease-based methods

These methods of enrichment rely primarily on the sensitivity of restriction endonuclease (RE) activity to DNA methylation (Figure 1-11), for example the inhibiting impact of DNA methylation on *HpaII* , or conversely the affinity toward methylated DNA of McrBC (Ordway *et al*., 2007). Restriction endonucleases have been used widely to distinguish methylated DNA from unmethylated DNA, because of their availability, ease of application, and usefulness for mapping entire genomes without a priori knowledge. Any downstream method using this approach either enriches the sample

with methylated DNA or provides a mirror image of unmethylated DNA. RE-cleaved DNA can be compared in three different ways; i) between a sample treated with a methylation-sensitive RE or a cocktail of methylation sensitive REs and an undigested control to profile DNA methylation across the genome; ii) between a sample treated with a methylation-sensitive enzyme compared with a control treated with a methylation-insensitive isoschizomer (*HpaII* / MspI) again to profile DNA methylation genome wide ; and iii) between two test samples, such as two tissue types or mutant and wild-type samples, both treated with the same RE or RE cocktail; to identify methylation differences specific to the experimental factor (Zilberman and Henikoff, 2007). The coverage of the genome using this approach is dependent on the frequency and distribution of the recognition site of the particular enzyme as well as the number of RE enzymes used (Zilberman and Henikoff, 2007). This approach has been criticized because of its low genomic coverage, since each RE addresses only 2–8 % of the genome. For example, methyl-sensitive *HpaII* covers only about 8 % of the CpG in the human genome (Schumacher *et al*., 2006).

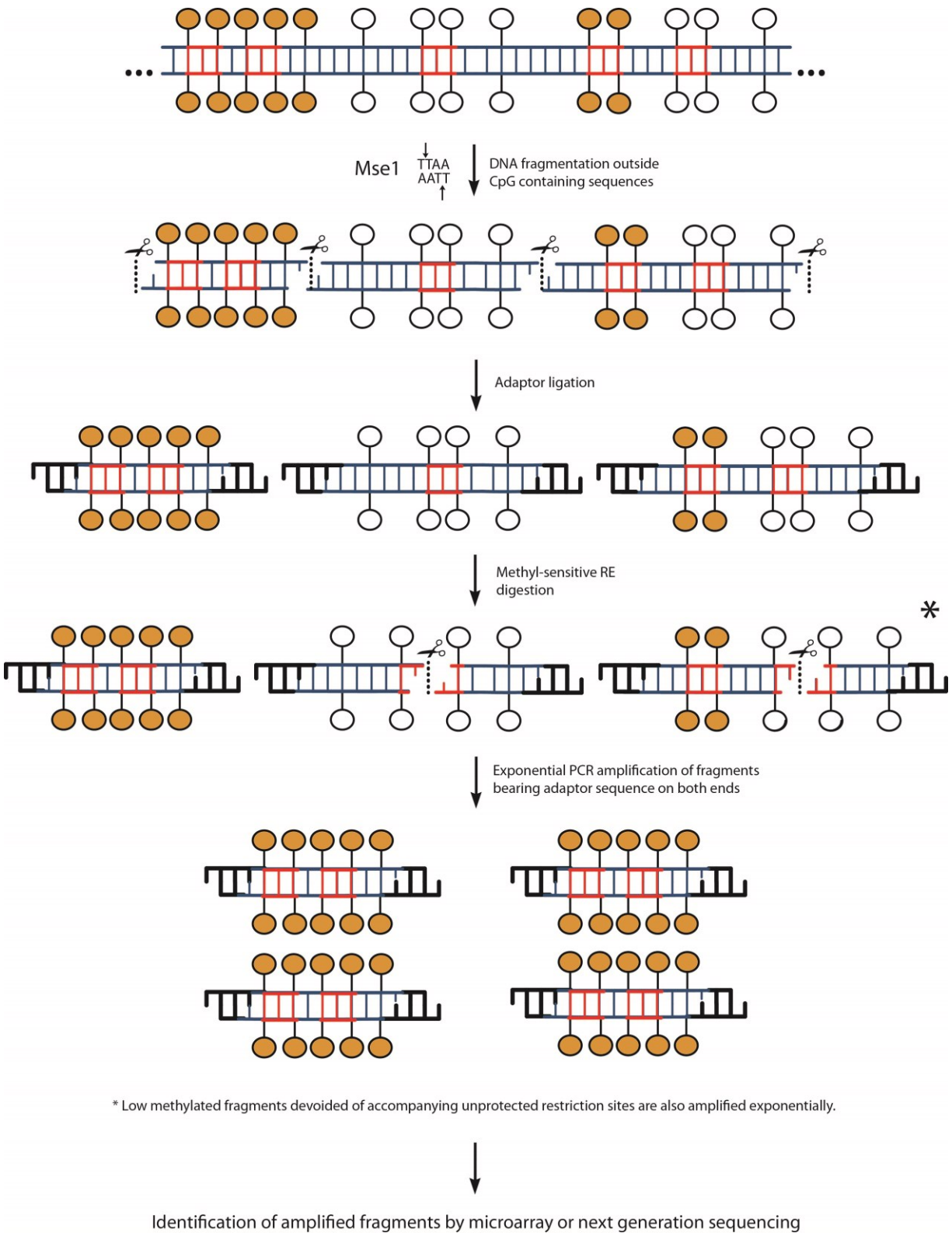

Figure 1-11 General flow of events of platforms for the study of DNA methylation based on methylation-sensitive restriction enzymes.

One way of increasing coverage is to combine several 5mC-sensitive enzymes (Suzuki and Bird, 2008; de Montera *et al*., 2013a). RE-based platforms thus provide at best moderate coverage, and are limited to the study of candidate loci bearing the RE recognition site within the targeted DNA sequences (Gupta *et al*., 2010). Small amounts of DNA can be analyzed as long as the completeness of the cleavage is confirmed by spiking samples with internal positive controls. False positives due to DNA polymorphisms (causing differences in methylation between samples) at enzyme recognition sites are always possible (Zilberman and Henikoff, 2007). Although most RE do not distinguish between 5mC and 5hmC marks, a few enzymes that target 5hmC specifically have been found and successfully used (Szwagierczak *et al*., 2011; de Montera *et al*., 2013a).

1.4.1.3. Affinity-based

Affinity-based procedures rely on two principles, namely i) the affinity and specificity of anti-5mC antibodies, and ii) the affinity of certain proteins for methylated (methyl-binding proteins; MeCP2 and MBD2) or unmethylated CpG sites. Affinity-based platforms in general provide a measurement of the relative enrichment of a sample in methylated DNA fragments (Figure 1-12). They offer the simplest way of reducing whole genome datasets to methylated loci. Data analysis and interpretation are easier than for bisulfite conversion. Since repeated elements are generally methylated in most cell types, enrichment approaches lead to their over representation in the dataset. Furthermore, the efficacy of the enrichment procedure depends on the affinity of the 5mC-capturing protein, which can be variable. Although all affinity-based

techniques lead to target enrichment, they appear influenced by 5mC density, and thus more useful for surveying methylation-rich elements such as CpG islands (Suzuki and Bird, 2008a) than for studying low-methylated DNA samples (Zilberman and Henikoff, 2007). Since capture of methylated DNA is generally achieved on randomly cleaved samples, downstream identification of the fragments does not indicate exact positions of methylation but rather a ~100 bp range (Beck, 2010; Down *et al*., 2008).

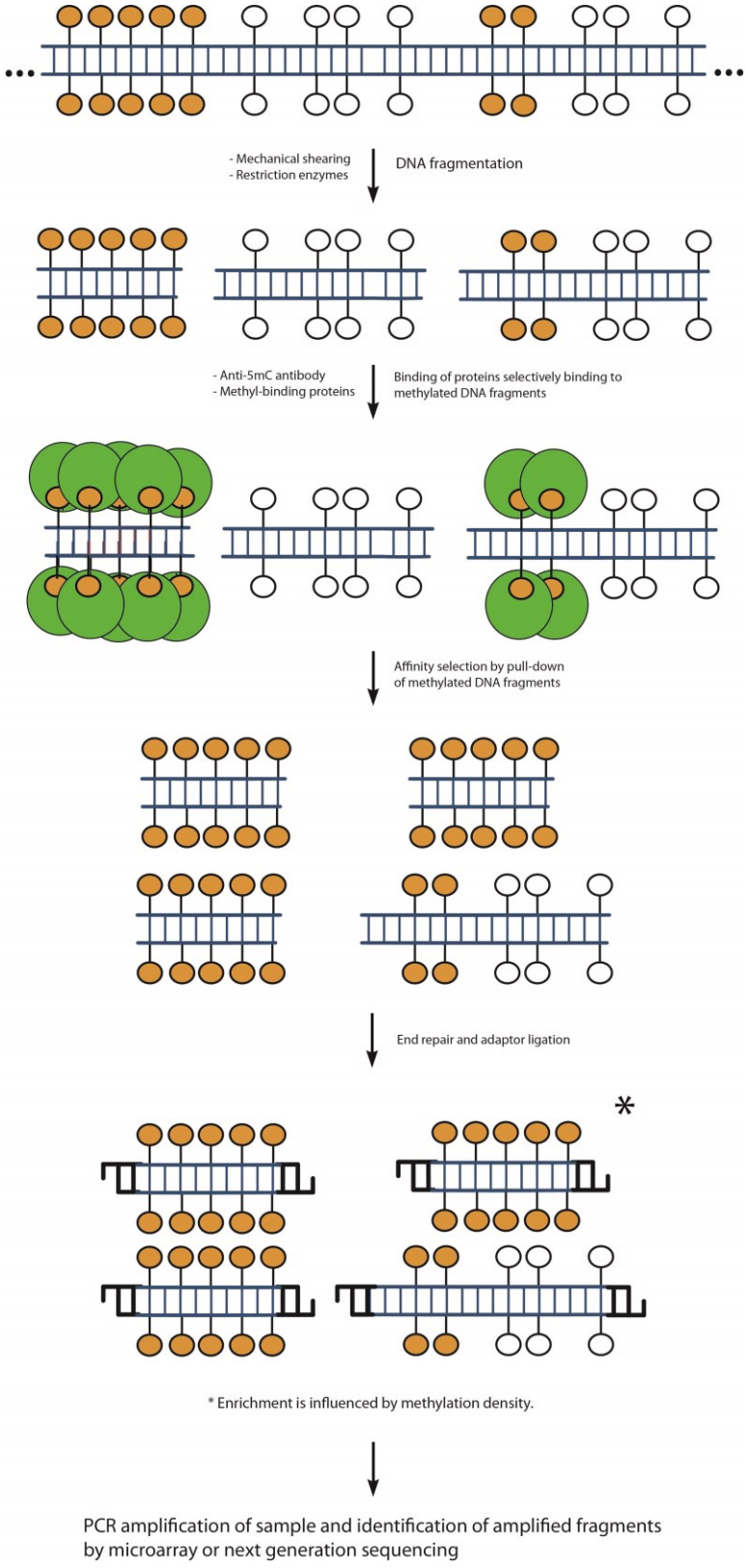

Figure 1-12 General flow of events of platforms for the study of DNA methylation based on affinity enrichment of methylated sequences.

The development of a genetically engineered binding protein with a multimerized MBD1 domain allows high-affinity capture of larger molecules (> 50-fold larger than with the monomeric MBD domain) for efficient pull-down (Jørgensen *et al*., 2006). The most recently introduced pull-down approach requires150–300 ng of input DNA (Taiwo *et al*., 2012), making it slightly better than other methods, which requires 0.3–5 µg (Beck, 2010). Combined to whole-genome amplification, authors were able to immunoprecipitated the methylated fractions from substantial pools of blastocysts (n=300) to map DNA methylation (Guibert *et al*., 2012; Borgel *et al*., 2010b). Hence, the use of affinity based methods for genome-wide embryo DNA methylation studies remains inappropriate or highly problematic when considering that a single blastocyst composed of 100 cells contains about 600 pg of DNA.

1.5. Downstream methods for identifying selected sequences

Cleaved, cytosine-converted, and enriched DNA is quantified and characterized using primarily one of four technological platforms. These are i) detection and quantification of candidate loci by quantitative PCR; ii) gel electrophoresis to determine cleavage fragment size distribution; iii) array-based detection and identification based on the designed oligoset; and iv) next-generation sequencing. A wide variety of methodological variants have been developed and successfully used to study candidate methylation loci (Table 1-2). A growing diversity of complementary methodological platforms also exists for genome-wide profiling of DNA methylation (Table 1-3). Methods targeting candidate loci have been used widely to study imprinted genes (Denomme and Mann, 2012), while electrophoresis-based identification of fragments such as RLGS are laborious and less precise due to incomplete digestion, and are being replaced by other techniques (Laird, 2010). The focus in this part of the introduction shall be genome-wide approaches, which identify targets either through array-based or next-generation sequencing-based platforms. The other methods are described in detail elsewhere, notably in reviews (Laird, 2010; Suzuki and Bird, 2008a; Umer and Herceg, 2012; Beck and Rakyan, 2008; Olkhov-Mitsel and Bapat, 2012; Gupta *et al*., 2010; Zhang *et al*., 2015).

Table 1-2 Survey of different methodological platforms developed to study DNA methylation of candidate loci.

Locus specific methods	Bisulf.	PCR	Dig.	Pr.Ext	IP	Sequencing		Mass spect.	5mC≠5hmC†	References
						Sanger	*Pyro*			
MSP	A	B	-	-	-	-	-	-	*No*	(Herman *et al.*, 1996)
MethylLight	A	B	-	-	-	-	-	-	*No*	(Eads *et al.*, 2000)
COBRA	A	B	C	-	-	-	-	-	*No*	(Brena and Plass, 2009)
BS	A	-	-	-	-	B	-	-	*No*	(Frommer *et al.*, 1992)
Pyro-Seq	A	-	-	-	-	-	B	-	*No*	(Uhlmann *et al.*, 2002)
HeavyMethyl	A	B	-	-	-	-	-	-	*No*	(Distler, 2009)
MS-SnuPE	A	-	-	B	-	-	-	-	*No*	(Kristensen and Hansen, 2009)
MS-MCA	A	B	-	-	-	-	-	-	*No*	(Worm *et al.*, 2001)
MS-HRM	A	B	-	-	-	-	-	-	*No*	(Wojdacz and Dobrovic, 2007)
MeDIP-PCR	-	B	-	-	A	-	-	-	*No*	(Laird, 2010)
EpiTYPER	A	B	C	-	-	-	-	D	*No*	(Ehrich *et al.*, 2006; Ehrich *et al.*, 2005)

A: first step, **B:** second step, **C:** third step, **D:** fourth step. **†:** discrimination between 5-methylcytosine and 5-hydroxymethylcytosine

Bisulf.: sodium bisulfite treatment; **BS:** bissulfite sequencing; **COBRA:** combined bisulfite restriction analysis; **Dig.:** restriction endonuclaese digestion; **IP:** immunoprecipitation; **Mass spec.:** mass spectrophotometry; **MS-HRM:** methylation-sensitive high-resolution melting. **MS-MCA:** methylation-sensitive melting curve analysis; **MS-SnuPE:** methylation-sensitive single nucleotide primer extension; **MSP:** methylation specific PCR; **Pr.Ext :** primer extension; **Pyro- seq.:** pyrosequencing.

Table 1-3 Survey of different methodological platforms developed to study genome-wide DNA methylation.

	DNA treatment options									
	Restriction endonuclease-based				Affinity-based			Bisulfite-based			
	Name	DNA (µg)*	RE	5mC ≠ 5hmC†	Name	DNA (µg)	5mC ≠ 5hmC	Name	DNA (µg)	5mC ≠ 5hmC	References
Earlier Methods											
	MS-RDA	*1*	*HpaII*	*No*	-	-	-	-	-	-	(Ushijima *et al.*, 1997)
	LUMA	*0.25-0.5*	*HpaII /MspI*	*No*	-	-	-	-	-	-	(Mohsen *et al.*, 2006)
Gel-based											
	RLGS	*1-5*	*NotI or AscI §PvuII, PstI*	*No*	-	-	-	-	-	-	(Costello *et al.*, 2002)
	MS-AP-PCR	*2*	*HpaII /MspI*	*No*	-	-	-	-	-	-	(Liang *et al.*, 2002)
	AIMS	*1*	*SmaI/XmaI*	*No*	-	-	-	-	-	-	(Frigola *et al.*, 2002)
Microarray based											
	CHARM	*20*	*McrBC*	*No*	MeDIP-Chip(A)	*1*	*No*	MSO (A)	-	*No*	(Irizarry *et al.*, 2008(Irizarry *et al.*, 2008); (Weber *et al.*, 2005);(Gitan *et al.*, 2002)
	DMH	*2*	*BstUI/HpaII - MseI*	*No*	MAP (A&B)	*100*	*No*	Infinium 27/ 450(D)	*0.5-1*	*No*	(Huang *et al.*, 1999);(Illingworth *et al.*, 2008);(Bibikova *et al.*, 2011; Bibikova *et al.*, 2009)
	HELP	*10*	*HpaII /MspI*	*No*	MIRA –Chip (A& B)	*5*	*No*	GoldenGate® assay (D)	*0.25*	*No*	(Khulan *et al.*, 2006);(Rauch *et al.*, 2009);(Bibikova and Fan, 2009)
	MCAM	*5*	*SmaI/XmaI*	*No*	mDIP	*10*	*No*	-	-	*No*	(Estécio *et al.*, 2007);(Keshet *et al.*, 2006)
	McrBC	*5*	*McrBC*	*No*	MethylCap-seq	*1*	*No*	-	-	-	(Lippman *et al.*, 2004{Nouzova, 2004 #181};(Bock *et al.*, 2010; Rodríguez-Ubreva *et al.*, 2012)

Table 1-3 continued.	DNA treatment options									
	Restriction endonuclease-based				Affinity-based			Bisulfite-based			
	Name	DNA (µg)*	RE	5mC ≠ 5hmC †	Name	DNA (µg)	5mC ≠ 5hmC	Name	DNA (µg)	5mC ≠ 5hmC	References
	MMASS	*1.2*	*McrBC/cocktail*	*No*	mCIP	*2*	*No*	-	-	-	(Ibrahim *et al.*, 2006);(Zhang *et al.*, 2006)
	PMAD	*0.5*	*HpaII /MspI*	*No*	-	-	-	-	-	-	(Fukasawa *et al.*, 2006)
	MethylScope	*15*	*McrBC*	*No*	-	-	-	-	-	-	(Ordway *et al.*, 2007)
	MSNP (A,C)	*0.25*	*XbaI- StyI/ HpaII / MspI*	*No*	-	-	-	-	-	-	(Kerkel *et al.*, 2008)
Sequencing- based											
	Methyl-seq	*5*	*HpaII -MspI*	*No*	MIGS	*5*	*No*	RRBS	*0.03-1*	*No*	(Brunner *et al.*, 2009);(Serre *et al.*, 2010);(Meissner *et al.*, 2008a)
	HELP-seq	*10*	*HpaII /MspI*	*No*	MeDIP-seq	*1-10*	*No*	BC-seq	*50-100*	*No*	(Oda *et al.*, 2009);(Down *et al.*, 2008);(Meissner *et al.*, 2005)
	MSCC	*2*	*HpaII*	*No*	MBD-seq	*25*	*No*	BSPP	*0.2-1*	*No*	(Ball *et al.*, 2009);(Lan *et al.*, 2011);(Berman *et al.*, 2009; Li *et al.*, 2009)
	MCA-seq	*5*	*SmaI / XmaI*	*No*	MethylCap-seq	*1*	*No*	WGSBS	*1-10*	*No*	(Toyota *et al.*, 1999);(Bock *et al.*, 2010);(Cokus *et al.*, 2008; Lister and Ecker, 2009)
	HMST-Seq	*1*	*HpaII /MspI*	*Yes*	MIRA-seq	*5*	*No*	MethylC-Seq	*5*	*No*	(Xia *et al.*, 2013);(Park *et al.*, 2011);(Lister *et al.*, 2008)
	-	-	-	-	-	-	-	ox-RRBS	*2g*	*Yes*	(Booth *et al.*, 2012a)
	-	-	-	-	-	-	-	oxBS-Seq	*1*	*Yes*	(Booth *et al.*, 2012a)
New introduced technology	**GLIB** *which uses different DNA treatment options (glucosylation/periodate oxidation/biotinylation)*								*20*	*Yes*	(Pastor *et al.*, 2012{Pastor, 2011 #112)

***:** Required input gDNA as reported in reference literature; **RE:** restriction endonuclease; **†:** discrimination between 5-methylcytosine and 5-hydroxymethylcytosine; **A:** oligonucleotide array, **B:** probe CGI array, **C:** SNP array, **D:** Beadchip array.

AIMS: amplification of inter-methylated sites; **BC-seq:** bisulphite conversion followed by capture and sequencing; **BSPP:** bisulphite padlock probes; **CHARM:** comprehensive high-throughput arrays for relative methylation; **-Chip:** chromatin immunoprecipitation; **DMH:** differential methylation hybridization; **GLIB:** glucosylation, periodate oxidation, biotinylation; **HELP:** *HpaII* tiny fragment enrichment by ligation-mediated PCR; **HELP-seq:** HELP sequencing; **HMST-Seq:** hydroxymethylation and methylation sensitive tag sequencing; **LUMA:** luminometric methylation assay; **MAP:** MBD affinity purification; **MBD:** methyl-binding domain; **MCAM:** methyl CpG island amplification (MCA) coupled with microarray; **MCA-seq:** methyl CpG island amplification followed by sequencing; **mCIP , mDIP, MeDIP:** methylated DNA immunoprecipitation**; MethylCap:** capture of methylated DNA with the MBD domain of MeCP2; **MethylC:** cytosine methylome; **MIGS:** MBD-isolated genome sequencing; **MIRA:** methylated CpG island recovery assay; **MMASS:** microarray-based methylation assessment of single samples; **MS-AP-PCR:** methylation-sensitive arbitrarily primed PCR; **MSCC:** methylation-sensitive cut counting; **MSO:** methylation-specific oligonucleotide microarray; **MSNP:** methylation single-nucleotide polymorphism; **MS-RDA:** methylation-sensitive representational difference analysis; **ox-RRBS:** oxidative RRBS; **oxBS-seq:** oxidative bisulphite conversion followed by capture and sequencing; **PMAD:** promoter-associated methylated DNA amplification DNA chip; **RLGS:** restriction landmark genome scanning; **RRBS:** reduced representation bisulfite sequencing; **–seq:** followed by sequencing; **WGSBS:** whole-genome shotgun bisulphite sequencing.

1.5.1. Array-based identification

In order to study DNA methylation at the genomic scale using an array-based platform, three main types of technologies are available, namely i) bead arrays (*e.g.* from Illumina); ii) short oligonucleotide arrays (25-mer oligonucleotides from Affymetrix), and iii) long oligonucleotide arrays (60-mer oligonucleotides, from NimbleGen and Agilent). These are all commercially available, and the bead array (Katari *et al*., 2009) and oligonucleotide arrays can be custom-designed to meet technical and financial constraints (Suzuki and Bird, 2008a; Zilberman and Henikoff, 2007). It should be noted that each of these arrays has its advantages, as well as limitations rendering them unsuitable for some experimental setups (Schumacher *et al*., 2006). Their utility in methylation analysis depends on their specific design and the applied technology (Zilberman and Henikoff, 2007). Bead arrays have been used for DNA methylation polymorphism discovery and analysis (Shen *et al*., 2005; Bibikova *et al*., 2006), while Affimetrix SNP arrays were useful for analyzing allele-specific processes such as imprinting and X inactivation associated with DNA methylation (Hellman and Chess, 2007). Both short and long oligonucleotide arrays can be used for whole-genome and large-scale methylation mapping as well as tiling genomic arrays (Zhang *et al*., 2006) and promoter arrays (Reinders *et al*., 2008). Although the short oligo array (Affymetrix) is only offered in a single-channel version (one fluorescent dye), long oligo arrays (NimbleGen and Agilent) use dual channels (two fluorescent dyes), allowing labeling of two samples with different fluorescent dyes. In addition, because of the length of the probe for long oligo

arrays, noise may be lessened and the balance between specificity and sensitivity may be improved compared to short oligo arrays (Kreil *et al*., 2006).

Considering the current cost and complexity of bioinformatics data analysis for deep sequencing (discussed below), at the moment, array-based techniques are more "affordable" than sequencing if data analysis time and costs are included and have a shorter sample to published data turnaround time. The major constraint of all array platforms is the limited sequence identification to the oligoset. In addition, except for SNP arrays, which query predetermined candidate cytosines in bisulfite-converted samples, oligonucleotide hybridization does not provide single nucleotide resolution. Microarrays are generally used in combination with cleavage by restriction endonuclease to allow parallel screening of numerous restriction fragments representing DNA methylation profiles over large segments of the genome (Schumacher *et al*., 2006).

1.5.2. Sequencing based identification

Three types of sequencing have been used for DNA methylation analysis. These are Sanger sequencing, pyrosequencing, and other NGS platforms (primarily Hiseq from Illumina). Unlike arrays, sequencing-based platforms do not require optimal probe design, are free of hybridization artifacts and are more flexible and powerful, allowing single base resolution and allele-specific DNA methylation analysis. Their drawbacks are the high cost per sample when library preparation (inherent to NGS), the sequencing run and

downstream bioinformatics are taken into account. Potential sequencing library biases (Dohm *et al*., 2008; Zhang *et al*., 2015) as well as the considerable expertise required in order to analyze the sequencing data obtained are additional challenges of DNA sequencing platforms. Sodium bisulfite conversion of cytosine in combination with DNA sequencing, especially through NGS, represents the current state of the art in the study of DNA methylation. For genome-scale DNA methylation studies, Sanger sequencing is barely feasible due to the low throughput but can be used for PCR amplified specific locus analysis. Whole-genome shotgun bisulfite sequencing (WGSBS) developed by Illumina for their Genome Analyzer platform is currently the most comprehensive single-base-pair resolution DNA methylation analysis technique. However, still approximately 90 % of all CpG dinucleotides in the mammalian genome align properly with bisulfite-converted reads (Lister *et al*., 2009). As mentioned, sequencing approaches are bioinformatics intensive, especially for bisulfite-converted samples with low degrees of methylation, because of their low sequence complexity (bisulfite conversion of low methylated sequences generates a three nucleotide code instead of the standard four bases) and high homopolymer content (Laird, 2010; Zilberman and Henikoff, 2007). In fact, bioinformatics is often the bottleneck preventing quick sample to publication turnover. In addition, it should be noted that due to the production of unprecedented amounts of data (terabytes), storage of the NGS dataset can rapidly become challenging even for facilities with considerable expertise in genomic data management (Park, 2009).

1.5.3. Other options for the study of DNA methylation and new technologies in development

Some bisulfite-free and enzyme-free techniques were introduced which were either based on direct-oxidation or chemical-oxidation cleavage analysis. The direct-oxidation worked based on oxidation of methylated cytosine through applying different oxidative product and the chemical-oxidation cleavage analysis was based on chemical oxidation cleavage for discrimination of 5-mC. Although these methods produced promising results and had the advantage of high throughput sample discrimination, but they require skillful operation is their main drawback making them inapplicable for being widely used (Zhang *et al*., 2015).

Nanopore-based epigenetic analysis is currently the most promising although developing method for DNA methylation analysis. Nanopore-based epigenetic analysis is a technology in which under electric field, individual DNA molecules can be read at single nucleotide precision when is forced through membrane containing a synthetic nanopore, which allows only one molecule to cross at a time. Therefore, from the change of voltage threshold and combined with processive enzymes, all five C5-cytosine variants including, C, 5-mC, 5-hmC, 5-foC and 5-caC can be distinguished with the accuracy range of 91.6–98.3%. This should be a useful complement to bisulfite-based methods due to several unique features of this emerging technology: including directly reading from gDNA, indefinite retaining of gDNA for redoing the analysis and increasing the accuracy,

plausible long reads of gDNA (>10kb) and detection of all five C5-cytosine variants known to occur at CpG dinucleotides in mammalian genomes in one assay (Wescoe *et al*., 2014).

1.6. Successful application of the methodologies to the study of DNA methylation in early embryos

The majority of genome-scale methods for studying DNA methylation are listed in Table 1-3. These include comprehensive high-throughput arrays for relative methylation (CHARM), methylated DNA immunoprecipitation profiles generated using NGS (MeDIP-seq), methyl-binding domain sequencing (MBD-seq), and methyl-seq (Kantlehner *et al*., 2011). It should be noted that many of these platforms require micrograms of DNA input, making them unsuited to the study of mammalian oocytes and early embryos.

A restriction-enzyme-based platform combined with a microarray containing an oligoset of long oligonucleotides (NimbleGen) has been used successfully to profile the methylation of 300–600 pg of gDNA recovered from a single mouse blastocyst (Wright *et al*., 2011). The authors combined methyl-sensitive enzyme digestion (HpyCh4IV) with ligation-mediated PCR to obtain methylated DNA fragment enrichment, and used ~ 72 000 oligonucleotides to assess relative methylation at about 16,000 loci distributed along mouse chromosome 7 for identification. Although this study was not genome wide, it provided evidence that *in vitro* embryo culture is associated with overall hypermethylation as well as increased locus-to-locus variability in methylation.

Affinity-based enrichment protocols still require substantial DNA input, making them less compatible with small samples. However, these pull-down assays may provide rapid and efficient genome-wide assessment of DNA methylation, while sequence identification can be performed using array hybridization or NGS (Down *et al*., 2008).

Bisulfite conversion of cytosine has been used for more than a decade to study DNA methylation of candidate loci, namely imprinted genes (Denomme and Mann, 2012; Manning *et al*., 2000; Engemann *et al*., 2001). However, since this harsh chemical treatment leads to sample loss, its use for genome-wide profiling of DNA methylation in very small samples has met with limited success. A genome-scale single-base-pair resolution DNA methylation map of a mammalian genome has been produced nonetheless using reduced representation bisulfite sequencing (RRBS) (Meissner *et al*., 2008a). Almost three years later, a group from the Babraham Institute reported the first study of pre-implantation embryos using the same approach. Using gDNA extracted from 54 blastocysts, these researchers were able to show dynamic CpG island methylation landscaping at single-nucleotide resolution in mouse pre-implantation embryos (Smallwood *et al*., 2011a). Since then, several studies using RRBS have yielded single base resolution genome-scale DNA methylation maps in mouse sperm, oocytes, and different stages of pre-implantation embryos (Smith *et al*., 2012b) or SCNT-reconstructed embryos (Chan *et al*., 2012). RRBS appears suitable for analyzing DNA methylation patterns in pre-

implantation embryos because of its compatibility with limited amounts of gDNA (Wu and Zhang, 2012).

As its name suggests, RRBS is used to reduce sequence redundancy by selecting candidate regions of the genome for sequencing (Figure 1-13). After extraction, gDNA is fragmented using the methylation-insensitive restriction enzyme MspI (C^CGG), which does not target specific genomic regions, although the produced fragments are enriched in CpG-containing segments. The cleaved gDNA is then processed for addition of methylated sequencing adaptors. The adaptor-ligated fragments are run on agarose gel for molecular weight selection (usually 40–150 and 150–270 bp). The size-selected MspI digested fragments are treated with sodium bisulfite and amplified by PCR from the conversion-protected adaptor sequences, which generates the RRBS sequencing library. Following quality control assessment, RRBS libraries are sent for NGS.

The genomic coverage obtained using RRBS is generally estimated at about 1 % of the genome, which translates to approximately a million CpG dinucleotides (5 % of all CpG), of which about half are located within promoters and CpG islands, while the rest are representative of various classes of repetitive elements or even relatively CpG-poor sequences (Meissner *et al*., 2008a).

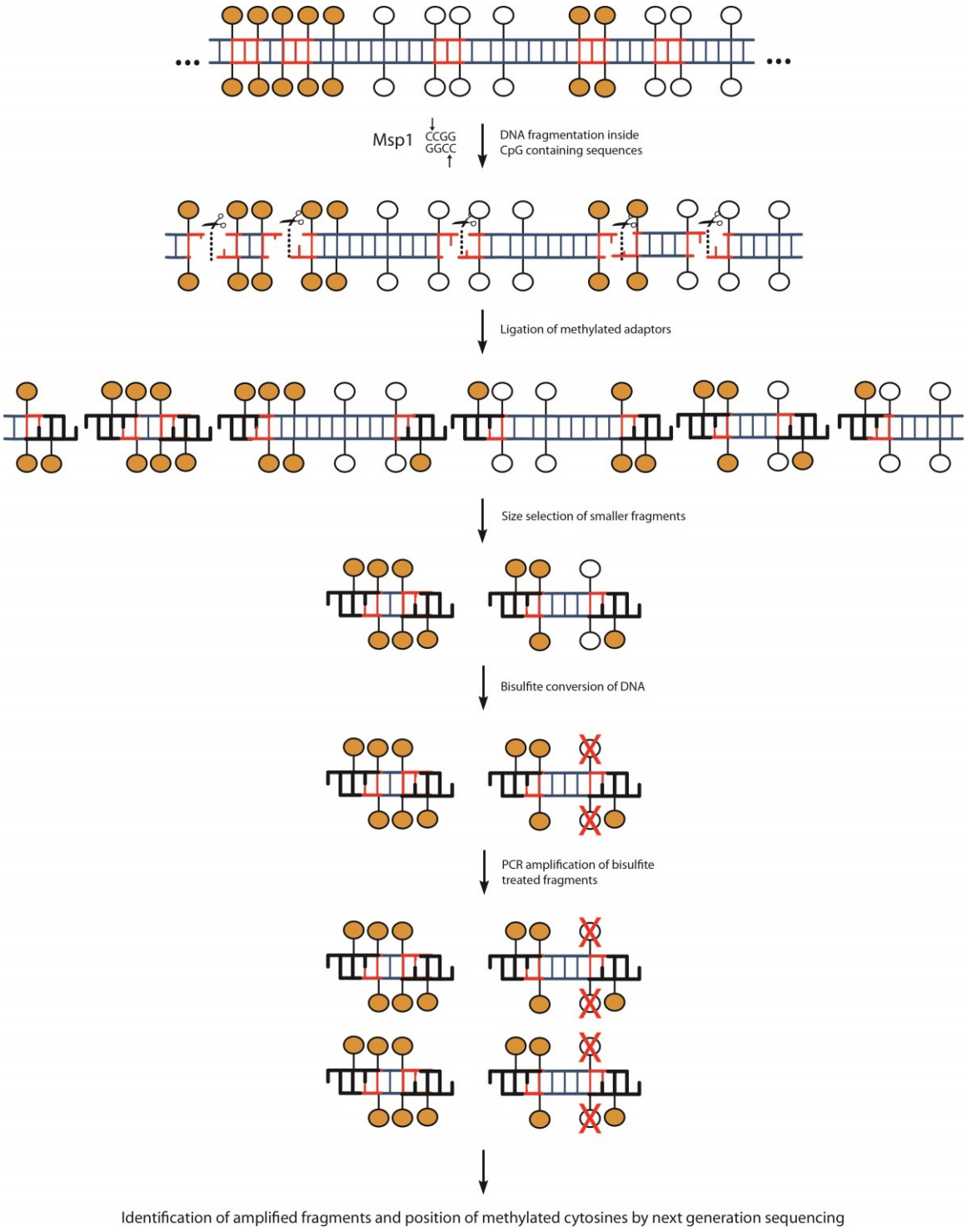

Figure 1-13 General outline of the reduced representation bisulfite sequencing (RRBS) platform dedicated to the study of DNA methylation. Red 'X' represents converted cytosine to uracil bases.

The advantages of RRBS include reduction of genome complexity and hence sequencing costs, DNA methylation information (at least one CpG dinucleotide) contained in every MspI-digested fragment, fragments enriched in gene promoters, and CpG islands in close proximity with the functional (*i.e.* gene-encoding) segment (Boyle *et al*., 2012).

A Modified RRBS protocol described as "gel-free", introduced by Meissner and colleagues, is called multiplexed RRBS or mRRBS (Boyle *et al*., 2012). Multiplexed RRBS allows simultaneous processing of 96 or more samples in remarkably short time at just over half the cost of the original RRBS protocol. Several laborious steps were removed and replaced with less onerous alternatives. Meanwhile, improvements in sample handling are continuing. For example, embedding samples in agar prior to bisulfite treatment reduces loss of gDNA (Denomme *et al*., 2012). Using this method, Denomme *et al*. successfully determined DNA methylation profiles of specific loci in a single oocyte. Combined with optimized sample handling procedures, rapid library generation using mRRBS will greatly increase throughput while substantially reducing the cost per sample and providing CpG coverage similar to that of the original RRBS protocol. Following optimization of the protocol by minimizing input DNA channel, adjusting size-selection of DNA fragments, and maximizing unmethylated DNA channel, a pipeline was formed and could analyze the methylation bioinformatics. Therefore, in comparison with other sequencing approaches, this technique can screen more samples with the ability to detect new epigenetic alterations at single nucleotide resolution (Zhang *et al*., 2015). It is therefore expected

that new studies of mammalian pre-implantation embryos will be conducted using this platform.

1.7. The challenges associated with the study of the epigenome in early embryos

The main challenges in the study of the epigenome in oocytes and early embryos are associated with the extremely limited quantities of biological sample (Wu and Zhang, 2012). In order to appreciate this challenge more fully, one need to only consider several key numerical characteristics of mammalian genomes:

- Based on sequencing studies, it is estimated that mammalian genomes contain roughly 21,000–22,000 genes, most of which are protein encoding (Frazer, 2012; Sato *et al*., 2009). These genes account for about 1–2 % of the entire genome (Biémont, 2010; Harrow *et al*., 2009; Consortium *et al*., 2007);
- A mammalian genome on average is composed of more than three billion base pairs (http://www.genomesize.com). If all of the DNA molecules making up a genome of a diploid cell were to be joined end to end, the resulting molecule would extent to more than two meters (Gregory, 2005a; Gregory, 2005b);
- This DNA is packed into a nucleus that is 10 µm in diameter in somatic cells;
- The nucleosomes are the basic unit of DNA packaging, each composed of four histone proteins; each chromosome contains several hundred thousand nucleosomes;

- The amount of DNA in a diploid cell is about six pg (Wells and Delhanty, 2000).
- The early embryonic developmental window in which most reprogramming events occur spans the first week, during which the embryo becomes a blastocyst, 50–150 cells on average, depending on the species (Hardy *et al*., 1989; Thouas *et al*., 2001).

Considering these facts, the technical challenges inherent in studying the embryonic epigenome are easily appreciated. For instance, considering that 0.3–0.9 ng of gDNA can be obtained per blastocyst and that current whole-genome profiling methods generally require one µg (CGH array) to 60 µg (ChiP) of gDNA input, thousands of embryos would be required making these platforms unsuitable (O'Neill *et al*., 2006; Wright *et al*., 2011). Such challenges are not new to the study of early mammalian development, transcriptomic profiling or pre-implantation genetic diagnosis having found solutions through whole transcriptome amplification (Robert, 2010) or whole genome amplification (Peng *et al*., 2007). In the cases of DNA methylation, histone modification or chromatin-related protein complexes, the problem remains daunting, since amplification strategies do not copy or preserve epigenetic marks. Until these methodological constraints are resolved, the unraveling of the epigenetic mechanisms in oocytes and early embryos will remains out of reach.

Another challenge inherent in the study of early development is data normalization. It is known that the epigenome is cell-type specific, which in the case of mammals means over

200 distinct epigenomes (Watt and Driskell, 2010; Hon *et al*., 2009). Upon this complexity is heaped the challenge of data interpretation when studying mixed tissues. A good example is the opposite DNA methylation patterns found for several pluripotent genes in trophectoderm cells comparatively to inner cell mass cells at the blastocyst stage. In the case of early embryos, the data normalization issue is often viewed differently, with many researchers assuming that early blastomers are equivalent, since it has been shown by embryo splitting that these early cells can be extracted to generate healthy twin offspring (Illmensee *et al*., 2005; Illmensee and Levanduski, 2010). However, early blastomere cell fate is not yet fully understood, and other researchers are challenging the homogeneity assumption, proposing that cell fate could be determined as early as the two-cell stage (Lorthongpanich, 2008). It has been suggested that blastomeres arising from certain cell division patterns may inherit epigenetic factors predisposing them to either an inner cell mass (ICM) or a trophectoderm (TE) fate (Parfitt and Zernicka-Goetz, 2010; Tabansky *et al*., 2013). In any case, if such cellular homogeneity exists, it is of short duration, since the blastocyst stage is composed of at three cell types; ICM, TE and primitive endoderm. The epigenome is also known to harbor sex-specific characteristics such as imprinted genes and a wide array of epigenetic events leading to X-chromosome inactivation (Gabory *et al*., 2009; Sasaki and Matsui, 2008). It may therefore be necessary in some situations to separate male and female embryos. However, sorting embryos according to sex and dissecting the cell types exacerbate issues associated with sample size. Furthermore,

obtaining samples of precisely staged embryos for all treatments is definitely a challenge when considering that the epigenome is expected to vary as embryogenesis progresses thus on daily if not on an hourly basis.

Another aspect of the data normalization challenge arises when profiling the epigenome across developmental stages. As noted for transcriptomic profiling throughout early development, developmental stages differ dramatically and stable references are very difficult to identify and likely do not exist (Robert, 2010; Gilbert *et al*., 2009a; Robert *et al*., 2002). Data normalization needs to be considered on a case per case basis, since when studying nuclear components such as DNA methylation or histone modification, it would be logical to report data on a per nucleus basis. However, reporting the abundance of non-coding transcripts or of non-nuclear restricted proteins per nucleus could decrease the physiological relevance of the data, since neither cytoplasmic accumulation nor cell size are stable factors, nor does either follow a linear progression throughout early development. These extreme fluctuations in the early embryo epigenome between developmental stages (Hales *et al*., 2011; Shi and Wu, 2009; Chason *et al*., 2011) make data normalization extremely difficult.

1.8. Functional analysis of DNA methylation

Research efforts so far have focused on describing the genome-wide distribution of DNA methylation (5mC) and hydroxymethylation (5hmC) in cells and tissues. Comparative analyses suggest the presence of diverging methylation patterns in response to specific stimuli, stresses or environmental conditions. In many cases, DNA methylation displays discrete patterns between treatment groups overall but also shows considerable variance between samples within the same treatment group, suggesting that DNA methylation has a certain degree of plasticity. The next step would be to provide evidence of functional involvement of DNA methylation in disease development. To achieve such goal, technological platforms allowing targeted and inducible DNA methylation are required.

With recent advances in protein engineering, it is now possible to target specific epigenetic editors (writers or erasers) to a specific gene or locus. This can be achieved using artificial transcription factors (ATF), various classes of engineered DNA-binding domains such as designer zinc finger proteins (ZFP), triplex-forming oligomers (TFO) and transcription-activator-like effector (TALE) domains (de Groote *et al*., 2012; Chaikind *et al*., 2012; Li *et al*., 2011). These specific DNA-targeting tools homing on candidate loci have applications to the currently recognized principal aspects of the epigenome (*i.e.* histone modification and DNA methylation). With respect to DNA methylation, the concept consists of combining a DNA-binding element specific for a candidate DNA sequence with an enzymatically active module bearing DNA methyltransferase activity,

which can be done using ZFP or TALE for example. Through the action of this complex, targeted genomic loci would be methylated. In addition to this functional analysis of DNA methylation, such targeted DNA methylation approaches have the potential for correcting disease-associated epi-mutations and suggest the possibility of "epigenetic therapy" (Jurkowska and Jeltsch, 2010; de Groote *et al*., 2012). The two currently emerging technologies are based on ZFP-methyltransferase and TALE-methyltransferase (TALEM).

1.9. Methods to analysis novel cytosine modifications

Recently, novel cytosine modifications with potential regulatory roles, such as 5hmC, 5fC, and 5caC, were discovered. Similar to 5mC, a number of analysis approaches have been developing to study and understand the functionality of these oxidative formats of methylated cytosine. Currently, based on the available developed strategies to study these novel cytosine modifications, it has been suggested that 5hmC may act as an epigenetic mark associated with transcriptional regulation, maintenance and differentiation of embryonic stem, possible regulatory roles in neurodevelopment and neurological diseases as well as aging and carcinogenesis. In addition, 5fC and 5caC suggested to have possible functional roles on replication and transcription (Yuan and Feng, 2014). Interestingly, very recently the presence of 5hmC and 5fC have been

demonstrated in mammalian RNA, implying another undiscovered aspect of these 5mC oxidative products in mammalian cellular regulation (Zhang *et al*., 2016).

It should be noted that within the genome, 5-mC has an abundance of 10-fold to 100-fold higher than that of 5-hmC, and 5-hmC being about 40-fold to 1000-fold higher than that of 5-fC and 5-caC (Song *et al*., 2012b; Yin *et al*., 2013; Ito *et al*., 2011). Therefore, analysis of these oxidative formats of 5mC would be challenging in terms of the required gDNA as staring material as well as the sensitivity of the methods. These current available analysis methods to study different oxidative formats of 5mC can mainly be divided into two categories: overall detection, and genome-wide distribution analysis of 5hmC, 5fC and 5caC.

The overall detection techniques rely on the release of DNA components, such as 2-deoxynucleotides (dNTPs), 2-deoxynucleosides, or nucleobases, through enzymatic or chemical treatment followed by determination of the 5-mC and its oxidation products. Liquid chromatography (LC), capillary electrophoresis (CE), LC-mass spectrometry (LC-MS), gas chromatography-MS (GC-MS), thin-layer chromatography (TLC), chemical derivatization-based detection, single-molecule detection, and immuno-based detection. These methods are currently are being used to determine the overall 5-mC and its oxidation products in the given sample (Yuan and Feng, 2014).

Considering the fact that traditional bisulfite sequencing cannot distinguish 5hmC from 5mC, nor can it differentiate 5fC or 5caC from unmodified cytosine, several approaches has been developed to make discrimination between the 5mC and 5hmC at genome-wide levels such as oxidative bisulfite sequencing (oxBS-Seq) strategy (Booth *et al*., 2012b), TET-assisted bisulfite sequencing (TABSeq)(Yu *et al*., 2012), selective chemical labeling-single-molecule real-time (SMRT)(Song *et al*., 2012a).

1.10. Conclusions and perspectives

It is now clear that technological innovations such as microarrays and ultra-high-throughput sequencing can provide tools for studying the epigenome. Combined approaches now make it possible to generate more comprehensive genomic maps of cytosine methylation and hydroxymethylation, and chromatin modifications at the genomic scale, in addition to related expression profiling of non-coding RNA. At the current rate of technological advancement, even the study of early embryogenesis is becoming more feasible. In fact, this application became reality less than five years ago. The next challenge is without any doubt data integration. This is already the case for transcriptomic platforms, for which the wealth of data generated far exceeds the needs of individual research teams, who tend to focus on a handful of preferred candidates. The transcriptome as a whole is derived from only 1–2 % of the genome, while epigenome profiling encompasses the entire genome, at least until science provides a basis for

narrowing the focus. Finding the key epigenetic signature thus amounts to a task similar to searching for a needle in a haystack. However, with the right tools, such a task is surmountable.

Integration of different datasets across technological platforms will be instrumental, although it poses certain challenges due to the current lack of parallel analyses (same samples run on different platforms), which are required in order to evaluate the extent of dataset compatibility and determine reference points for dataset merging. Integration of the ever-growing mass of data in the public domain relating to every aspect of the epigenome in very diverse physiological contexts will eventually provide means of pinpointing key epigenetic signatures on key genomic loci. These candidates will become prime targets for downstream functional assessment through the use of the upcoming tools allowing targeted epigenetic modifications.

It is known that interactions between DNA methylation, histone modification, nucleosome remodeling, histone variants and non-coding RNA all introduce meaningful variation into the chromatin fiber and thus collectively contribute to epigenetics (Geiman and Robertson, 2002; Goldberg *et al*., 2007). In fact, the true physiological perspective relies on transversal integration of data, of which the ultimate comprehensive map will require considering the different components that drive gene expression. The latter includes the full complement of the more traditional transcriptional regulators (*e.g.* transcription factors) involved primarily in acute transient cellular responses to stimuli, in

combination with epigenomic effectors that are involved in such transient response as well as in long-term adaptive responses. Insight into these mechanisms is required in order to understand how embryos cope with environmental changes and insults, and how these types of stress affect the initiation of cellular differentiation and sometimes lead to long-term effects. It is expected that in the next few years, high-throughput analytical methods of genome-wide DNA methylation and its oxidation products may be commercialized and become widely available in a more user friendly, cost effective and higher sensitivity and specificity. Therefore, it would also not unexpected that in the line with the technology advancements for genome-wide 5mC and its oxidative formats analysis, early embryo compatible methods which require very minimum gDNA will be developed and introduced, such as the recent developed method for histone modifications analysis compatible with very small cell number (Brind'Amour *et al*., 2015).

2. Hypothesis, Rationales, and Objectives

2.1. Hypothesis

Compared to *in vivo*-derived embryos, assisted reproductive technologies (ART)-derived treatments not only cause deviations in transcription of early embryos, but also impact the establishment of DNA methylation patterns in early embryos and gametes. Furthermore, such deviations in DNA methylation patterns are associated with responses to stimuli that have both short- and long-term impacts on embryo development.

2.2. The first rationale

In addition to transcriptome deviations, there is increasing evidence that assisted reproductive technologies (ARTs) are associated with aberrant DNA methylation in imprinted genes, suggesting that ART may cause long-term impacts on treated embryos or gametes. In the beef and dairy cattle industries, ART such as *in vitro* embryo production and manipulations are widely used. Nevertheless, there has apparently not been any report regarding genome-wide epigenetic profiling of bovine embryos and gametes to identify mechanisms by which embryonic stress and deviation in gamete epigenetics causes these long-term impacts, or to the extent they can alter DNA methylation as the most stable and heritable epigenetics effectors. Although numerous platforms already exist to study genome-wide methylation profiles, none were functional with the small amount of starting material such as early embryos, in particular in parallel with

transcriptome analysis of the same sample. It is noteworthy that overlaying methylomic and transcriptomic data has provided complementary information to reveal highly relevant targets and potential associations between the DNA methylome and transcriptome.

Therefore, there is a need to develop a genome-wide DNA methylation platform for cattle, which efficiently analyzed very small samples such as early embryos in parallel with transcriptome analysis.

2.3. The first specific hypothesis

A different approach using methyl-sensitive enzymes rather than bisulfite sequencing could provide a compromise platform (more robust and sensitive but lower resolution) for study genome-wide DNA methylation from very low input samples.

2.4. The first objective

Develop a user-friendly, cost effective and array-based technological platform that is complementary to existing platforms, and compatible with limitations imposed by the small amount of starting material (*e.g.* early embryos) to perform parallel analysis of genome-wide DNA methylation and transcription profiles of a given sample.

2.5. The second rationale

Splitting early bovine embryos is currently one ART method to generate monozygotic (MZ) twin bulls. Despite being genetically identical, for unexplained reasons, progeny testing of the generated monozygotic twin bulls (produced by embryo splitting) yielded inconsistent outcomes. Therefore, daughters of MZ twin bulls were not comparable for milk production, fertility, etc. Therefore, despite the identical genetic sequences shared by MZ twin bulls, they seemed to pass different traits to their daughters via their sperm. Hence, there was a need to use the developed platform to study the impact of early embryo splitting on DNA methylome of the MZ twin bulls' sperm.

2.6. The second specific hypothesis

It was observed that twin bulls do not get the same estimated values of genetics merit. Therefore, they are not transmitting the same production capacity. We hypothesised that if it was caused by an epigenetic mechanism, it would imply that the DNA methylome would be different between twins.

2.7. The second objective

Investigate deviation of the genome-wide DNA methylation profile of sperm between monozygotic twin bulls generated with embryo splitting which have distinct progeny performance (daughters' performance records) despite identical genetic materials.

2.8. The third rationale

Folic acid supplementation has been shown to prevent and decrease the incidence of neural tube defects in children. Hence, adding folic acid to human diets is mandatory in more than 70 countries. However, more recently, concerns have been raised regarding high concentrations of folic acid supplementation, both prior to and during pregnancy. Recent studies have reported potential adverse effects of high folic acid on early embryos and adults. Since folic acid can impact methylation processes through one-carbon metabolism by generating S-adenosyl methionine (SAM), the global methyl donor, we investigated impacts of high-dose supplementation of SAM on early embryos. It was expected that this would elucidate how early embryos coped with excess global methyl donor and if early embryos would not react to supraphysiological dose of global methyl donor, the observed consequences of high folic acid would need to be conferred in later stages of development.

2.9. The third specific hypothesis

Bovine early embryos would react at both transcriptome and DNA methylome levels when placed in a microenvironment rich in global methyl donor.

2.10. The third objective

Study effects of high dose supplementation of global methyl-donor, S-adenosyl methionine, on phenotype, transcriptome and epigenome (DNA methylation) of *in vitro* -produced bovine early embryos.

3. An integrated platform for bovine DNA methylome analysis suitable for small samples

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Authors' contributions

HASS worked on platform development, performed bovine sperm and blastocyst experiments, and designed the analysis pipeline. AOD performed pyrosequencing for

candidates. DG was involved in platform development. EF performed bioinformatic analyses, array design and programmed the plot generation pipeline. JG performed the *in silico* survey of CpG islands. MAS and CR conceived the general strategy for the platform's development. CR designed and supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

3.1. Résumé

Contexte :

Les ovocytes et les embryons précoces contiennent d'infimes quantités d'ADN, d'ARN et de protéines, complexifiant l'étude du développement précoce des mammifères. L'étude de l'épigénome de l'embryon, en particulier la méthylation de l'ADN, a été rendue accessible grâce à la possibilité d'amplifier des séquences spécifiques en fonction de leur statut initial de méthylation. Cet article décrit une nouvelle plateforme dédiée à l'étude pangénomique de la méthylation de l'ADN bovine, incluant une méthode complète d'analyse des données et leur visualisation. La plateforme permet le traitement et l'intégration de la méthylation de l'ADN et des données de transcriptomique à partir du même échantillon. Les procédures ont été optimisées pour l'analyse du génome entier à partir de 10 ng d'ADN (10 blastocystes bovins). Des échantillons de spermatozoïdes et de blastocystes bovins ont été comparés afin d'évaluer la capacité de la plateforme.

Résultats :

L'hyperméthylation de l'ADN spermatique bovin comparativement au génome de l'embryon a été confirmée. Les régions différentiellement méthylées ont été distribuées entre différentes classes de fonction génomique du spermatozoïde bovin, dont les promoteurs, les régions introniques et exoniques, les régions non-ilots-CpG (côte, plateau et pleine mer ; de l'anglais shore, shelf and open-sea) et les îlots CpG avec une densité

faible à intermédiaire. Il a été observé que le génome du blastocyste portait plus de marques de méthylation que l'ADN spermatique dans les îlots CpG de densité élevée en CpG. Les rétrotransposons à séquence terminale longue répétée (LTR), les LINE et les SINE étaient plus méthylés dans l'ADN spermatique, tout comme les éléments répétitifs de faible complexité dans des blastocystes.

Conclusions :

La plateforme développée dans cette étude est la première du genre qui permet l'étude de la méthylation pangénomique pour les embryons précoces bovins. Cette plateforme a le potentiel d'améliorer l'étude des risques épigénétiques possibles en lien avec les technologies de reproduction assistée (TRA), l'établissement des séquences de lignées cellulaires embryonnaires ainsi que les écarts potentiels dans l'expression des gènes et de la méthylation d'ADN, dont il est possible d'observer un impact à long terme.

Mots-clés :

Épigénome, méthylation de l'ADN, Embryon bovin, Méthylome et analyse combinée du transcriptome, Méthode d'analyse des données, Enrichissement CpG, Éléments répétitifs, Étude d'association épigénétique à large échelle

3.2. Abstract

Background: Oocytes and early embryos contain minute amounts of DNA, RNA and proteins, making the study of early mammalian development highly challenging. The study of the embryo epigenome, in particular the DNA methylome, has been made accessible thanks to the possibility of amplifying specific sequences according to their initial methylation status. This paper describes a novel platform dedicated to the genome-wide study of bovine DNA methylation, including a complete pipeline for data analysis and visualization. The platform allows processing and integrating of DNA methylome and transcriptome data from the same sample. Procedures were optimized for genome-wide analysis of 10 ng of DNA (10 bovine blastocysts). Bovine sperm and blastocysts were compared as a test of platform capability.

Results: The hypermethylation of bovine sperm DNA compared to the embryo genome was confirmed. Differentially methylated regions were distributed across various classes of bovine sperm genomic feature including primarily promoter, intronic and exonic regions, non-CpG-island regions (shore, shelf and open-sea) and CpG islands with low-to-intermediate CpG density. The blastocyst genome bore more methylation marks than sperm DNA only in CpG islands with high CpG density. Long-terminal-repeat retrotransposons (LTR), LINE and SINEs were more methylated in sperm DNA, as were low-complexity repetitive elements in blastocysts.

Conclusions: This is the first early embryo compatible genome-wide epigenetics platform for bovine. Such platforms should improve the study of the potential epigenetic risks of assisted reproductive technologies (ART), the establishment sequence of embryonic cell lines and potential deviations in both gene expression and DNA methylation capable of having long-term impact.

Keywords: Epigenome, DNA methylation, Bovine embryo, Methylome and transcriptome parallel analysis, Analysis pipeline, CpG enrichment, Repetitive elements, Epigenome-wide association study.

3.3. Background

The study of early embryonic development continues to pose formidable technical challenges due in large part to the limited amounts of sample material. However, high-throughput high-fidelity amplification of nucleic acid is making the macromolecular study of embryonic physiology more accessible. Microarray platforms, and more recently RNAseq, have made studying the early embryo transcriptome almost routine. Our group has been developing bovine and porcine microarrays based on transcriptomic platforms that include standardized sample preparation procedures and a complete user-friendly software suite for data normalization and analysis, allowing efficient processing of samples from extraction through to the generation of publishable graphs (Robert *et al.*, 2011; Tsoi *et al.*, 2012). Transcriptomic platforms have been used to study how early embryos of many different species interact with their immediate microenvironment (Gad *et al.*, 2012; Cagnone and Sirard, 2013; Cagnone *et al.*, 2012; Zhang *et al.*, 2009; Hamatani *et al.*, 2004; Léandri *et al.*, 2009). Although very useful, the transcriptome has not allowed us to determine whether or not deviant gene expression, that is observed in embryos, is a transient adaptation to surrounding conditions that later yields to normal expression without any long-term impact on development. In order to provide a more complete picture of embryo adaptation and its potential long-term consequences, study of the epigenome is necessary (Szyf, 2009; Guerrero-Bosagna and Skinner, 2011). The epigenome is the sum of all epigenetic information (Suzuki and Bird, 2008) and refers more precisely to the complete description of chemical changes to DNA and histones

(Bernstein *et al.*, 2007), including histone tail modifications, chromatin remodelling proteins, and ncRNA. Epigenomics and transcriptomics are closely interrelated in terms of gene function and regulation (Baccarelli *et al.*, 2010; Shi and Wu, 2009) and together modulate gene expression.

Among epigenomic effectors, DNA methylation is believed to be a strong primary molecular mark having a major impact on intergenerational gene silencing (Bird, 2002; Raynal *et al.*, 2012). DNA methylation patterns are known to be relatively stable and established in a tissue-specific manner (Talens *et al.*, 2010; Fraga *et al.*, 2005). However, following fertilization and during mammalian pre-implantation development, the DNA methylation pattern is dynamic and undergoes reprogramming in the form of a wave of genome-wide de-methylation and re-methylation (Wu and Zhang, 2012; Seisenberger *et al.*, 2013b; Reik *et al.*, 2001; Morgan *et al.*, 2005; Seisenberger *et al.*, 2013a; Santos *et al.*, 2002), thus putting the embryo at risk of programming errors (van Montfoort *et al.*, 2012). Furthermore, the study of how the DNA methylome can be modified by changes in the embryo microenvironment such as *in vivo/vitro* culture, uterine conditions, or maternal nutritional regimen has represented a major challenge and continues to do so, due mainly to sample scarcity offering input DNA well below minimal recommendations.

Numerous platforms already exist to study methylation of targeted loci or to obtain genome-wide methylation profiles. For the study of very small samples, determining DNA methylation at targeted loci has so far been more successful than genome-wide

approaches (McGraw *et al.*, 2013). The main advantages of general survey are the possibility of describing physiological responses at the genome-wide scale and the potential for novel discovery.

The aim of the present work was to develop a technological platform that is complementary to existing platforms, in order to provide a whole-genome view of DNA methylation in bovine early embryos. Since a diploid mammalian nucleus contains about 6.8 picograms of DNA (www.genomesize.com) and the expanded blastocyst of large mammalian species is composed of about 150 cells, a single bovine blastocyst contains approximately 1 ng of DNA. The current benchmark for minimal sample size is around 10 ng, therefore corresponding to a pool of 10 expanded blastocysts. The other criterion is ease of use, at both the sample handling and data processing steps. We thus sought to identify an existing methodological approach which would be best suited to analyze very small samples of DNA. The platform was tested and validated using experimental samples.

3.4. Results

3.4.1. Platform Design

The EmbryoGENE (http://embryogene.ca) DNA Methylation Analysis (EDMA) platform was designed for high-throughput methylation profiling of bovine genome using limited amounts of input material. It combines four independent methodological principles: i) restriction endonuclease-based RE (*MseI)* genomic DNA fragmentation; ii) targeting methylated regions using a cocktail of methyl-sensitive restriction endonucleases; iii) amplification of methylated (thus protected) fragments using ligation-mediated PCR; and iv) identification of the amplified methylated fragments using a microarray. The EDMA workflow is presented in Figure 2-1.

Sample treatment protocol and microarray design were optimized in parallel. Our laboratory has previously conducted a survey of DNA methylation in bovine embryos using various reduced representation approaches (de Montera *et al*., 2013). This allowed us to identify a collection of loci at which DNA methylation varies in association with early development. These loci included CpG islands, gene bodies, intergenic regions and repetitive elements. The oligo design accounted for the sample preparation steps in which genomic DNA was fragmented using the *MseI* restriction enzyme, which recognizes 5'-T/TAA-3', thus avoiding methylated cytosine residues. In silico digestion of the bovine genome shows that *MseI* yields fragments averaging 160 bp in length (see Figure 2-12 (Supplementary) A). A second layer of in silico analysis located the methyl-sensitive

restriction endonucleases (MSRE) restriction sites, namely C/CGG (*HpaII)*, GC/GC (*HinP1I)* and C/CGC (*Aci1I)*. Distribution of common and unique MSRE sites within *MseI* fragments is shown in Figure 2-12 (Supplementary)B. Furthermore, the information regarding the number of CpG sites per restriction fragments and the number of MSREs restriction sites per restriction fragments are provided in Figure 2-13 (Supplementary) A-D. The 60-mer oligo design was based, in part, on a collection of *MseI* fragments containing MSRE sites within the genomic loci that we previously found to bear methylation or hydroxymethylation marks in early bovine embryos (de Montera *et al*., 2013), to which were added CpG islands determined by in silico analysis. Additional oligos were designed by tiling the *MseI* fragments adjacent to this initial set of targets until the capacity of a single microarray slide was reached (1x1 M oligos). Preliminary hybridizations allowed selection of a subset of 400 K oligos that performed well, based on sequence specificity and signal strength variations across the set of test hybridizations (data not shown). The final probe collection queries a variety of different genomic features not limited to CpG islands. A summary of the genomic targets surveyed by the microarray is shown in Figure 2-2 and Tables 2-2; 2-3; 2-4 (Supplementary). As illustrated for two bovine imprinted genes (NNAT, PEG10) in Figure 2-3, the EDMA probes were distributed across various genomic features (intergenic, promoters, gene body, and repetitive elements).

Since the platform uses a cocktail of MSREs to target genomic locations bearing methylation marks, the extent of cleavage is a critical factor. Incomplete cleavage will lead

to false positive results. For quality control purposes, control DNA templates were designed to account for extent of cleavage both by *MseI* and the MSREs cocktail. A pair of DNA templates was designed for each MSRE (Figure 2-4A). All spiked-in controls had internal *MseI* sites at their ends, and for each pair, one template was methylated *in vitro* for protection against MSRE activity. The controls were thus subjected to the same cleavage conditions as the sample. Following genomic fragmentation, adaptor ligation and MSRE treatment, sample quality was determined using qPCR, with calculation of the extent of cleavage for each MSRE. The difference in threshold cycle (Ct) between protected (hypermethylated) and unprotected (hypo/unmethylated) control templates can be calculated, while sample uniformity can be visualized from the amplification curves (Figure 2-4B, 4C). Only samples displaying uniformity with the other samples cleaved > 97 % in the same cohort were retained for downstream treatment. Insufficiently cleaved samples were in some cases subjected to a second treatment with additional MSRE digestion.

In EDMA platform, after hybridization on the microarray and data analysis there are two types of quality control (QC) plots which the examples are shown in Figure 2-5. The first plot, which assesses the completeness of the genomic digestion, shows nearly complete cleavage by *MseI,* with most control probes showing signal below the background noise (Figure 2-5A). The second quality control assess the quality of detected signals. The plot uses signals from probes corresponding to the spiked-in controls, and confirms that

unprotected templates led to low signals and vice versa for protected fragments (Figure 2-5B).

3.4.2. The Analysis Pipeline

For each dye-swaps microarray hybridization, the quantification of methylation measurements are based on M values (the log differential- expression ratio of the two channels) which further normalized (inter & intra-array) and statistical analysis is performed as described in methods. This means larger values represented more evidence of relative methylation similar to other microarray-based genome-wide DNA methylation analysis platforms (Irizarry *et al*., 2008). To support data mining, an extensive analysis pipeline was designed with the goal to sort the data according to defined structural characteristics (*e.g.* near known genes, within gene body components, distance from CpG islands, etc.). We designed a user-friendly and comprehensive bioinformatics data analysis pipeline to complement our developed platform (EDMA). A complete schematic of our data analysis pipeline is shown in Figure 2-6. The data analysis pipeline was designed to be compatible with results obtained from our array-based gene expression platform (Robert *et al*., 2011). It enables to identify alterations of DNA methylation in bovine genomic regions under various enrichment outputs in parallel to deviations in transcription. The pipeline comprises several analysis steps for data QC and differential analysis, generating a list of DMRs as well as downstream sequence-based enrichment

analyses, Hot spot detection and concordant analysis in search of loci where both transcriptomic and DNA methylation are affected. The information is binned according to the set of annotations given to every genomic locus (see methods for details). Typical enrichment analysis account for CpG islands neighbourhood both in length and density, gene body structures (promoter, exons, introns) and classes of bovine repetitive elements (low complexity, SINE or LINE elements). Since EDMA is not based on bisulfite conversion and sequencing, it does not provide a relative value of the extent of DNA methylation. This information needs to be determined by targeted pyrosequening of the regions of interest.

The data analysis pipeline output is a large contingent of plots that serve five different objectives (Figure 2-7): i) to document the quality of the samples through the quality control plots ; ii) to provide visual aid to determine where the MSRE-protected fragments were found for each sample type; iii) to provide an overall perspective of the extent of the differences in methylation between the samples; iv) to mine differentially methylated regions (DMRs) data according to genomic features; v) and lastly, the Circos plot which can be used to integrate the epigenomic and transcriptomic data (or present a chromosomal overview of either type alone).

3.4.3. DNA methylation profiles of bovine sperm and early embryos

3.4.3.1. Genome-wide overview of DNA methylation profiles

Platform performance was evaluated on the basis of the contrast in genomic DNA methylation patterns between bovine sperm and blastocysts. Similar amounts of input DNA from both sample types were processed, and microarray data was analyzed using the pipeline described above. Using a predetermined significance criterion *i.e.* Fold-change > 2^1.5, p-value < 0.05, 811 DMRs were identified, most of which (> 85 %) were due to hypermethylation in sperm (Figure 2-8). Large numbers of the methylated loci found in the embryo genome were also found in sperm DNA. Differences can also be observed using the genome-wide view provided by Circos plot, which shows a prevalence of hypomethylation in the sperm for chromosome X compared to blastocysts DNA. Nearly all (97 %) of the top 100 identified differentially methylated regions (DMRs) were found to be hypermethylated in sperm DNA (Figure 2-9). This reveals that the majority of DMR genes in bovine sperm DNA lose their methylation after fertilization and during early embryo development, at least until the blastocyst stage.

3.4.3.2. Characteristics of differentially methylated regions (DMRs).

Our data revealed that sperm DNA shows a tendency to hypermethylation compared to blastocysts in all types of promoter, intronic and exonic regions, non-CpG islands regions (shore, shelf and open sea) and CpG islands with low-to-intermediate CpG density and small-to-intermediate length (Figure 2-10). Only in high-density CpG islands in the blastocyst genome demonstrated a tendency to hypermethylation compared to sperm.

Amongst DMRs containing repetitive elements, bovine sperm DNA tended to be hypermethylated in the majority of the repetitive element classes, in particular long-terminal-repeat (LTR) retrotransposon, LINE and SINE. However, low-complexity repetitive elements showed more hypermethylated in the blastocyst genome.

3.4.3.3. DMR validation

Seven candidate DMR loci from different regions of the bovine genome were selected and primers were designed (see Table 2-5 (Supplementary)) for measurement of DNA methylation levels by pyrosequencing. These results confirmed those obtained using our platform and showed very high levels of methylation with high reproducibility in sperm DNA compared to the blastocyst genome (Figure 2-11). However, as expected there was not any linear correlation between the EDMA fold changes for the selected DMRs and their corresponding measured fold changes after the pyrosequencing confirmation (see Figure 2-14 (Supplementary)).

3.4.4. Discussion

3.4.4.1. Development of EDMA

The study of the epigenome has become a prime focus in the effort to understand the expression of complex phenotypes such as obesity, diabetes (Slomko *et al*., 2012; Youngson and Morris, 2013), mental disorders (Saab and Mansuy, 2014; Tsankova *et al*., 2007) and cancers (Iacobuzio-Donahue, 2009; Dawson and Kouzarides, 2012; Rodriguez-Paredes and Esteller, 2011; Feinberg, 2007). Vast epigenetic erasure and reprogramming

events during early embryogenesis make this phase of development a window for perturbations, potentially having long-term impact on phenotype in adulthood. Methods for monitoring candidate loci using as little as single cell (Kantlehner *et al*., 2011), one oocyte (Denomme *et al*., 2012) or single cell blastomere (Lorthongpanich *et al*., 2013) have been developed. The aim of the present work is to develop a robust technological platform to study genome-wide DNA methylation, suitable for the small amount of sample material obtainable from early embryos and to complement this platform with a comprehensive suite of tools for quality control and data analysis. The EmbryoGENE DNA methylation analysis (EDMA) platform was developed for compatibility with our previously published transcriptome analysis platform (Robert *et al*., 2011) with the aim of integrating data of both types from the same sample.

Among the three sample treatment options currently employed to study genomic DNA methylation (McGraw *et al*., 2013), we opted for methyl-sensitive restriction enzymes (MSREs) over bisulfite conversion and affinity selection because it allowed for robust processing of DNA input of a few nanograms as well as a more straightforward data processing based on the well-established tools dedicated to microarray data analysis. An optimal procedure was sought for isolating intact genomic DNA for subsequent specific fragmentation by nucleases, which offers better repeatability than mechanical shearing (data not shown). The selection of methylated fragments thus obtained is performed by means of successive PCR reactions, while amplicon identification is achieved using a

microarray, which brings the added benefit of mature data processing and analysis procedures. In addition, each microarray slide is composed of two arrays that hold two samples each (two-color arrays), allowing for the necessary inclusion of biological replicates in the experimental design. All platforms must include biological replication, since the extent of DNA methylation is naturally variable among samples of similar origin.

The procedures involved in EDMA, namely restriction endonuclease genomic fragmentation, adaptor ligation, cleavage by MSREs, selection of protected fragments by PCR and identification of fragments by microarray, are essentially those proposed for nanoHELP and for array-comparative genomic hybridization, which have been applied successfully to inputs of respectively 10 ng and a single cell (Klein *et al*., 1999). Ten nanograms is the approximate quantity of DNA obtainable from 10 expanded blastocysts. Comparing 1 µg and 10 ng genomic DNA input, Oda and colleagues demonstrated that the smaller input provided higher reproducibility ($R > 0.96$), due probably to more complete cleavage, suggesting that low input of genomic DNA would likely be more suitable for MSRE-based platforms (Oda *et al*., 2009). Sample processing used in EDMA was tested with lower gDNA inputs as low as 15 cells and genomic coverage was measured using the 50K bovine SNP Chip which showed important loss of information due to allele drop-out when input were lower than 1 ng (data not shown). When dealing with single cell DNA methylation analysis, partial sample loss is often observed. This loss may not be problematic when aiming for targeted loci but is definitely problematic for

genome-wide profiling. As such, 10 ng was set the minimal input leading to a robust DNA methylation profiling.

In order to prevent the introduction of false positives that would arise from incomplete cleavage of sample DNA (Hashimoto *et al*., 2007; Laird, 2010), spiked-in controls were designed to account for extent of cleavage both during the initial *MseI* fragmentation and by MSREs. Samples were tested before fragment amplification by PCR to ensure that cleavage was sufficient. When cleavage is incomplete, the MSREs treatment can be repeated. For each MSRE, a pair of synthetic DNA controls was designed, one of which was methylated *in vitro and* used as a control for fragment protection, while the unprotected counterpart acted as the cleavage target.

One of the main concerns with any genome-wide approach is the actual genomic coverage. Since the *MseI* recognition site is T/TAA, CpG dinucleotides are left intact and most CpG islands are conserved (Schumacher *et al*., 2006). These sites are very abundant throughout the genome, resulting in small fragments well suited for LM-PCR (Cross *et al*., 1994; Rauch *et al*., 2006; Klein *et al*., 1999). By fragmenting the entire genome in this manner, methylation status outside CpG islands can be queried. This provides valuable information, since evidence is mounting for potential important roles for 5mC in non-CpG islands, which have been found more dynamic than CpG islands with respect to methylation-based regulation (Jones, 2012). This is potentially even more important in early embryos where overall demethylation just occurred.

Since detection based on restriction enzymes is limited to fragments bearing the recognition sites (Suzuki and Bird, 2008; Zilberman and Henikoff, 2007), adequate genomic coverage by MSRE requires a combination of nucleases (Schumacher *et al*., 2006; de Montera *et al*., 2013). In silico analysis showed that compared to *HpaII* alone, the combination of *HpaII, HinP1I* and *Aci1I* increased the coverage of CpGs sites in the probes designed for EDMA from 2.3 % to 8.6 % and genomic coverage from 4.8 % to 6.1 % (see Table 2-2 (Supplementary)). This coverage accounts for a little more than 2.3 M CpG sites, which corresponds to about 10 % of all CpG sites in the bovine genome. Similar coverage is obtained using reduced-representation bisulfite sequencing (RRBS), which has been shown to be efficient with small DNA input (Wu and Zhang, 2012; Smith *et al*., 2012; Smallwood *et al*., 2011). Both EDMA and RRBS have several steps in common, including genomic fragmentation by restriction enzyme, adaptor ligation and PCR amplification. The main benefits of reduced-representation bisulfite sequencing are that it allows quantitative evaluation of methylation at single-base resolution and is applicable to all species since it is based on DNA sequencing, which does not require a priori knowledge of the genome (Harris *et al*., 2010). However, EDMA similar to any other MSREs enrichment-based approaches generate a list of DMRs ranked in order of significance but does not provide information regarding the extent of DNA methylation which must be determined by targeted pyrosequencing. Furthermore, enrichment-based approaches using antibody or methyl-binding proteins have not been thoroughly tested with samples

containing only a few ng of DNA. With larger DNA input, these methods have been shown to provide moderate resolution, since the capture depends on fragment methylation density (Suzuki and Bird, 2008; Fouse *et al*., 2010).

While epigenome-wide association studies and the development of technological platforms suitable for low input DNA are broadening in scope, improvement to the standardization of experimental assays across samples and to data analysis and interpretation remains slow (Michels *et al*., 2013). Although amplicons obtained by LM-PCR could be processed into sequencing libraries, we opted for microarray-based identification, which is more restrictive (being limited to the probes printed on the array) but has the major benefit of compatibility with an established, robust and user-friendly data analysis pipeline. These features may limit genomic coverage but definitely increase sample turnover rate by decreasing the time required to interpret data.

In comparison to gene expression, which is most often limited to the study of protein encoded transcribed elements which account for little more than 1 % of the eukaryote genome, profiling overall DNA methylation considers a vastly more complex diversity of sequences, which in turn complicate data analysis. We therefore sought to support data interpretation by binning the information according to genomic features. Such an approach increases the statistical power to identify subtle alterations in genome regions by avoiding P value dilution, through multiple-testing corrections that include the vast majority of regions considered a priori unlikely to be differentially methylated (Gu *et al*.,

2010). The data analysis pipeline built in to our platform accounts for: i) site specificity (*e.g.* promoter, intron, and exon), ii) region (*e.g.* shore, shelf, open-sea) and iii) sequence composition (*e.g.* CpG island density and length). This is in accordance with recent recommendations for epigenome-wide association studies (Michels *et al*., 2013).

The enrichment categorization implemented in the data analysis pipeline is based on previous studies (Meissner *et al*., 2008; Smith *et al*., 2012; Weber *et al*., 2007; Elango and Yi, 2011; Krausz *et al*., 2012; Doi *et al*., 2009; Bock *et al*., 2012) and provides a full complement of graphic outputs. Even though the graphs might not all be relevant to all users, depending on the experimental design and biological hypothesis, are all produced automatically at no additional cost in time or resources on the part of the user. Overall, EDMA was developed as a cost-effective standardized platform that robustly profiles DNA methylation across the entire bovine genome. This sample processing platform is also complemented with bioinformatics supports for data analysis with the specific aim to aid data interpretation.

3.4.4.2. The importance of genome-scale parallel analysis of the DNA methylome and transcriptome.

Current technological advances and the exponential growth of epigenetics studies in the past few years, in particular genome-wide studies, are advancing our knowledge and providing more evidence for the interdependence of epigenetic and genetic variations

(Michels *et al*., 2013). By providing genome-wide parallel survey of the DNA methylome and the transcriptome for the same sample, EDMA offers a powerful tool for revealing highly relevant targets and potential associations between the DNA methylome and the transcriptome. In addition, overlaying methylomic and transcriptomic data has been shown to provide complementary information (Bock *et al*., 2012).

3.4.4.3. Genome-wide profiles of bovine sperm and blastocyst DNA methylomes

As proof of concept, we compared the DNA methylation profiles of bovine sperm and blastocysts and performed validation by pyrosequencing on selected DMRs. All selected candidates were found to be substantially more highly methylated in sperm DNA, corroborating the results obtained with EDMA. Physiologically relevant data were also generated and compared to the current literature. The sperm genome was found hyper-methylated compared to the embryo, which is consistent with the de-methylation process known to occur after fertilization in bovine (Dean *et al*., 2001) and other species such as the mouse (Smallwood *et al*., 2011; Smith *et al*., 2012) and the zebra fish (Andersen *et al*., 2012). The data also indicated that large numbers of loci methylated in bovine embryos are also methylated in sperm DNA, which also corroborates a previous report (Andersen *et al*., 2012). As previously observed in mice (Smallwood *et al*., 2011), we found that from the DMRs enrichment, that short length/low density CpG islands showed a higher tendency to changes (*i.e.* being a DMR) than other regions. The direction of change is predominantly toward hypermethylation in sperm, but not always. In

addition, we observed a high level of methylation in many repetitive elements, which is also in agreement with the patterns observed in mice (Smallwood *et al*., 2011; Smith *et al*., 2012).

We observed marked differences (more hypermethylated DMRs in sperm) in the extent of methylation in long-terminal-repeat (LTR) retrotransposon, similar to previous findings with mice (Smallwood *et al*., 2011). Furthermore, it has been shown that the sperm contributed DMRs in pre-implantation embryos at LTRs were associated with reduction of DNA methylation and the most drastic methylation changes (reduction) in the sperm-to-zygote transition observed in some families of LINE and LTR retrotransposon (Smith *et al*., 2012). In our previous report, we also observed a transition in DNA methylation in LTR during bovine embryo development from Day 7 to Day 12 (de Montera *et al*., 2013).

The reason for the observed large difference in LTR methylation during early development and, as shown specifically in this study, between sperm and blastocysts is not yet clear, although these differences might reflect the importance of *de novo* establishment of genome-wide methylation in the bovine early embryo. In this regard, robust LTR transposon up-regulation has been shown during activation of the bovine embryo genome (Bui *et al*., 2009) and found to be a general requirement for progression through to the cleavage stage in mouse embryos (Peaston *et al*., 2004; Kigami *et al*., 2003). However, the nature of its role in mammalian early development remains elusive. Furthermore, it has been suggested that LTR re-methylation occurs in the early, rather

than late, pre-implantation mouse embryo (Peaston *et al*., 2004). Furthermore, the observation that a higher number of DMRs are present in low-complexity simple repeats in bovine blastocysts, in comparison with sperm, might represent notable dynamic changes in 5mC in these specific class of repetitive elements during bovine early embryo development and differentiation (Kim *et al*., 2013).

3.5. Conclusion

By developing EDMA, we are providing a unified and reliable approach to analyze small amounts of genomic DNA, one that offers a good balance between genomic coverage and data turnaround time. The use of a microarray for fragment identification is robust, efficiently minimizing sample-to-data time. The built-in data analysis pipeline provides efficient means for data interpretation. The integrated data analysis pipeline could be a good option for researchers with limited bioinformatics resources. The platform is at the present time specific for the bovine genome, but a similar approach could be adapted easily to any species of which the entire genome is known. Such platforms enable the study of the potential epigenetic risks associated with assisted reproductive technologies (ART) or to highlight the sequence of events occurring during the establishment of embryonic cell lines.

3.6. Methods

3.6.1. Ethics statement

Experiments took place in compliance with the guidelines of the Canadian Council on Animal Care and supervised by the Animal Protection Committee of Université Laval. These guidelines are strictly followed by the local abattoir and L'Alliance Boviteq who provided all the tissues and samples. The study did not require handling animals on university premises.

3.6.2. Microarray design

The design of the EmbryoGENE (http://embryogene.ca) DNA Methylation Array (EDMA) was based on a compilation of methylation-sensitive genetic loci found previously to be involved in early embryonic development (de Montera *et al*., 2013). To maximize genomic coverage, all probes on the EDMA array were designed on the assumption that genomic cleaving using the *MseI* (T/TAA) restriction endonuclease would be nearly complete. All probes therefore targeted a specific *MseI* -*MseI* fragment within the bovine genome. Target loci were selected on the basis of their proximity to either the putative sites identified in our previous study (de Montera *et al*., 2013) or to known CpG islands. Tiling of fragments neighboring the selected loci was then carried out until enough loci were selected to fill one Agilent SurePrint 1x1M slide (Agilent Technologies). Test hybridizations were carried out, and the best-performing 400,000 probes were selected for placement on the final Agilent SurePrint 2x400K array. Probe quality was measured by assessing sequence specificity and signal strength variation across the set of test hybridizations. The

final EDMA array (EDMA.V2) contains 414,566 probes targeting 359,738 loci, surveying 20,355 genes and 34,379 CpG islands. The microarray also contains 10,388 control probes accounting for 2.5 % of all the total probes, representing 5,610 Agilent proprietary spiked-in controls, 4,634 genomic cleavage controls and 144 EDMA spiked-in methylation controls. Controls were designed with an *MseI* restriction site at their center and were tiled at every 1 M base pairs throughout the bovine genome. These were used to assess the degree of genomic digestion. The EDMA spiked-in controls are exogenous DNA fragments (Solanum lycopersicum) chosen for their lack of homology with the bovine genome and for the presence of specific *HpaII,* AciI and *HinP1I* restriction sites within their sequence. They were artificially methylated or left unmethylated to provide positive and negative controls for the methylation-sensitive cleavage. Probe design for the EDMA microarray was carried out by Genotypic Inc. (Bangalore, India). The arrays were printed using the SurePrint technology (Agilent Technologies). The details of the EDMA platform array have been deposited in NCBI's Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO, Platform accession number: GPL18384.

3.6.3. Sample production and genomic DNA extraction

Bovine blastocyst production in synthetic oviduct fluid (SOF) media was performed as described previously (de Montera *et al*., 2013). The ovaries were collected at a local abattoir which is complying with the guidelines provided by the Canadian Council on

Animal Care. Only expanded-blastocyst-stage embryos were collected, in four pools of ten embryos (n=40). All embryos were washed three times with RNAse-free phosphate-buffered saline (PBS) prior to snap-freezing and storage at -80 °C. Extraction of genomic DNA was carried out using an AllPrep DNA/RNA Mini Kit (QIAGEN, Mississauga, ON, Canada), and samples were eluted in 30 µL. Straws containing frozen semen from Holstein bulls were obtained from L'Alliance Boviteq Inc (Saint-Hyacinthe, QC, Canada). L'Alliance Boviteq Inc is a commercial service provider also complying with the guidelines provided by the Canadian Council on Animal Care. Sperm genomic DNA was extracted using lysis buffer, followed by ethanol precipitation. The quality and quantity of extracted DNA were analyzed by optical absorbance using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and by electrophoresis on 0.5 % agarose gel at 45 volts for 2 h.

3.6.4. Sample treatment

The same DNA input (10 ng) was used for both embryo and sperm samples. For quality control purposes, all samples were spiked in with a mixture of six synthetic DNA constructs harbouring *MseI* restriction sites at each end. One pair of constructs was designed for each methyl-sensitive restriction enzyme (*HpaII, HinP1I* and *Aci1I)* to be used in the fragment selection process. For each pair, one control DNA fragment was methylated *in vitro* using CpG methyltransferase *M.SssI* (New England Biolabs). The difference between the extents of cleavage obtained with each pair was used to calculate

cleavage efficiency. Residual *MseI* sites within the fragments were measured after processing to determine the extent of genomic fragmentation.

Sample (in 30 µL of elution buffer) plus 0.5 µL of bovine serum albumin (New England Biolabs), 5 µL of 10X Buffer 4 (New England Biolabs) and 28 µL of DNAse/RNAse free water were divided into two equal fractions and fragmented using 10 U of *MseI* (New England Biolabs) as follows, 16 h at 37 °C followed by 65 °C for 20 min. The two fractions were combined and DNA was concentrated by ethanol precipitation following addition of 5 µg of linear acrylamide solution (Ambion) as carrier and 5 µL of sodium acetate buffer (3 M, pH 5.5, Ambion Inc.). The pellet was washed twice with 70 % ethanol, air-dried and resuspended in 5 µL of DNAse/RNAse free water.

3.6.4.1. Fragmented genome DNA adapter ligation

Adaptors were ligated to the *MseI* digested genomic fragments as described previously (Klein *et al*., 1999). Briefly, 5 µM of each primer (MseLig 21: 5'-AGT GGG ATT CCG CAT GCT AGT-3', MseLig 12: 5'-TAA CTA GCA TGC-3', IDT DNA), 0.5X One-Phor-All plus Buffer (Pharmacia Biotech) and 1.5 µL of nuclease-free water were added to the sample. Annealing was initiated at 65 °C for 1 min, and the temperature was ramped at 1 °C/min down to 15 °C. T4 DNA ligase (5 units, Boehringer Mannheim) and 10 nmol of ATP were added and the reaction and incubated for 16 h at 15 °C.

3.6.4.2. *HpaII* tiny fragment enrichment by ligation-mediated PCR (HELP) cocktail cleavage of ligated genomic DNA

The ligated sample was triple-cleaved with FastDigest™ methyl-sensitive restriction endonucleases (MSRE) *HpaII* (C/CGG), *HinP1I* (GC/GC), *Aci1I* (C/CGC) (Fermentas Thermo Fisher Scientific) using a sequential digestion. Samples were first digested in 50 µL reactions that included 10 µL of sample, 0.5 µL of *HpaII,* 0.5 µL of *HinP1I,* 5 µL of 10X FastDigest buffer and 34 µL of nuclease-free water for 12 h at 37 °C. A second cleavage was performed by adding 0.5 µL of *Aci1I,* 5 µL of 10X FastDigest buffer and 44.5 µL of nuclease-free water and incubating at 37 °C for 4 h, followed by thermal inactivation at 85 °C for 10 min.

3.6.4.3. Verification of cleavage: qPCR of spiked-in templates

The extent of cleavage by MSREs was determined using qPCR detection of spiked-in controls. A master mixture (19 µL) containing 2mM MgCl2, 1X LightCycler FastStart DNA Master SYBR Green mix (Roche Diagnostics Canada, Laval, QC, Canada), 14.4 µL of nuclease-free water and 1 µL of cleaved sample (template) was divided into three fractions to which 0.25 µM of each forward and reverse primer designed to target the appropriate control template was added (see Table 2-1 (Supplementary)). Each qPCR run also included a positive control (non-digested spiked-in, 1/1000 dilution) and negative control (no template). The qPCR conditions were as follows: initial denaturation was carried out at 95 °C for 10 minutes, followed by 50 amplification cycles at 95 °C for 5 seconds, 52 °C for 5 seconds and 72 °C for 20 seconds. Melting curve analysis was

performed for 1 cycle with a ramp rate of 0.2 °C per second, starting at 94 °C for 5 seconds, 72 °C for 30 seconds, and back to 94 °C for 0 seconds, and cooling at 40 °C.

Amplicon specificity was determined from the shape of the melting curve and the difference in cycle threshold (Δ Ct) between methylated (MSRE cleavage protected) and unmethylated (MSRE cleavage unprotected) templates was used to calculate cleavage efficiency. The DNA samples were then precipitated by ethanol, washed and dissolved in 10 µL of nuclease-free water.

3.6.4.4. Fragment selection by ligation-mediated PCR

Selective amplification of methylated fragments was performed using two rounds of ligation-mediated PCR (LM-PCR) as described previously (Klein *et al*., 1999), with some modifications. The first LM-PCR amplification was carried out in 50 µL using 10 µL of sample to which 1 µM of primers (MseLig12 and MseLig21) was added, 0.1X One-Phor-All plus Buffer (Pharmacia Biotech), 0.6X Ex Taq™ buffer without Mg2+ (TaKaRa), 0.1 U Ex Taq™ enzyme (TaKaRa), 1.5 mM MgCl2, 0.4 mM dNTP, and 23.5 µL of nuclease-free water. The mixture was then subjected to thermal cycling as follows: 94 °C (40 sec), 57 °C (30 sec) and 72 °C (1 min 15 sec) for 15 cycles; 94 °C (40 sec), 57 °C (30 sec) and 72 °C (1 min, 45 sec) for 34 cycles; and 94 °C (40 sec), 57 °C (30 sec) and 72 °C (5 min) for the final cycle. The PCR products were resolved on 1 % agarose gel to assess the quality.

To obtain sufficient DNA for downstream processing, three PCR reactions were carried out using 0.75 µL aliquots from the first LM PCR, to which 1.5 µM MseLig21 primer was

added, plus 1X Buffer 1 (Roche/Boehringer Mannheim, Expand Long Template), 0.2 mM dNTPs, 0.8 µL of Expand Long Template Enzyme Mix (Roche/ Boehringer Mannheim) and 38.7 µL of nuclease-free water. The following four-step programs were used: 94 °C (60 sec), 65 °C (30 sec), and 72 °C (2 min) for 1 cycle; 94 °C (40 sec), 65 °C (30 sec) and 72 °C (90 sec) for14 cycles; 94 °C (40 sec), 65 °C (30 sec) and 72 °C (2 min) for 9 cycles and 72 °C (5 min) as the final cycle. The quality and concentration of DNA were evaluated as described above.

3.6.4.5. Adaptor removal

The amplified product from the second LM-PCR was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions with minor modifications. Final elution volume was 41.5 µL, of which 1.5 µL was used for quantification by absorbance measurement. The rest was divided in two separate 20 µL aliquots. Adaptors were removed by digestion for 16 h at 37 °C with 1 U of *MseI* (New England Biolabs). The mixture also contained 1X bovine serum albumin (New England Biolabs, Ipswich, MA, USA), 1 X Buffer 4 (New England Biolabs), and 24 µL of nuclease-free water. The reaction was terminating by heating for 20 min at 65 °C. The sample was then purified using a QIAquick PCR Purification Kit (Qiagen), eluted in 23.5 µL of nuclease-free water and quantified by absorbance.

3.6.4.6. Sample labeling and hybridization

For each sample, 2.5 µg of DNA was labeled using the Universal Linkage System (ULS) labeling kit (Kreatech Biotechnology) according to the manufacturer's instructions with minor modifications: 1 µL of Cy-ULS dye was added per 1 µg of genomic DNA adjusted with 10X labeling buffer. The labelling mixture was then held for 30 min at 85 °C in a thermocycler, followed by 3 min on ice. Non-reacted ULS-Cy3/5 was removed by purification using a QIAquick PCR Purification Kit and samples were eluted in 23.5 µL nuclease-free water. A 1.5 µL aliquot was used to determine DNA concentration and dye incorporation using the ND-1000 NanoDrop. Hybridizations were performed according to the microarray manufacturer's instructions (Agilent Technologies). Briefly, 1 µg of labeled sample (in 40 µL) was mixed with 158 µL of hybridization master mix containing 25 µL of bovine Cot-1 DNA (1.0 mg/mL, Bovine Hybloc competitor DNA, Applied Genetics Laboratories), 2.6 µL of Agilent 100x Blocking Agent and 130 µL of Agilent 2x HI-RPM Hybridization Buffer (Agilent Technologies). Samples were held at 95 °C for 3 min and at 37 °C for 30 min followed by addition of 65 µL of Agilent-CGHBlock (final volume 260 µL). The samples were loaded onto the microarray and hybridization was carried out in a hybridization oven (Shel Lab) for 40 h at 65 °C and 20 rpm. Washing was carried out according to the microarray manufacturer's instructions and slides were scanned with the PowerScanner (Tecan) and analyzed with Array-Pro Analyzer 6.3 software (MediaCybernetics).

3.6.5. Bioinformatics

3.6.5.1. Data analysis pipeline

To complement our transcriptomic platform (Robert *et al*., 2011), a complete suite of data analysis tools was added and integrated into the EmbryoGENE LIMS and Microarray Analysis (ELMA) gateway (http://elma.embryogene.ca/). This pipeline processes sample-associated information, experimental design and protocols, as well as microarray data analysis for identification of differential gene/loci lists and further data mining for enrichment analyses. EDMA data was analyzed using the Limma package from Bioconductor (Smyth *et al*., 2005; McCarthy and Smyth, 2009). First, Loess intra-array normalization followed by quantile inter-array scale normalization were applied. Normalized data was then fitted to a linear model and Bayesian statistics of differential expression were obtained. Differences in DNA methylation were considered significant when the P value was < 0.05 and the absolute log2 fold-change was at least 1.5. The inter-treatment comparisons were conducted using between-group analysis based on Eigen values using the Bioconductor package MADE4 (Culhane *et al*., 2005). Visualization tools were developed to generate plots of the extent of cleavage of spiked-in controls and overall genomic cleavage for quality control purposes. Different plots including Volcano plots were generated to visualize the amount of differentially methylated regions among treatments. Normalized data and a list of differentially methylated loci can be exported

as text files for downstream data mining. Different enrichment analyses for genome-scale DNA methylation data are performed through a string of integrated scripts that categorize the information based on CpG island density (Smith *et al*., 2012), CpG island length (Elango and Yi, 2011), CpG island distance (Krausz *et al*., 2012), genomic location and types of repetitive elements (Jurka *et al*., 2005). The pipeline also pinpoints methylation hot spots, generates bedgraph files appropriate for visualization within a genome browser, and uses Circos plot (Krzywinski *et al*., 2009) to generate a circular graph representing overall methylation levels and correlating these to transcription levels, if the latter data are available.

3.6.5.2. Parameters definitions

The CpG island lengths, density and positions for the UMD3.1 (ftp://ftp.cbcb.umd.edu/pub/data/assembly/Bos_taurus/) build of the bovine genome was obtained from the UCSC genome browser (Meyer *et al*., 2013). For any given gene in EDMA, there are five types of annotation "windows" including; 1) Distal promoter, 2) Promoter, 3) Proximal Promoter, 4) Exons and 5) Introns. The "Proximal Promoter", "Promoter" and "Distal Promoter" regions are defined as the first 1 kbp, 5 kbp and 50 kbp 5' of the transcription start site (TSS). For each probes, genes are added to the appropriate columns if their *MseI* -*MseI* fragment overlap those windows. Genomic locations which were not part of a CpG island were further split into three types based on their distance from the nearest CpG island: "CpG shores" for regions within 2,000

nucleotides of an island, "CpG shelves" for regions between 2,000 and 4,000 nucleotides of an island and "Open Sea" for regions further away (Krausz *et al*., 2012). Probes whose fragment overlaps a CpG island were annotated using the descriptive characteristics of that island. Of all CpG island surveyed by the array, those in the bottom 20 percentile of length were classified as "short", those at the top 80 percentile were classified as "long" and all others were classified as "intermediate" (Elango and Yi, 2011). The same scheme was applied to CpG island density, with the bottom 20 percentile being labelled "low density", the top 80 percentile being labelled "high density", and all others being labelled "Intermediate density" (Smith *et al*., 2012). Bovine repetitive elements content were identified by RepeatMasker (http://www.repeatmasker.org/) with build 20120418 of the RepBase database (Jurka *et al*., 2005) and then used the repeat classes attribute (LINE, SINE, etc.) as a basis for category enrichment. The “methylation hot spots” is calculated as the averages p-values of differential methylation over windows of 100K nucleotides. More specifically, for all probes on the array, we look up all other probes within 100K nucleotides upstream and downstream, and average the p-values thus obtained. The averaged p-values have no statistical meaning, but can be used as an indicator for regions of interest, which we called a "methylation hot spot".

3.6.6. Data validation

Pyrosequencing was performed as described previously (O'Doherty *et al*., 2012). Briefly, blastocyst and sperm DNA samples were bisulfite-converted using an EZ DNA methylation direct KIT, (ZYMO Research) followed by amplification of target loci by PCR prior to sequencing. For all pyrosequencing assays, PyroMark Assay Design software (Qiagen) was used to design three oligonucleotide primers (forward, reverse and sequencing), synthesized by Integrated DNA technologies (Coralville). All reverse primers were biotinylated at the 5' terminus and purified by HPLC. PCR amplification was carried out in 25 µL containing 0.2 µM of each forward and reverse 5'-biotinylated primer, 1.25 U of Platinum Taq DNA polymerase (Invitrogen), 1X Taq DNA polymerase buffer, 0.2 mM dNTPs, 3–4 mM MgCl2, 2 µL of bisulfite-treated DNA and 17.5 µL of nuclease-free water. The following three-step program was used: 95 °C (5 min) for 1 cycle, 95 °C (30 sec), 48 °C for DMR1b, 49 °C for DMRs 2 and 3a, 50 °C for DMRs 1a, 3b, 5-8 and 54 °C for DMR 4 (30 sec) and 72 °C (30 sec) for 35 cycles, and finally 72 °C (5 min). The specificity of the amplification was verified by electrophoresis on 2 % w/v agarose gel at 90 V for 45 min. Templates were purified by adding 2 µL of streptavidin-coated Sepharose beads (GE Healthcare) in the presence of 40 µL of binding buffer (Qiagen) and then pyrosequenced (Tost and Gut, 2007). Pyrosequencing reactions were conducted using a PyroMark Q24 apparatus (Qiagen).

3.6.7. Availability of supporting data

The data analysis pipeline user manual is available at; [EDMA user manual]: http://emb-bioinfo.fsaa.ulaval.ca/bioinfo/html/epigenetics/Epigenetics%20Analysis%20Pipeline.pdf

The dataset of microarray results has been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number: GSE57709 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57709).

3.6.8. Competing interests

The authors declare they have no competing interest.

3.6.9. Acknowledgements

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3.8. Figures

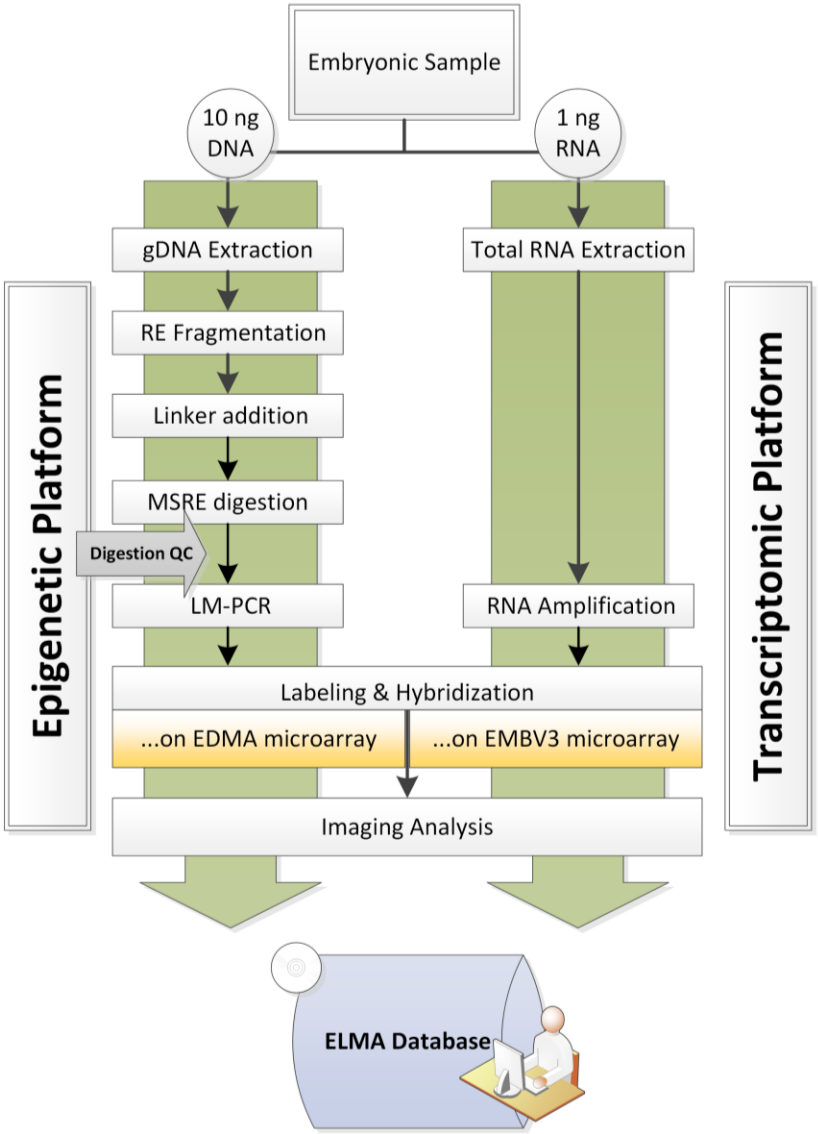
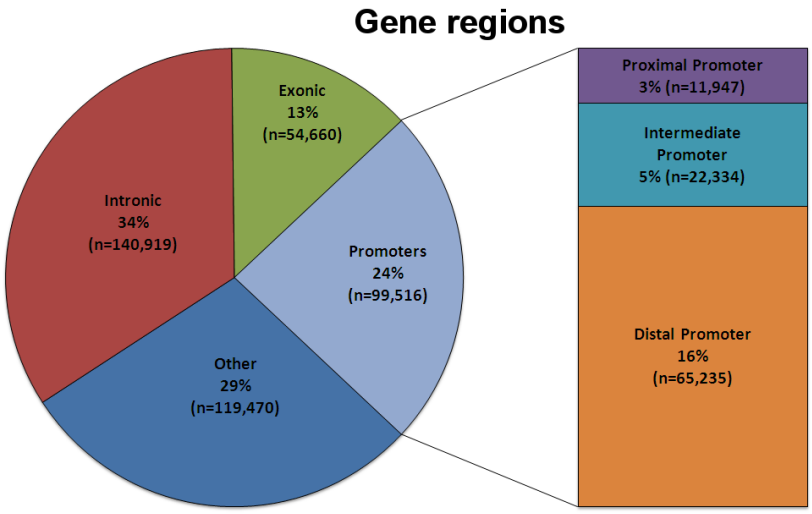
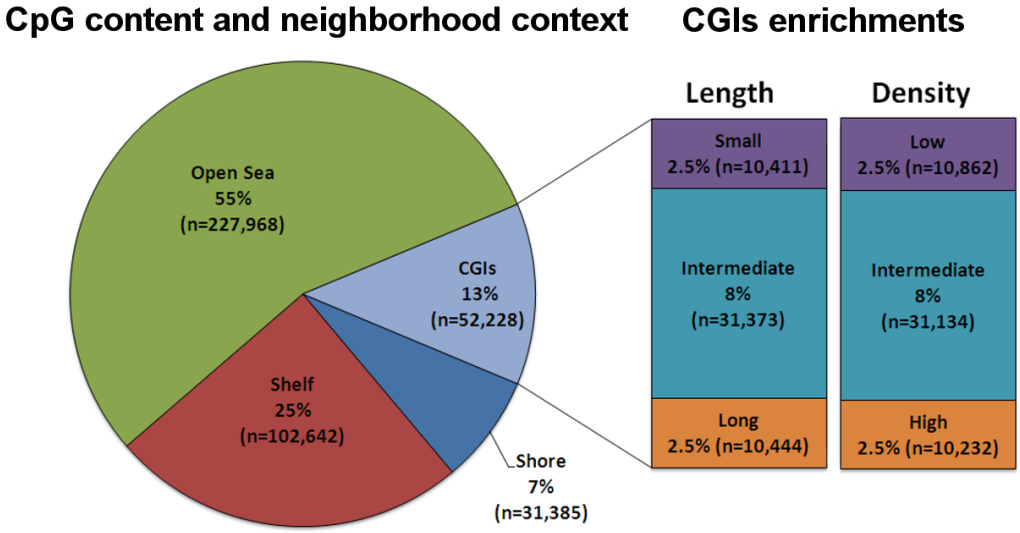


Figure 3-1 Sequence of steps involved in the generation of methylome and transcriptome data from the same sample using EDMA platform. A quality control step prior to LM-PCR allows evaluation of the efficiency of cleavage by MSREs for methylome analysis. **EDMA**: EmbryoGENE DNA methylation analysis platform, **ELMA**: EmbryoGENE LIMS and Microarray Analysis, **EMBV3**: EmbryoGENE bovine transcriptomics microarray Version 3, **LM-PCR**: Ligation-mediated PCR, **MSRE**: Methyl-sensitive restriction endonuclease, **RE**: Restriction endonuclease (*MseI*).

A


Gene regions
Exonic
13%
(n=54,660)
Intronic
34%
(n=140,919)
Promoters
24%
(n=99,516)
Other
29%
(n=119,470)
Proximal Promoter
3% (n=11,947)
Intermediate
Promoter
5% (n=22,334)
Distal Promoter
16%
(n=65,235)

B


CpG content and neighborhood context
CGIs enrichments
Open Sea
55%
(n=227,968)
CGIs
13%
(n=52,228)
Shelf
25%
(n=102,642)
Shore
7%
(n=31,385)
Length
Density
Small
2.5% (n=10,411)
Intermediate
8%
(n=31,373)
Long
2.5% (n=10,444)
Low
2.5% (n=10,862)
Intermediate
8%
(n=31,134)
High
2.5% (n=10,232)

C

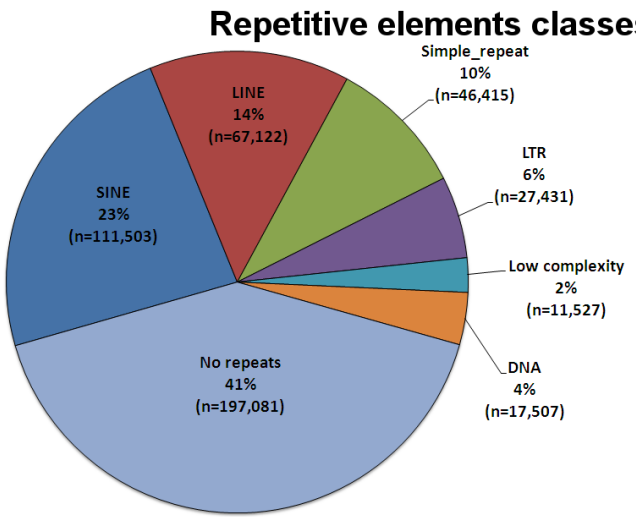

Repetitive elements classes
Simple_repeat
10%
(n=46,415)
LINE
14%
(n=67,122)
SINE
23%
(n=111,503)
LTR
6%
(n=27,431)
Low complexity
2%
(n=11,527)
No repeats
41%
(n=197,081)
DNA
4%
(n=17,507)

Figure 3-2 The characteristics of EDMA array. (A) Gene region coverage by the probes. The single greatest proportion (34%) corresponds to intronic regions. **(B)** Probe distribution based on proximity to CpG islands as well as CpG islands-related enrichments. More than half of the probes target fragments in the open-sea region. **(C)** Proportions of different classes of bovine repetitive elements detectable by the EDMA platform.

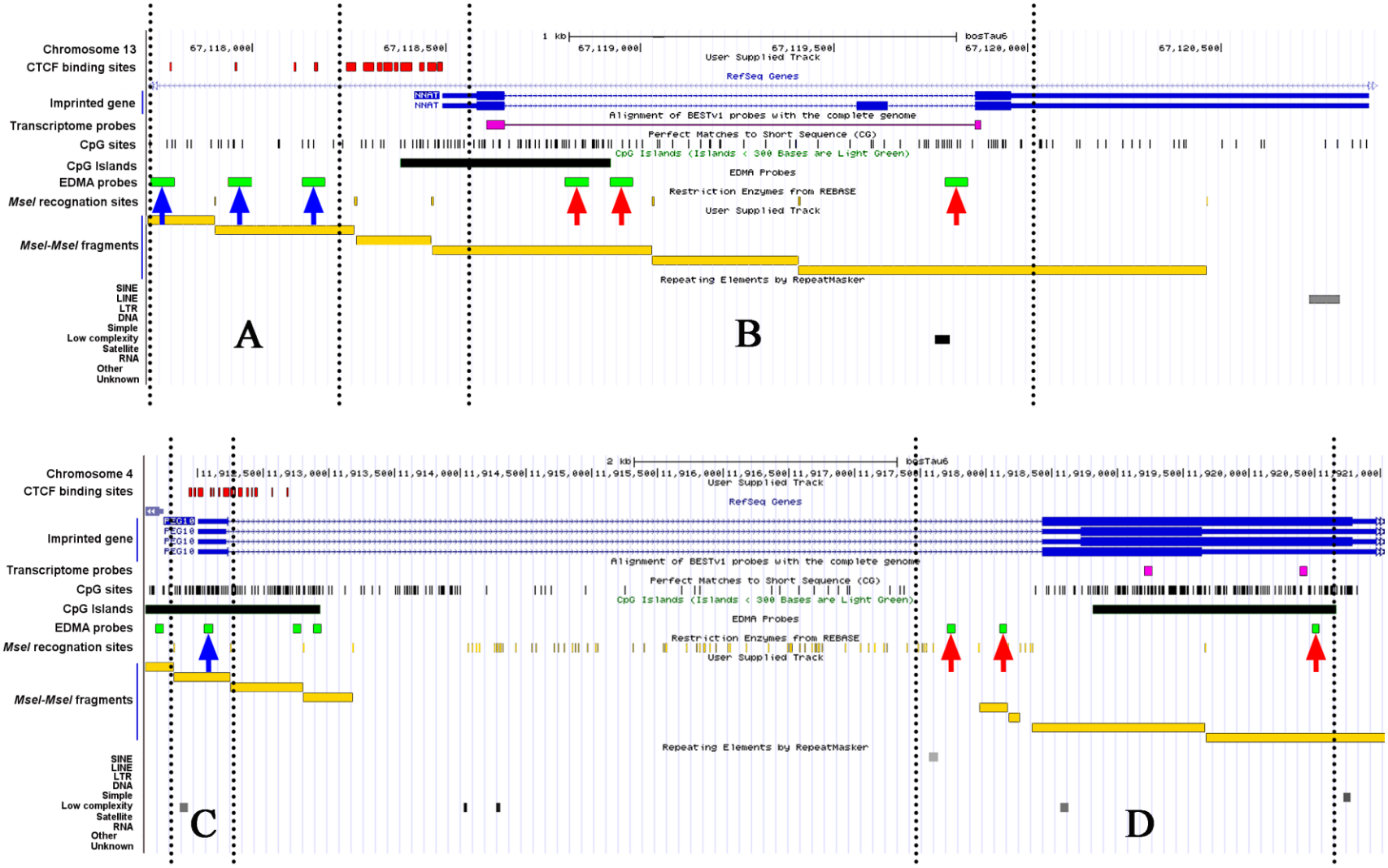

Chromosome 13
CTCF binding sites
Imprinted gene
Transcriptome probes
CpG sites
CpG Islands
EDMA probes
MseI recognation sites
MseI-MseI fragments
SINE
LINE
LTR
DNA
Simple
Low complexity
Satellite
RNA
Other
Unknown
67,118,000
67,118,500
1 kb
67,119,000
67,119,500
bosTau6
67,120,000
67,120,500
User Supplied Track
RefSeq Genes
NNAT
Alignment of BESTv1 probes with the complete genome
Perfect Matches to Short Sequence (CG)
CpG Islands (Islands < 300 Bases are Light Green)
EDMA Probes
Restriction Enzymes from REBASE
User Supplied Track
Repeating Elements by RepeatMasker
A
B
Chromosome 4
CTCF binding sites
Imprinted gene
Transcriptome probes
CpG sites
CpG Islands
EDMA probes
MseI recognation sites
MseI-MseI fragments
SINE
LINE
LTR
DNA
Simple
Low complexity
Satellite
RNA
Other
Unknown
2 kb
bosTau6
11,913,000
11,913,500
11,914,000
11,914,500
11,915,000
11,915,500
11,916,000
11,916,500
11,917,000
11,917,500
11,918,000
11,918,500
11,919,000
11,919,500
11,920,000
11,920,500
User Supplied Track
RefSeq Genes
PEG10
Alignment of BESTv1 probes with the complete genome
Perfect Matches to Short Sequence (CG)
CpG Islands (Islands < 300 Bases are Light Green)
EDMA Probes
Restriction Enzymes from REBASE
User Supplied Track
Repeating Elements by RepeatMasker
C
D

Figure 3-3 Snapshots from the UCSC genomic web-browser describing the genomic locations of two bovine imprinted genes (NNATand PEG10), positioning of the probes (methylome and transcriptome) and other associated bovine genomic features. *MseI* recognition sites on genomic DNA result in *MseI -MseI* fragments. CpG islands may be absent (Area **A**) or present (Areas **B-D**). EDMA probes may target CTCF binding sites (blue arrows), which may be proximal (Area **A**) or within (Area **C**) the gene body. Other EDMA probes (red arrows) also may overlap with probes the transcriptomic (EMBV3) array or cover CpG islands or non-CpG islands. Probes (red arrows) may also cover intronic or exonic portions of the genome (Area **D**).

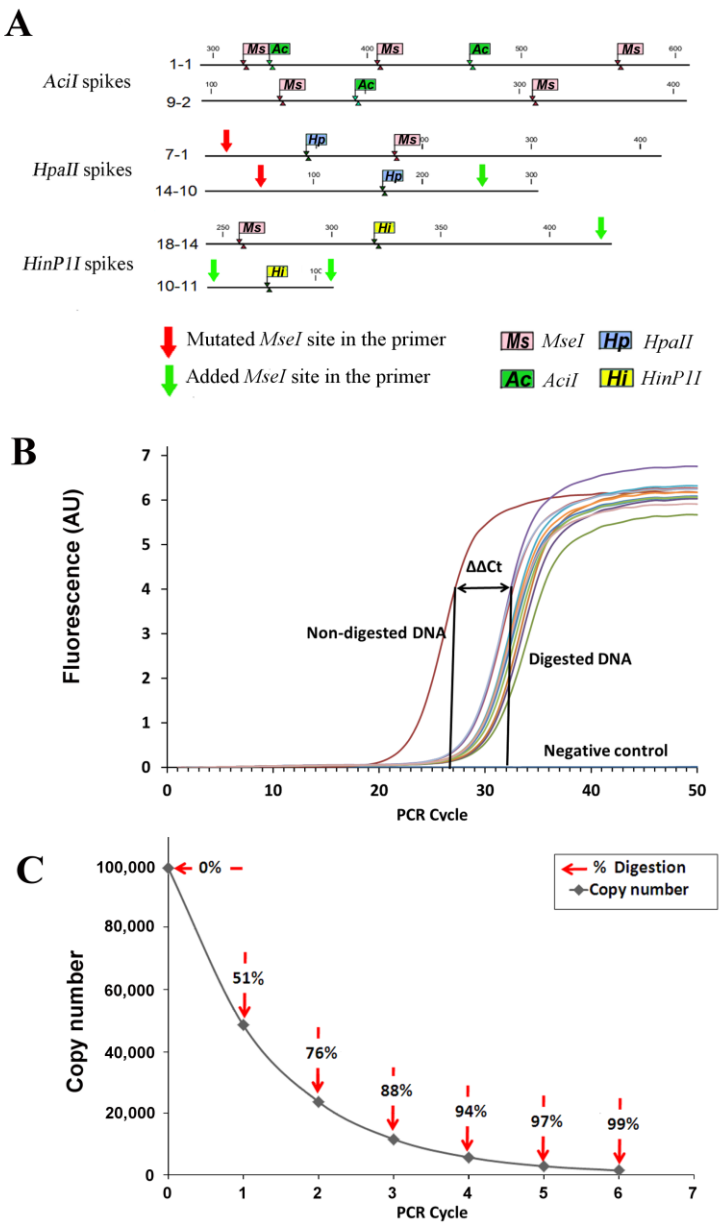


Figure 3-4 The feature of EDMA spiked-in controls and the quality control process of MSREs digestion assessment.(A) Location of sites cleaved by endonucleases within exogeous control DNA. **(B)** Difference in PCR threshold cycle (ΔΔCt) between un-cleaved (positive control) and cleaved samples. **(C)** Plot of residual number of copies and corresponding efficiency of cleavage by MSRE versus PCR cycle number, indicating that satisfactory cleavage (97%) is reached at the end of five PCR cycles.

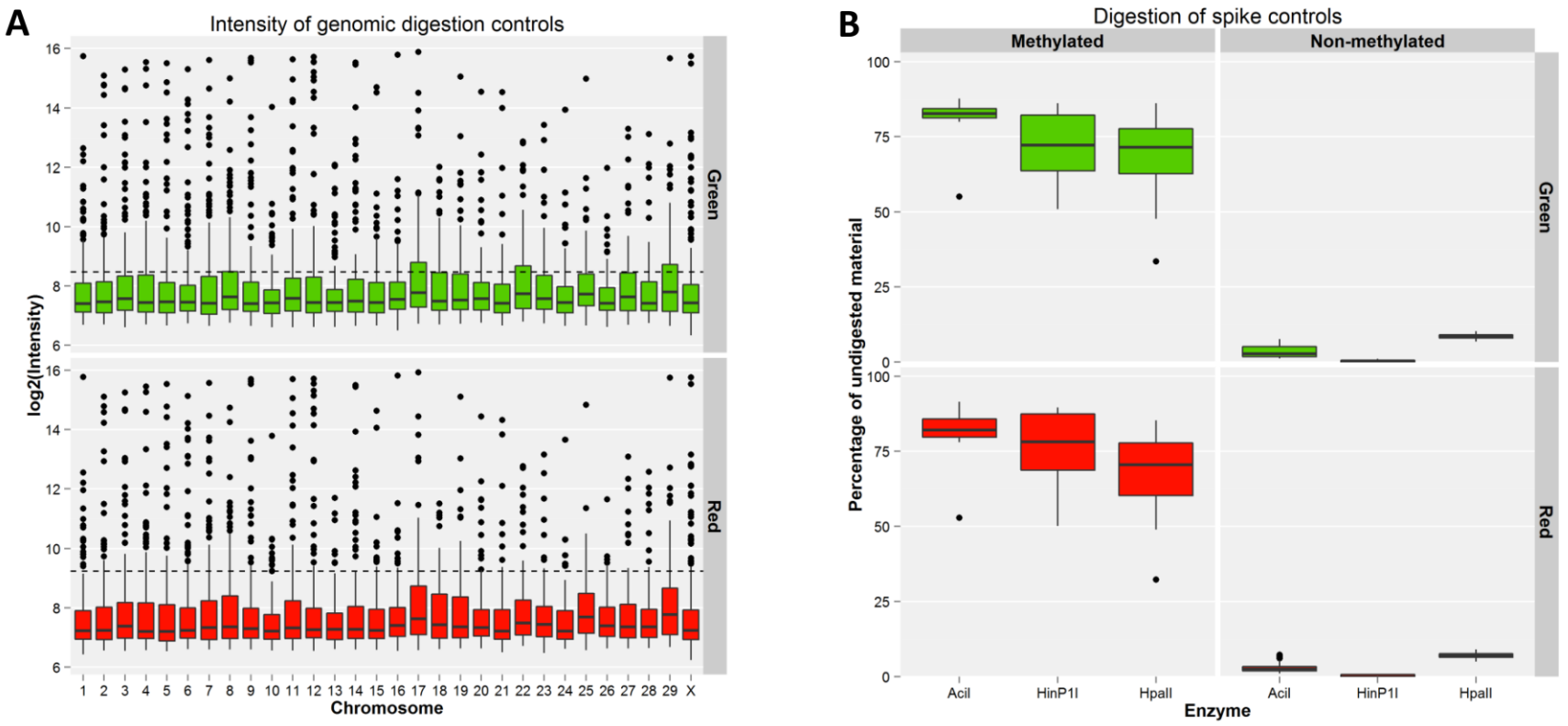


Figure 3-5 The two types of quality control (QC) plots generating after EDMA microarray hybridization and data analysis. (A) Signal intensity of control probes designed to overlap an *MseI* site. Probes corresponded to loci distributed throughout the bovine genome. The dashed horizontal line represents the limit of detection (mean intensity of negative controls plus four standard deviations). Intensities below this line indicate successful genomic fragmentation by *MseI* . **(B)** Cleavage of synthetic spiked-in control DNA pairs by MSRE, based on microarray signal. Signals from unprotected (unmethylated) fragments (right) are near background values.

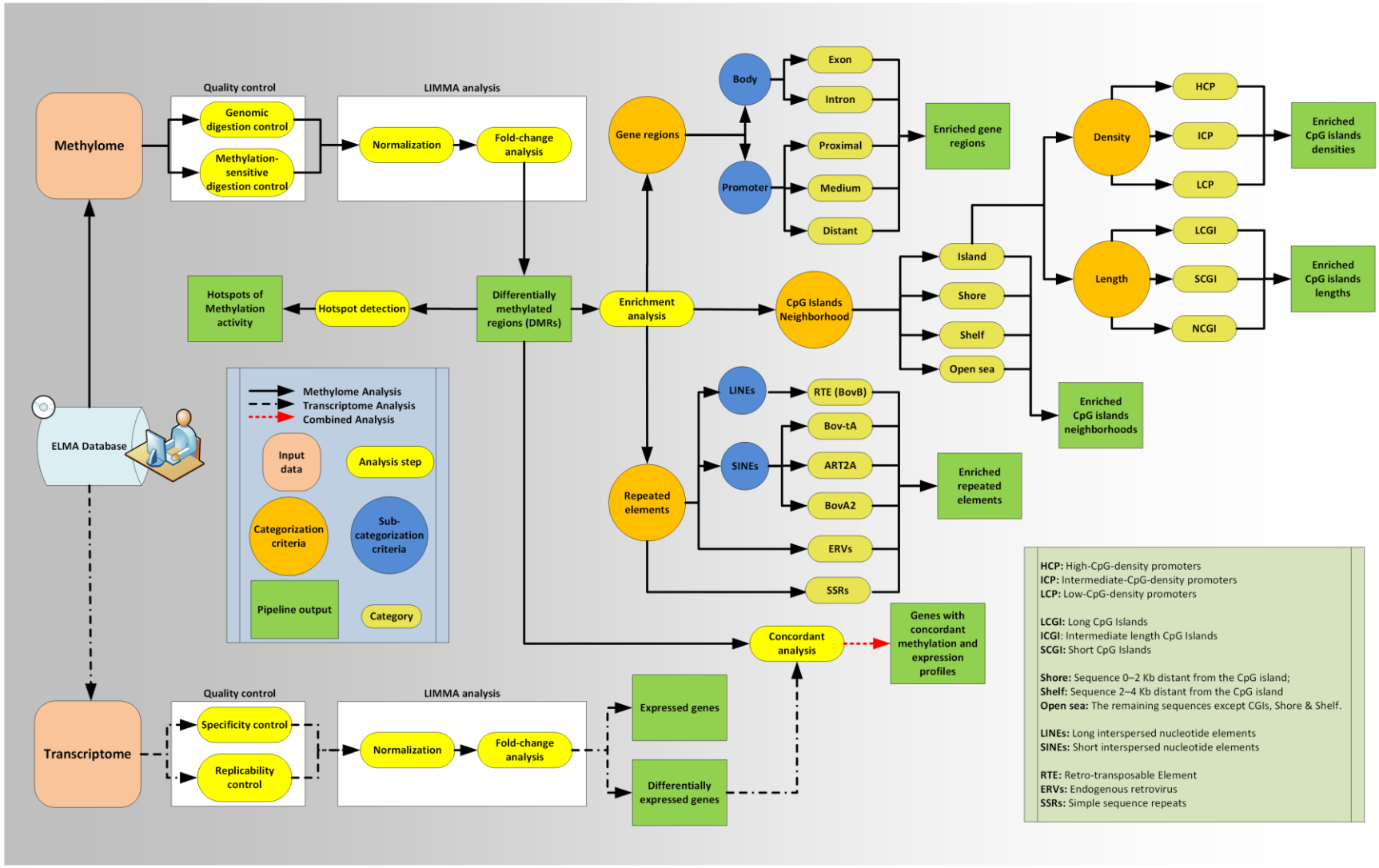

Methylome
Quality control
Genomic digestion control
Methylation-sensitive digestion control
LIMMA analysis
Normalization
Fold-change analysis
Gene regions
Body
Promoter
Exon
Intron
Proximal
Medium
Distant
Enriched gene regions
Density
HCP
ICP
LCP
Enriched CpG islands densities
Length
LCGI
SCGI
NCGI
Enriched CpG islands lengths
Island
Shore
Shelf
Open sea
Enriched CpG islands neighborhoods
CpG Islands Neighborhood
Enrichment analysis
Differentially methylated regions (DMRs)
Hotspot detection
Hotspots of Methylation activity
ELMA Database
Methylome Analysis
Transcriptome Analysis
Combined Analysis
Input data
Analysis step
Categorization criteria
Sub-categorization criteria
Pipeline output
Category
Repeated elements
LINEs
SINEs
RTE (BovB)
Bov-tA
ART2A
BovA2
ERVs
SSRs
Enriched repeated elements
Concordant analysis
Genes with concordant methylation and expression profiles
Transcriptome
Quality control
Specificity control
Replicability control
LIMMA analysis
Normalization
Fold-change analysis
Expressed genes
Differentially expressed genes
HCP: High-CpG-density promoters
ICP: Intermediate-CpG-density promoters
LCP: Low-CpG-density promoters
LCGI: Long CpG Islands
ICGI: Intermediate length CpG Islands
SCGI: Short CpG Islands
Shore: Sequence 0–2 Kb distant from the CpG island;
Shelf: Sequence 2–4 Kb distant from the CpG island
Open sea: The remaining sequences except CGIs, Shore & Shelf.
LINEs: Long interspersed nucleotide elements
SINEs: Short interspersed nucleotide elements
RTE: Retro-transposable Element
ERVs: Endogenous retrovirus
SSRs: Simple sequence repeats

Figure 3-6 The EDMA DNA methylation analysis pipeline. This genome-wide methylome analysis pipeline was designed to provide a comprehensive set of plots to ease interpretation of both methylome and transcriptome raw data generated from the same sample. Information is binned according to known functions or genetic features.

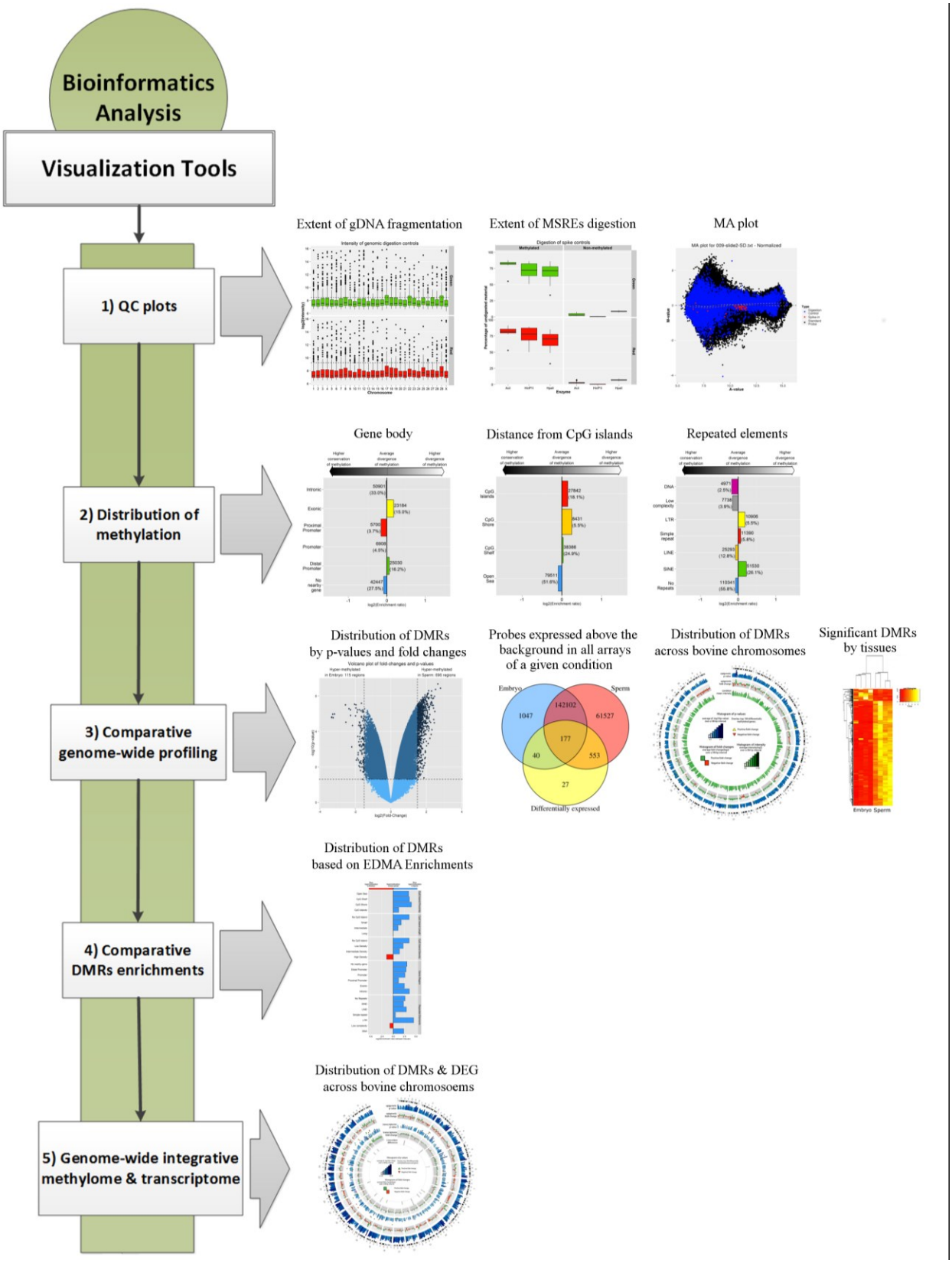


Figure 3-7 List of the graphs generated by EDMA analytical pipeline.

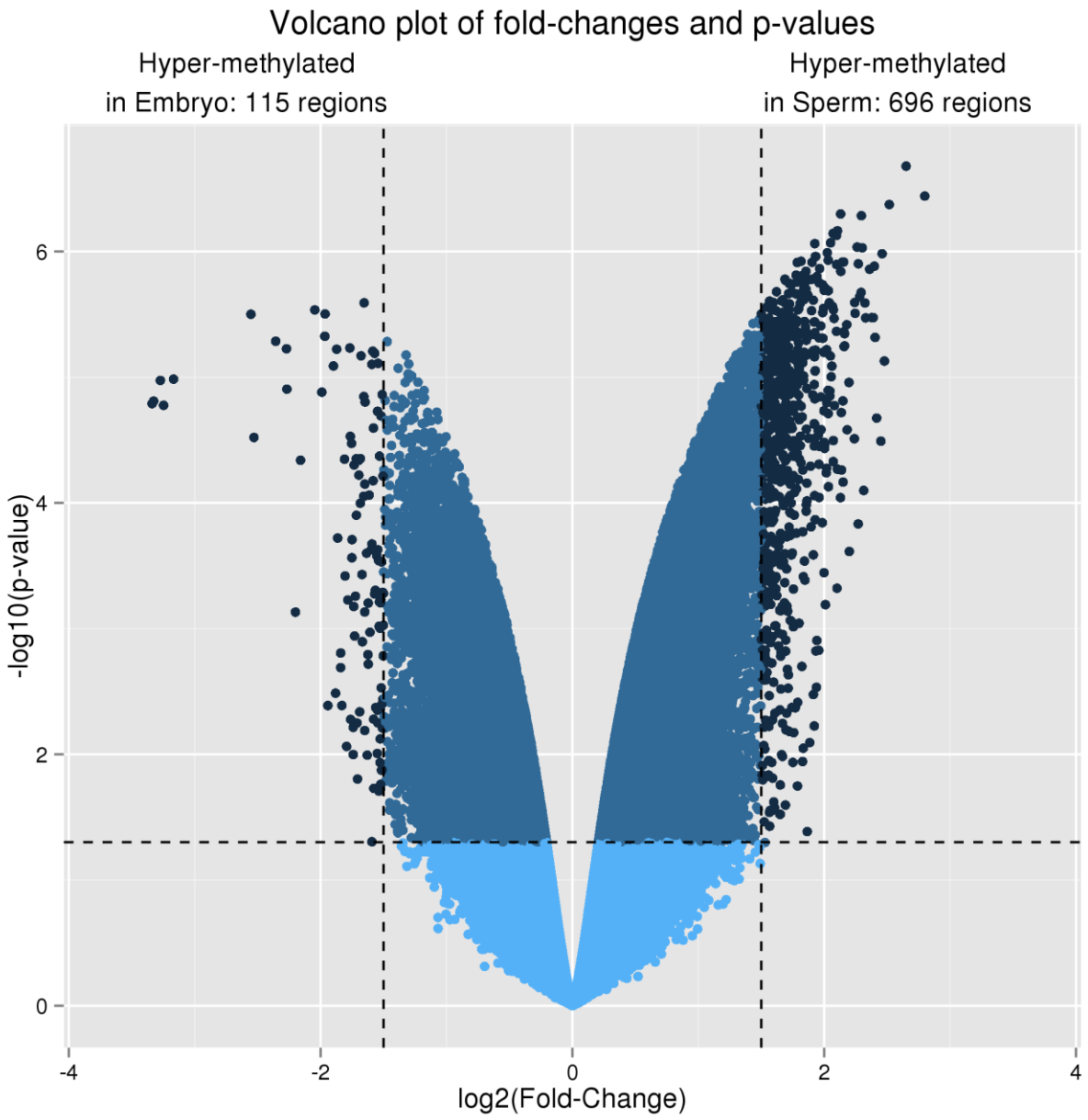


Figure 3-8 Volcano plot of genome-wide DNA methylation. This plot shows clearly that bovine sperm DNA was more methylated than embryo DNA.

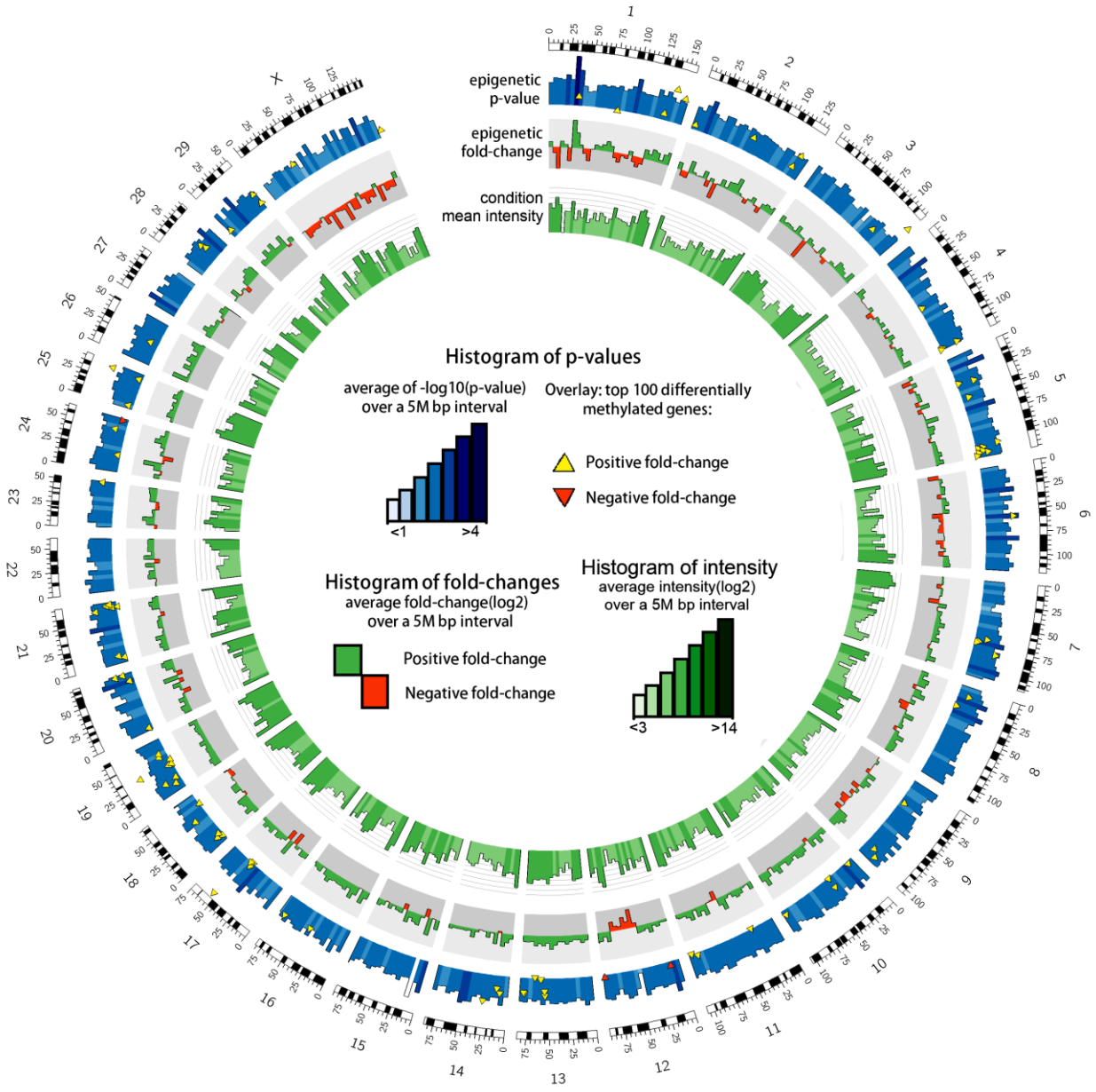


Figure 3-9 The Circos plot showing the genome-wide DNA methylation profile of bovine sperm DNA and the blastocyst genome, by chromosome. The mean p-values of 5 M bp windows are displayed along with the 100 most significant DMRs. Positive fold-changes represent hypermethylation in sperm while negative fold-changes represent hypermethylation in blastocysts. The inner circle depicts probe mean values across treatments to show the completeness of the coverage generated from the microarray signals.

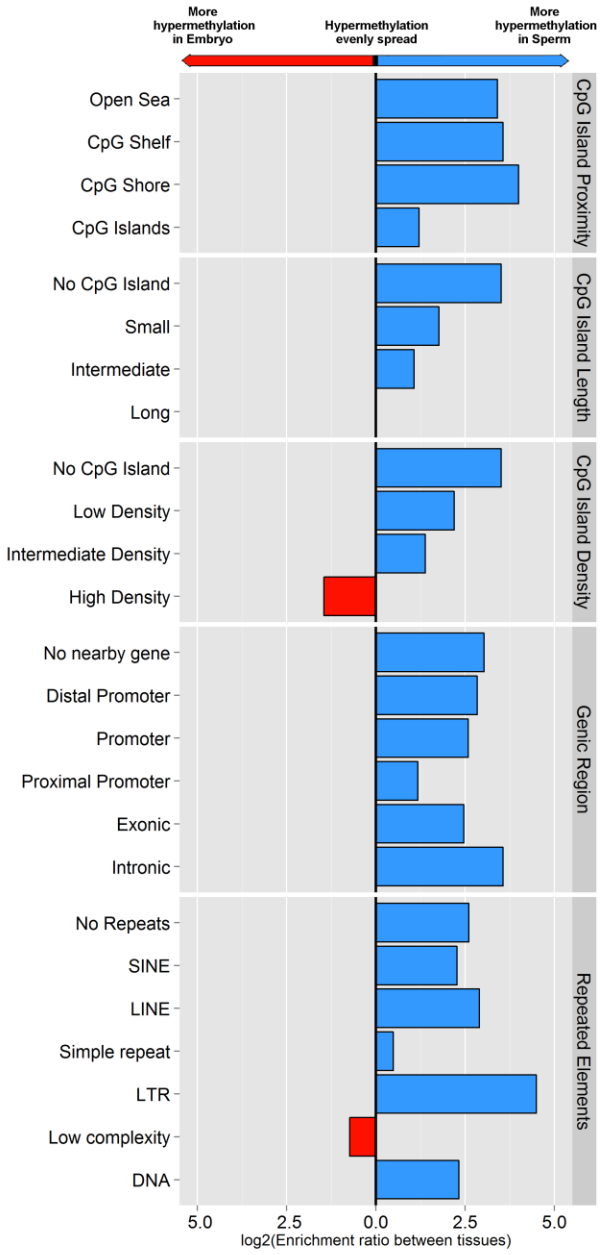


Figure 3-10 The comparative analysis of differentially methylated regions (DMRs) enrichments between bovine sperm and blastocysts. This figure shows the log2 enrichment ratios between the numbers of the DMRs found to be hypermethylated in bovine sperm compared to the number of DMRs found to be hypermethylated in bovine blastocysts, broken down by different types of genomic features. The majority of the DMRs showed hypermethylation in bovine sperm DNA.

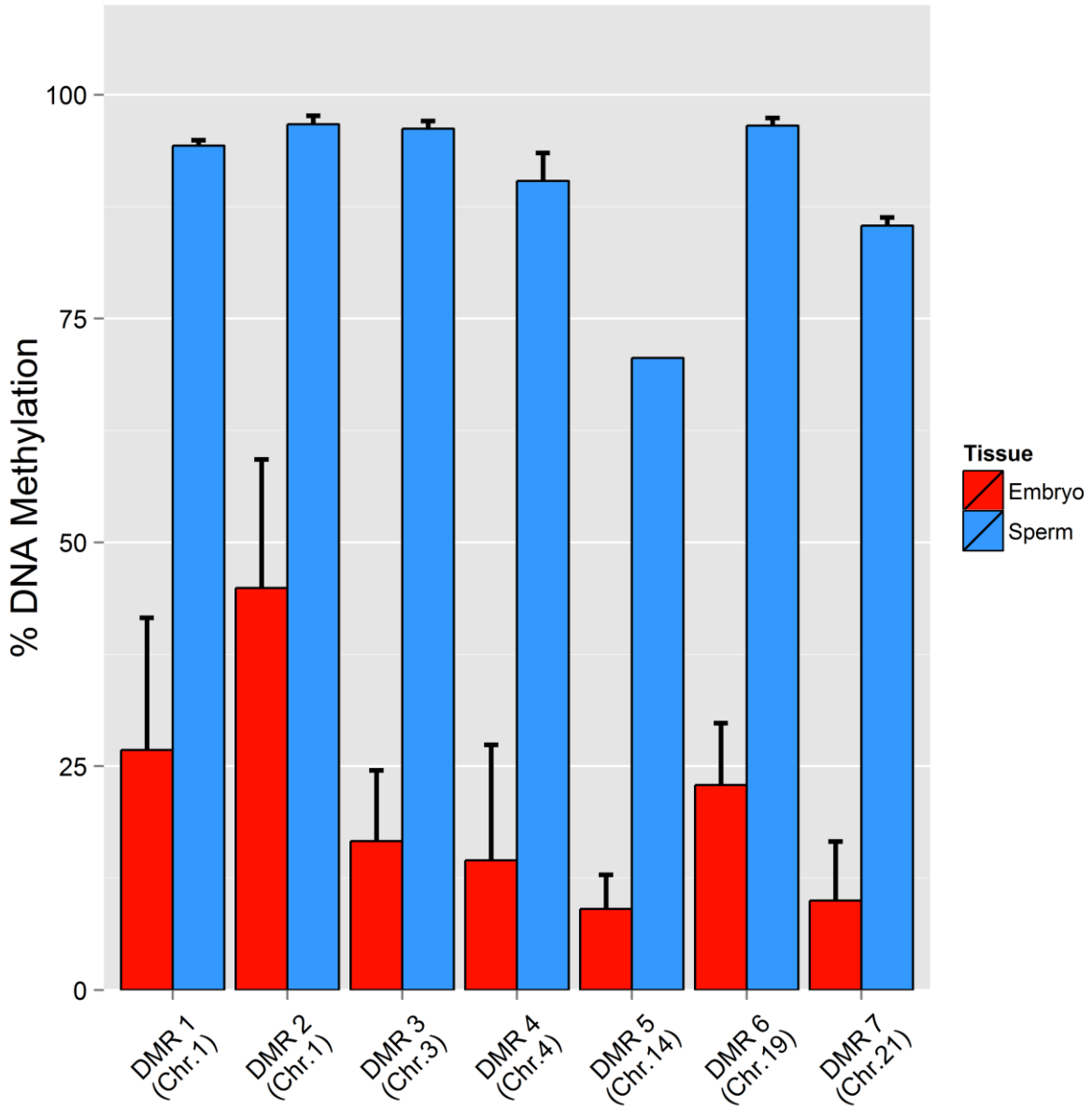


Figure 3-11 Validation of the selected DMRs by pyrosequencing.

3.9. Supplementary Figures

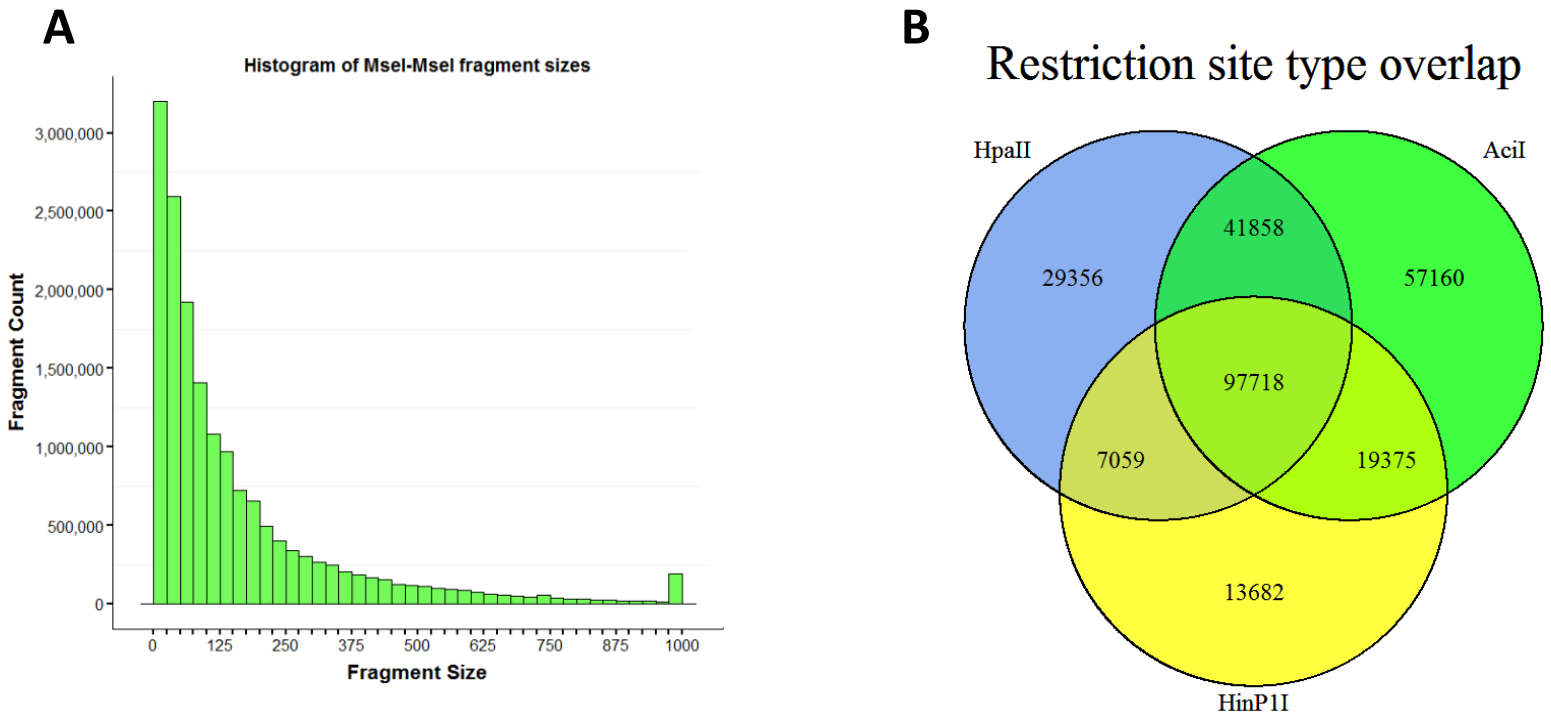


Figure 3-12 (Supplementary) (A) This histogram shows the *in silico* nalysis of the lengths of *MseI /MseI* fragments across the bovine genome. Higher frequencies of shorter fragments with an average size <160 bp are observed. **(B)** The Venn diagram shows the overlaps between restriction sites of the HELP cocktail MSREs within the genomic *MseI* fragments targeted by EDMA probes.

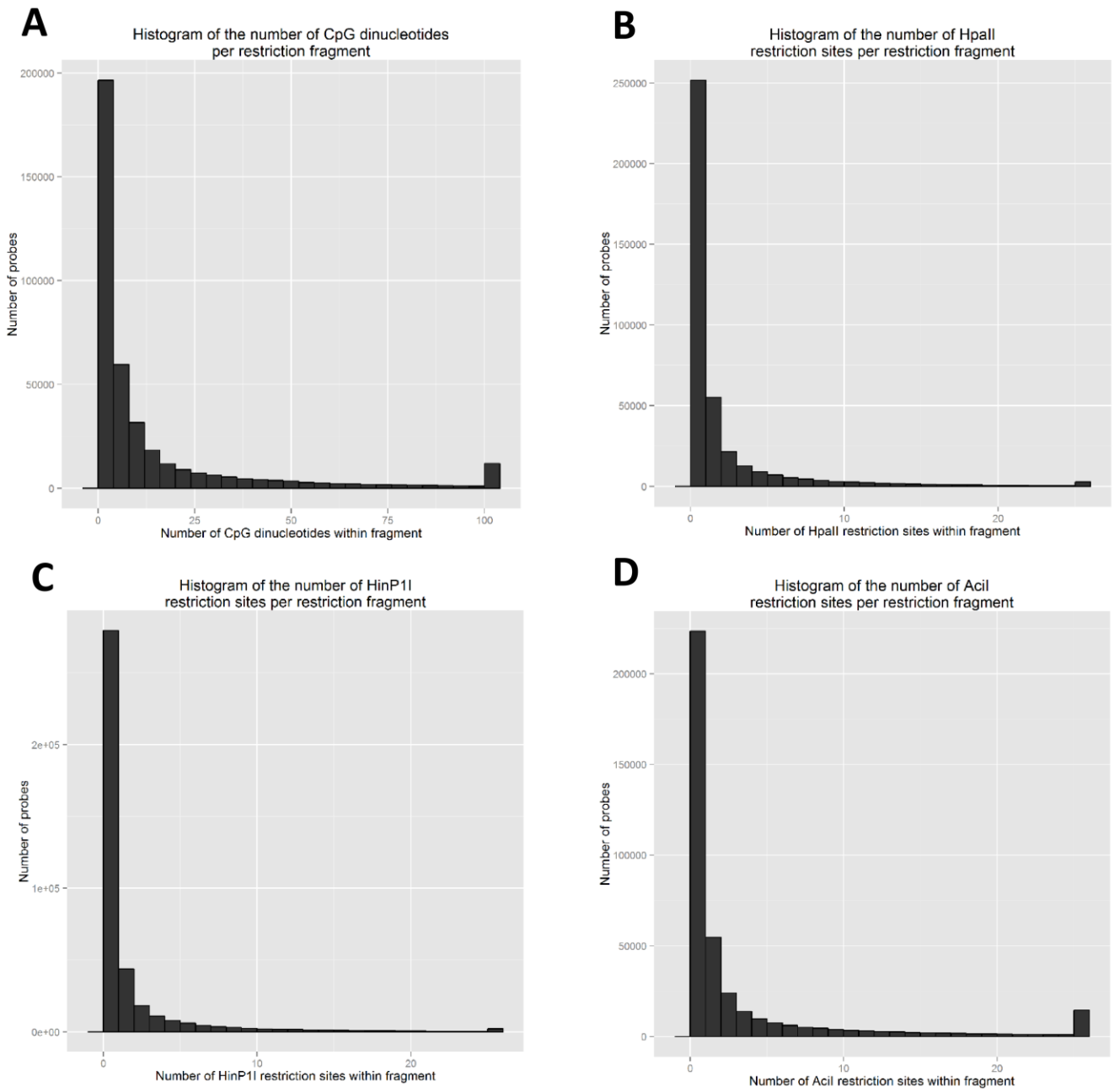


Figure 3-13 (Supplementary) This figure shows the histogram number of **(A)** CpG dinucleotide per *MseI* restriction fragments and histograms number of MSREs (**(B)** *HpaII* ; **(C)** *HinP1I* ; **(D)** *AciI*) restriction sites per restriction fragments in EDMA.

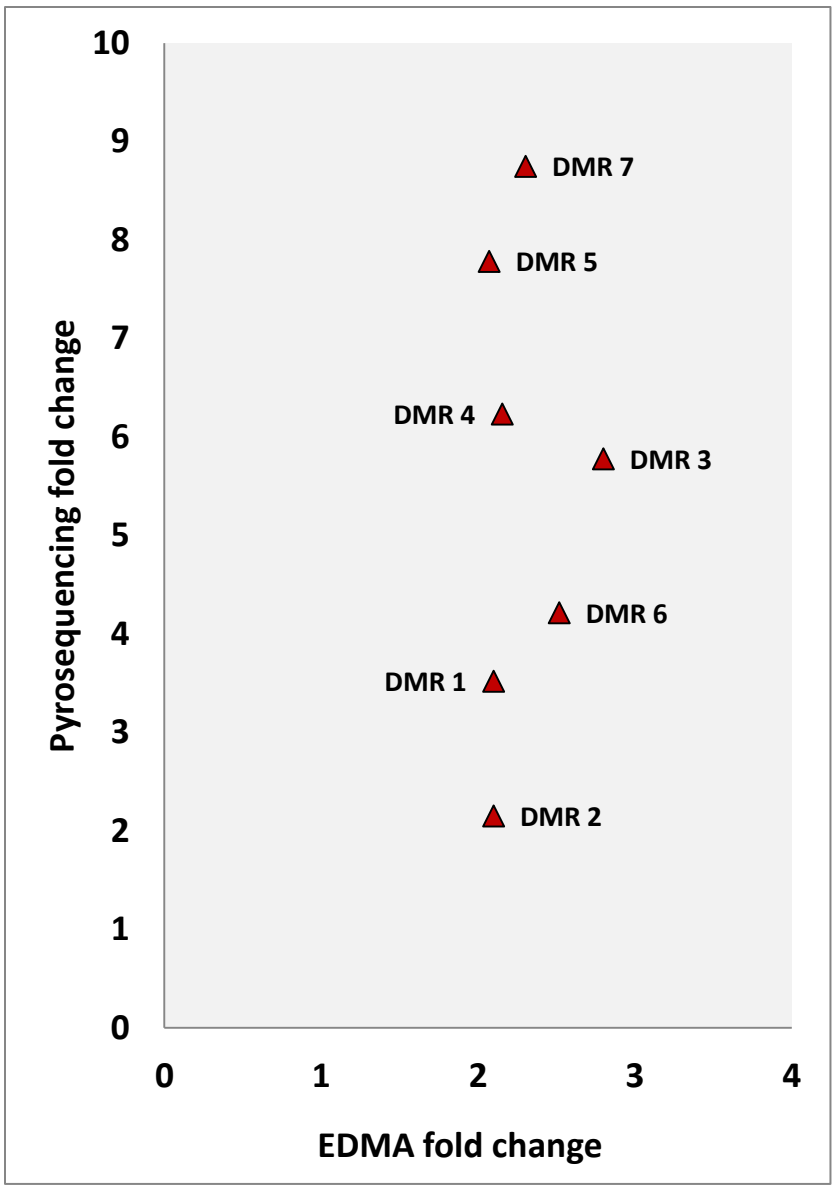


Figure 3-14 (Supplementary) This figure clearly shows that there is not any correlation between the determined EDMA fold change and Pyrosequening results due to the enrichment-based nature of the applied protocol.

3.10. Supplementary Tables

Table 3-1 (Supplementary) The designed Primers for the MSRE digestion quality control step.

***Solanum lycopersicum* selected genes**	**MSRE[a]**		**Spike-in (QC)[b] primers**	**Sequence**	***GC%***	***Length***	***Temp. (^0C)[c]***
Rubisco Large subunit	***AciI***	***Methylated***	*1-1*	5'- CATTCCGAGTAACTCCTCAACC-3'	50	22	54
			Forward/reverse	5'- CTTTTGCTAATACCCGGAAGTG-3'	45	22	
Phytochrome B1		***Non-methylated***	*9-2*	5- 'CGGGTATGACAGGGTTATGG -3'	55	20	54
			Forward/reverse	5'- GGACCGAACAGAAGTGTGGT-3'	55	20	
			9-2 (QC primers)	5'- AGCAGAACAGGGTGAGAATGAT-3'	45	22	52
			Forward/reverse	5'- AGACCCCATATTTGCCATGT-3'	45	20	
Glutamine synthetase	***HpaII***	***Methylated***	*7-1*	5'- CAGCTGGTGATTAAGTGTGG -3'	50	20	54
			Forward/reverse	5'- TGCAATCATGGAGGTAACGA-3'	45	20	
Nitrite reductase		***Non-methylated***	*14-10*	5'- CGAGTTAATCAGGAATCCAGTTGGAAATC-3'	41	29	54
			Forward/reverse	5'- CGAGTTAATGCAAGATCATTGATATGAGG-3'	37	29	
			14-10 (QC primers)	5'- TCTGGCTGGAATTGATCCTG-3'	50	20	52
			Forward/reverse	5'- TCATAAAGATCATGAGACCCT-3'	38	21	
TOMWIPIG	***HinP1I***	***Methylated***	*18-4*	5' - AAATCTCCCGCTTCGCCCTTAT-3'	50	22	54
			Forward/reverse	5'- CAGTTAAGCCATGAGAGTTTCAAAGGCTGTCG AT-3'	44	34	
Nitrate reductase		***Non-methylated***	*10-11*	5'- CGAGTTAATGGAGGAGAGACATGG-3'	50	24	54
			Forward/reverse	5'- CGACTTAACAACCAACTCGAAGAACCCTAC-3'	46	30	
			10-11 (QC primers)	5'- ACATGGAGTGTTCAGTTGTTG-3'	43	21	52
			Forward/reverse	5'- CGACTTACCACCCAACTCGAAGAACCCTAC-3'	53	30	

a) MSRE: Methyl-sensitive restriction endonuclease; **b)** ***QC primers:*** *Quality control primers;* ***c) Temp. (^0C):*** *Annealing temp.*

Table 3-2 (Supplementary) Genomic and CpG coverage by *MseI* fragments targeted by EDMA probes as a function of the MSRE sites present within those fragments

	Bovine genome (No.)	All probes (No.)	All enzymes (No.)	*HpaII* (No.)	*AciI* (No.)	*HinP1I* (No.)
Genomic coverage of fragments	-	7.5 % (200,653,194)	6.1 % (163,284,790)	4.8 % (129,228,720)	5.4 % (1,449,615,52)	4.1 % (109,590,310)
Coverage CpG per fragments	-	20.7% (5,640,598)	19.7 % (5,368,673)	17.7 % (4,808,063)	18.8 % (5,110,353)	16.4 % (4,448,586)
Coverage CpG per enzymes	-	8.6 % (2,343,216)	8.6% (2,343,216)	2.3 % (630,128)	4.6 % (1,246,095)	1.7 % (466,993)

MSRE: Methyl-sensitive restriction endonuclease.
*The bovine genome (*Bos Taurus*) has an average genome size of 2,670,422,299 bp and 27,203,575 CpG sites in entire genome.

Table 3-3 (Supplementary) Gene and CpG Island coverage by EDMA probes.

	Genes			CpG Islands	
Coverage	Covered features (#)	Covered features (%)	**Coverage**	Covered features (#)	Covered features (%)
Genes covered (Body or promoter)	16,912	77.8%	CGIs covered	44,182	87.3%
Genes covered (Distal promoter)	20,361	93.6%	Total CGIs	50,633	-
Total genes	21,747	-			

Table 3-4 (Supplementary) Breakdown of the location of EDMA probes in relation to annotated features of the bovine genome

Probe. Category	Probe. No.	Probe %
CpG Island	52,228	12.6%
4k of a CpG island	186,255	44.9%
Gene	195,580	47.2%
Gene or Its Promoter	207,527	50.1%
50k of a Gene	295,096	71.2%
Repeated Elements	217,362	52.4%

Probe Category: The probes within the specified category
Probe No: Number of Probes
Probe %: percentage of all probes in the microarray

Table 3-5 (Supplementary) The properties of the selected hypermethylated DMRs and their primers designed used for pyrosequencying

DMR[a]	Chr.[b]	Bovine genomic sequence number	Pyrosequencing primers	Sequence	*Temp.*[c]	*CpG*[d]	*Length*[e]
1	1	155133370-155133821	Forward Reverse (5'- Biotinated) Sequencing	5'- TTAGTAATTTAGATGGGGAAGTTTAAT-3' 5'- TCTTACCAAAAAAATTTCCAAAATAACAC-3' 5'- AGATGGGGAAGTTTAATT-3'	50 ^{o}C	5	56
2	1	155133370-155133821	Forward Reverse (5'- Biotinated) Sequencing	5'- TGGAAATTTTTTTGGTAAGAATAGTTAAT-3' 5'- CTCCCCTTTAAAATAATTAAAACTTAAATC-3' 5'- AAGAATAGTTAATTTTATGTTTGT-3'	48 ^{o}C	4	53
3	3	119346133-119346235	Forward Reverse (5'- Biotinated) Sequencing	5'-TTTTTTGTAGGAAATGGGTTTGTT-3' 5'- TTCCCCCTATTCTATTTCTCCTAATAA-3' 5'- AGGAAATGGGTTTGTTT-3'	49 ^{o}C	5	61
4	4	118285867-118285998	Forward Reverse (5'- Biotinated) Sequencing	5'- TGGAGAGTTTATAAAGTTAGGGTTAGA-3' 5'- ATAATTCTTAAACTCCAAAAACTAATTCAC-3' 5'- GAGTAGGTTTTTTTTATTTTTAGAT-3'	49 ^{o}C	2	22
5	14	27808884-27809309	Forward Reverse (5'- Biotinated) Sequencing	5'- GTAAGGTAGTAGGGGTTTTAGTTGAT-3' 5'- CTCTACTCCCTCCATCTACAAACCAAATAC-3' 5'- GTTTTAGTTGATGTTAAAAGTT-3'	54 ^{o}C	7	57
6	19	32804128-32804528	Forward Reverse (5'- Biotinated) Sequencing	5'- GTTTGGAGAGTTAGAAGTAAAAGTTAGAT-3' 5'- ACCTCAAAACCTTCAAACAAATAAA-3' 5'- TAGGTAGGGGTGGTT-3'	50 ^{o}C	4	56
7	21	62757397-62757852	Forward Reverse (5'- Biotinated) Sequencing	5'- GTTTGGAGAGTTAGAAGTAAAAGTTAGAT-3' 5'- ACCTCAAAACCTTCAAACAAATAAA-3' 5'- TAGGTAGGGGTGGTT-3'	50 ^{o}C	4	56

a) DMR: Differentially methylated region; **b): Chr.:** Chromosome number**; c): *Temp. (^{o}C):*** *annealing temperature**; d) CpG:*** *The number of CpG in the pyrosequenced fragment;* ***e) Length:*** *The length of pyrosequenced fragment.*

4. Genome-wide analysis of sperm DNA methylation from monozygotic twin bulls

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Authors' contributions

HASS worked on bovine sperm intact gDNA extraction development, performed semen and sperm analysis, MZ twin sperm DNA methylation experiments. EF performed bioinformatic analyses. CV and PB provided the MZ information and samples. JB provided

the CASA analysis system. CR designed and supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

4.1. Résumé

Les jumeaux monozygotiques (MZ) sont d'un grand intérêt pour élucider les contributions des facteurs environnementaux pré et post-natales sur l'épigénétique de l'expression des caractères et des maladies complexes. Les tests de descendance ont récemment révélé que les taureaux jumeaux MZ n'obtiennent pas nécessairement des estimations de la valeur génétique identiques (c.-à-d. valeurs d'élevage). Par conséquent, pour expliquer les différences de productivité des descendants de taureaux jumeaux MZ en dépit de leurs origines génétiques identiques, nous avons formulé l'hypothèse que les épigénomes spermatiques paternels varient entre taureaux jumeaux MZ. Dans cette étude, les caractéristiques de la semence et le méthylome global de l'ADN du spermatozoïde ont été étudiés pour quatre paires de taureaux jumeaux MZ. Quelques paires de jumeaux MZ avaient une qualité divergente de leur semence au niveau de la morphologie des spermatozoïdes, leur motilité et leur viabilité. L'étude comparative pangénomique du méthylome de l'ADN a été effectuée par une méthode favorisant un enrichissement de régions sensibles à la méthylation suivi d'une identification par puces à ADN. Les résultats démontrent qu'entre 2 et 10% des sondes présentent sur la puce (400 K) et 580 loci étaient différentiellement méthylées entre les paires de jumeaux MZ. En outre, les analyses ont indiqué un enrichissement significatif pour les QTLs (de l'anglais Quantitative Trait Loci) associé à la fertilité ($p = 0,033$). En conclusion, les différences observées dans l'épigénome spermatique peuvent contribuer à des performances divergentes incongrues des filles engendrées par des taureaux jumeaux MZ.

Mots clés : Jumeaux monozygotiques, Méthylation de l'ADN pangénomique, Taureau, Spermatozoïde, Sperm 45 DNA extraction, QTL.

4.2. Abstract

Monozygotic (MZ) twins are of great interest to elucidate contributions of pre- and post-natal environmental factors on epigenetics in expression of complex traits and diseases. Progeny testing recently revealed that MZ twin bulls do not necessarily lead to identical genetic merit estimates (*i.e.* breeding values). Therefore, to explain differences in offspring productivity of MZ twin bulls despite their identical genetic backgrounds, we hypothesized that paternal sperm epigenomes vary between MZ twin bulls. In this study, semen characteristics and global sperm DNA methylome were profiled for four pairs of MZ twin bulls. Some MZ twin pairs had divergent semen quality (sperm morphology, motility and viability). Comparative genome-wide DNA methylome surveys were performed using methyl-sensitive enrichment and microarray identification. Between 2 and 10% of all probes (400 K) were differentially methylated between MZ twin pairs. In addition, there were 580 loci differentially methylated across all pairs of MZ twins. Furthermore, enrichment analysis indicated a significant enrichment for fertility associated Quantitative Trait Loci (QTL) (p-value=0.033). In conclusion, differences in the sperm epigenome may contribute to incongruous diverging performances of daughters sired by bulls that are MZ twins.

Key words: Monozygote twins, Genome-wide DNA methylation, Bull, Sperm, Sperm DNA extraction, QTL.

4.3. Background

When studying expression of complex traits and diseases, experimental designs involving genetic copies, *e.g.* produced by somatic cell nuclear transfer cloning (SCNT) or embryo splitting to produce monozygotic (MZ) twins, are of great interest to delineate contributions of the inherent genomic sequence versus epigenetic effects induced by environmental conditions in expression of complex traits and diseases (Bell and Spector, 2011; Fraga *et al*., 2005; Kaminsky *et al*., 2009; Tan *et al*., 2015). In the cattle industry, the desire to disseminate exceptional genetic merit for breed improvement has made embryo splitting a very common assisted reproduction technology (ART) to duplicate elite bulls (Shojaei Saadi and Robert, 2015). However, based on progeny testing, it was recently noted that production of MZ twin bulls by embryo splitting did not necessarily lead to generation of MZ twin bulls with identical genetic merit estimates (*i.e.* breeding values). We have data (unpublished) that some daughters sired by MZ twin bulls had incongruous diverging performances. To explain such variance in offspring productivity of MZ twin bulls despite their identical genetic backgrounds, we hypothesized that the paternal (sperm) epigenome varies between MZ twin bulls. Therefore, in this study, global sperm DNA methylome was profiled for four pairs of MZ twins.

4.4. Materials and Methods

4.4.1. Sample collection and sperm characteristics

Four pairs of MZ twin bulls were generated by embryo splitting (morula stage), transferred to separate recipient cows, and rearing in different environments. The MZ twin embryos were produced *in vivo* by ovarian stimulation of estrous synchronized cow followed by artificial insemination with sexed semen. The embryos were collected by uterine flush 5 days post-insemination and splitting was done on site. Each hemi-section was transferred to a synchronized recipient cow. Each twin pair recipients were kept in the same facilities and each pairs were kept by contracted dairy producers for gestation, calving and rearing until the calves were 8 months of age after which they were transferred to the AI center. Management and rearing were done under standard recommendations from local practitioners. The MZ twin pairs were born 0 to 3 d apart. After puberty, these bulls were subjected to progeny testing. Semen was collected and cryopreserved and used for analyses. Age (in months) at semen collection of MZ twin pairs were: Pair #1: 28 and 41; Pair #2: 51 and 59; Pair #3: 13 and 13; Pair #4: 14 and 15 mo old, respectively. Semen characteristics (viability, motility and morphology) were determined using standard methodologies, as described (Shojaei Saadi *et al*., 2013; Campagna *et al*., 2009). Briefly, motility parameters were assessed by Computer Assisted Sperm Analysis (CASA; Hamilton Thorne, Beverly, MA, USA) and sperm morphology was determined with phase contrast microscopy. Statistical analyses were conducted using multivariate ANOVA within the R environment.

4.4.2. Genome-wide DNA methylation analysis

High-quality intact gDNA was extracted from these semen samples using a protocol developed in-house. A single straw containing ~5 x 107 sperm was diluted with 1 ml phosphate-buffered saline (pH 7·4, containing 2% SDS) in a 1·5 ml microcentrifuge tube and then vortexed (5 s) followed by centrifugation at 15,500 x g for 5 min to pellet the sperm. The supernatant was discarded and 450 µL lysis buffer (10 mM Tris pH 8·0, 10mM EDTA pH 8·0, 1.5% SDS, 100 mM NaCl) was added, followed by addition of 30 µL 1M dithiothreitol (DTT), and 20 µL Proteinase K solution (20 mg/ml). The sperm solution was placed in an incubator at 56 °C (Inkubator 1000, Heidolph, Schwabach, Germany) with an integrated orbital shaker (155 rpm, Unimax 1010, Heidolph) for 12-16 h. Following incubation, 160 µL of saturated NaCl solution were added and the mixture was vortexed 3X for 5 s. The solution was then centrifuged for 10 min at 15,500 x g. The supernatant from the liquid pellet was carefully removed and transferred to a 2 ml microtube. Then, 1 ml of chilled (4˚C) ethanol was added and gently mixed by hand shaking (15-20 times) to allow the extracted gDNA to precipitate and collected by centrifugation at maximum speed (17,200 xg) for 15 min at 4˚C. The supernatant was discarded and the gDNA pellet was washed with 500 µL 70% ethanol and centrifuged at maximum speed (17,200 xg) for 5 min at 4˚C. After discarding the supernatant, a final wash was done by adding 200 µL 50% ethanol and centrifuging at maximum speed (17,200 xg) for 5 min at 4˚C. The purified extracted bovine sperm gDNA was re-suspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8·0) or nuclease-free water. Quality and purity of the extracted gDNA

were tested on Nanodrop ND100 (Thermo Scientific, Wilmington, DE, USA) and integrity of gDNA was assessed by running samples on 0.5% agarose gel (45 volts for 2 h).

The gDNA samples were processed through our bovine genome-wide DNA methylation analysis platform (Shojaei Saadi *et al*., 2014). Briefly, genomic DNA was fragmented by restriction digestion and a linker was ligated to the cohesive ends. Methylated fragments were targeted using methyl-sensitive restriction enzymes (MSREs) and protected fragments were enriched by two rounds of successive PCR amplifications. The two samples corresponding to each pair of MZ twins were labeled either with Cy3 or Cy5, and hybridized on a custom-designed microarray slide containing 400K probes tiling across the entire bovine genome. Data handling used a built-in pipeline to perform pre-processing (data quality control and normalization) and analysis steps (statistical analysis and data sorting), as described (Shojaei Saadi *et al*., 2014). Array information and raw data have been deposited in NCBI's Gene Expression Omnibus (GSE74200).

4.5. Results and discussion

4.5.1. Semen end points

Some pairs of MZ twins produced semen with similar characteristics, whereas other pairs had divergent semen quality. However, regarding extent of divergence within and between MZ twin pairs, there was no association between studied parameters and genetic relationship (Figure 3-1). For instance, statistical analysis using multivariate ANOVA failed to detect greater similarity in-between sperm from twin bulls compared to sperm from unrelated bulls, neither for any single characteristic nor for the set of principal components of variation identified through PCA analysis. Despite being genetically identical, MZ twin bulls had different sperm characteristics. Such divergence in sperm quality between MZ twin bulls has been reported (Lessard *et al*., 2003; Braun *et al*., 1990; Galli *et al*., 1997). Since sperm morphology has a substantial heritable component (Storgaard *et al*., 2006), it has been proposed that the observed differences might be due to environmental or stochastic factors (Ollikainen *et al*., 2010; Galli *et al*., 1997; Czyz *et al*., 2012). We therefore hypothesized that such differences could also be detected at the level of sperm DNA methylation.

4.5.2. Sperm DNA methylation profiles

A genome-wide profile of changes in sperm DNA methylation was performed for each pair of MZ twin bulls. Under the premise that DNA methylation may act through a regional increase or decrease in methylation density (rather than impose its effect through a single methylation site), genomic blocks of 5 Mb were considered by averaging the intensity

ratios of probes within the genomic window. In doing so, data analysis revealed that patterns of identified differentially methylated regions (DMRs) in sperm were not conserved between pairs of MZ twins (Figure 3-2). However, assessing data from an individual-probe perspective, between 8,861 and 44,334 probes (corresponding to approximately 2 and 10% of all probes on the microarray, respectively) were differentially methylated between pairs of MZ twins (Figure 3-3A). Each probe covered an average of 525 bp (Shojaei Saadi *et al*., 2014). Among four MZ twin pairs in this study, only one (pair#1) had marked age difference (13 mo). Assuming the potential association between the age difference between MZ twin brothers and the divergence in their sperm DNA methylation profile, data analysis revealed no significant associations between degree of divergence in global sperm DNA methylation pattern and age of MZ twin bulls (at semen collection); therefore, older MZ twin bulls did not have more divergent global sperm DNA methylation pattern than younger MZ twin bulls. In addition, there was no significant association between extent of divergence in DNA methylation and sperm characteristics, perhaps due (at least in part) to limited sample size.

Furthermore, category enrichment analysis revealed that probes divergent in sperm DNA methylation among the MZ twin pairs was not associated with any specific types of genomic elements (*i.e.* intronic, exonic or promoter regions; Figure 3-3B). Moreover, very few probes within long and high density CpG islands were differentially methylated, indicating that this type of region might be more resilient to disturbances in methylation

patterns (Figure 3-3B). Approximately 40% of the identified top 1% of probes with the highest coefficients of variation (4,209 probes) were in CpG island shores and shelves (Figure 3-4 (Supplementary)) regions of bovine genome. This observation suggests that impacts of individual-specific environmental and stochastic factors account for more variations in DNA methylation within CpG-poor regions (*i.e.* CpG islands shore and shelf), similar to reports in human MZ twins (Czyz *et al*., 2012; van Dongen *et al*., 2014) .

Using a stringent selection (absolute symmetrical fold-change > 2, Benjamini-Hochberg adjusted p-value < 0.01), 580 probes were commonly differentially methylated across all pairs of MZ twin bulls (Figures 3-2 and 3-3A). This overlap was much higher than what would be expected from random sampling (between 2 and 8 probes at a 99% confidence interval). Matching the data randomly to remove the genetic link between the bulls did not allow us to identify more differences. These results support the presence of differentially methylated regions in the sperm DNA of twin bulls but the limited sample size does not allow to determine if the differences are caused by environmental exposures or random effects.

With the large numbers of reported quantitative trait loci (QTLs) spanning large genomic intervals, all probes on the array overlapped with at least one QTL. Considering that these common differentially methylated fragments could be associated with genomic "hotspots" responsive to environmental conditions, we performed an enrichment analysis for QTLs associated with these bovine genomic regions. Therefore, the

percentage of probes associated with each QTL within the subset of 580 common DMRs was compared to that self-same percentage for all probes of the array using Fisher's exact test. Data analysis revealed that only QTLs associated with the Fertility Index had significant enrichment (4.31 times more regions than expected, p-value=0.033). This is of interest considering dairy cattle fertility has been declining (Chagas *et al*.) and that fetal programing has been shown to influence offspring fertility (Evans *et al*., 2012). Fertility could be a trait involving an increased epigenetic control compared to other traits.

4.6. Conclusions

There were differences between pairs of MZ twin bulls in sperm characteristics and in genome-wide DNA methylation profiles. Remarkably, the extent of these differences between MZ twin pairs was just as variable as the extent of differences between non-related individuals, with no obvious association between global sperm DNA methylation patterns and semen parameters or age at semen collection. In this study, the use of MZ twin bulls allowed us to match bulls for genetics, age and sex. The origin of spermatic epigenetic discordance between MZ twin bulls remains unknown. However, matching the data randomly did not lead to the identification of more differentially methylated regions arguing that spermatic DNA methylation is not highly influenced by sequence. Although twins share the same genetic background, different pre-natal cues from developing in a different uterine environment or other post-natal effects from environmental exposures and conditions may induce the observed epigenetic differences. It is known that fetal programming can carry effects in adulthood (Brenseke *et al*., 2013; Hogg *et al*., 2011; Gluckman *et al*., 2008) and post-natal conditions such as nutrition can affect puberty and semen quality (Loke *et al*., 2013; Dance *et al*.; Gaskins *et al*., 2012; Vujkovic *et al*., 2009). Regardless, many key questions remain regarding true potential of these observed differences to transfer an impact to the offspring. To do so, these marks would need to persist following genomic reprogramming occurring post-fertilization (Daxinger and Whitelaw, 2012; Heard and Martienssen, 2014; Wei *et al*., 2015). Furthermore, it is presently unknown if differential methylation on a few localized CpGs is sufficient to

influence gene expression. Although these observations need to be substantiated with a larger sample size and longitudinal analysis, the present study represented a basis for highlighting a potential contribution of the epigenome to the incongruous, diverging performances of daughters sired by MZ twin bulls.

4.7. Competing interests

The authors declare they have no competing interest.

4.8. Acknowledgements

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4.10. Figures

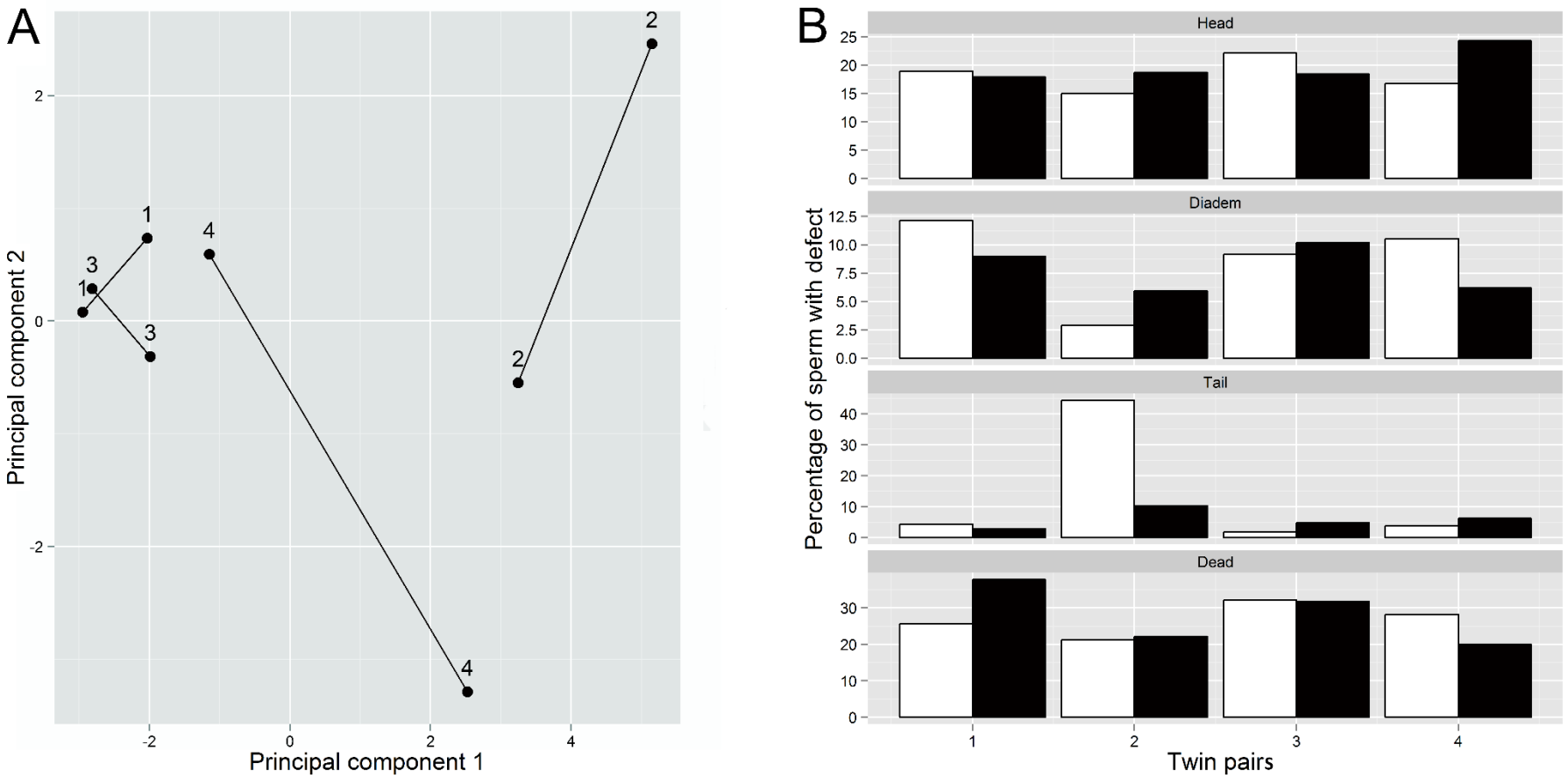

Figure 4-1 Sperm samples phenotypic characteristics. Analysis of sperm morphology and viability of MZ twin bulls.

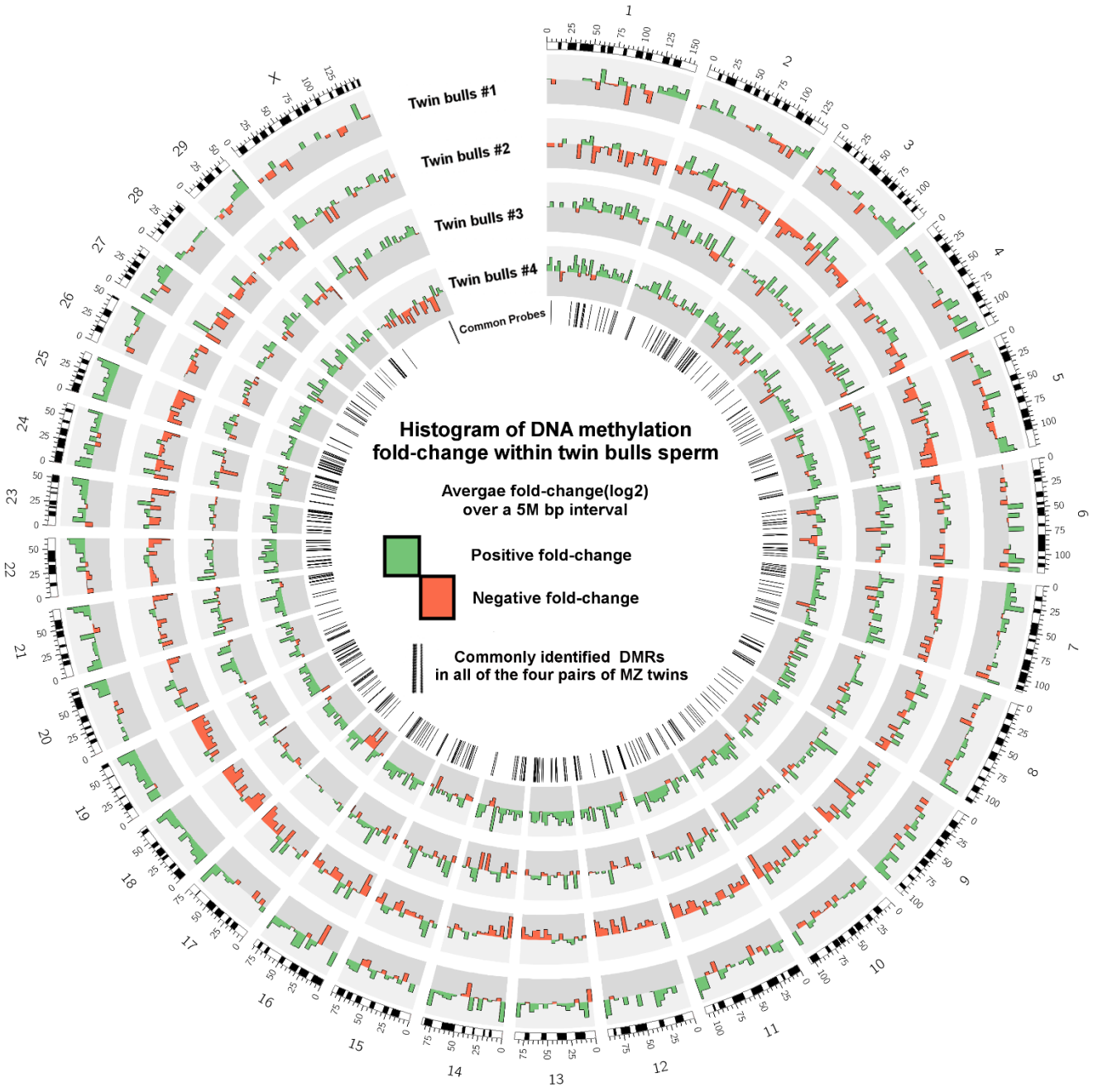
Twin bulls #1
Twin bulls #2
Twin bulls #3
Twin bulls #4
Common Probes
Histogram of DNA methylation
fold-change within twin bulls sperm
Avergae fold-change(log2)
over a 5M bp interval
Positive fold-change
Negative fold-change
Commonly identified DMRs
in all of the four pairs of MZ twins

Figure 4-2 Genome-wide perspective of sperm DNA methylation profiles of MZ twin bull pairs. Circos plot illustrating comparative distribution of DNA methylation on the genome of each pair of monozygotic (MZ) twin bulls. Each bar represents the average mean fold-changes of all probes in a 5 M bp window. Positive fold-changes (green) represent hypermethylation in one MZ twin, whereas negative fold-changes (orange) represent hypermethylation in the corresponding MZ twin brother. The innermost track (common probes) illustrates the 580 probes commonly differentially methylated in all four pairs of MZ twins. Each probe on the microarray targeted a single genomic fragment (average size of 525 bp).

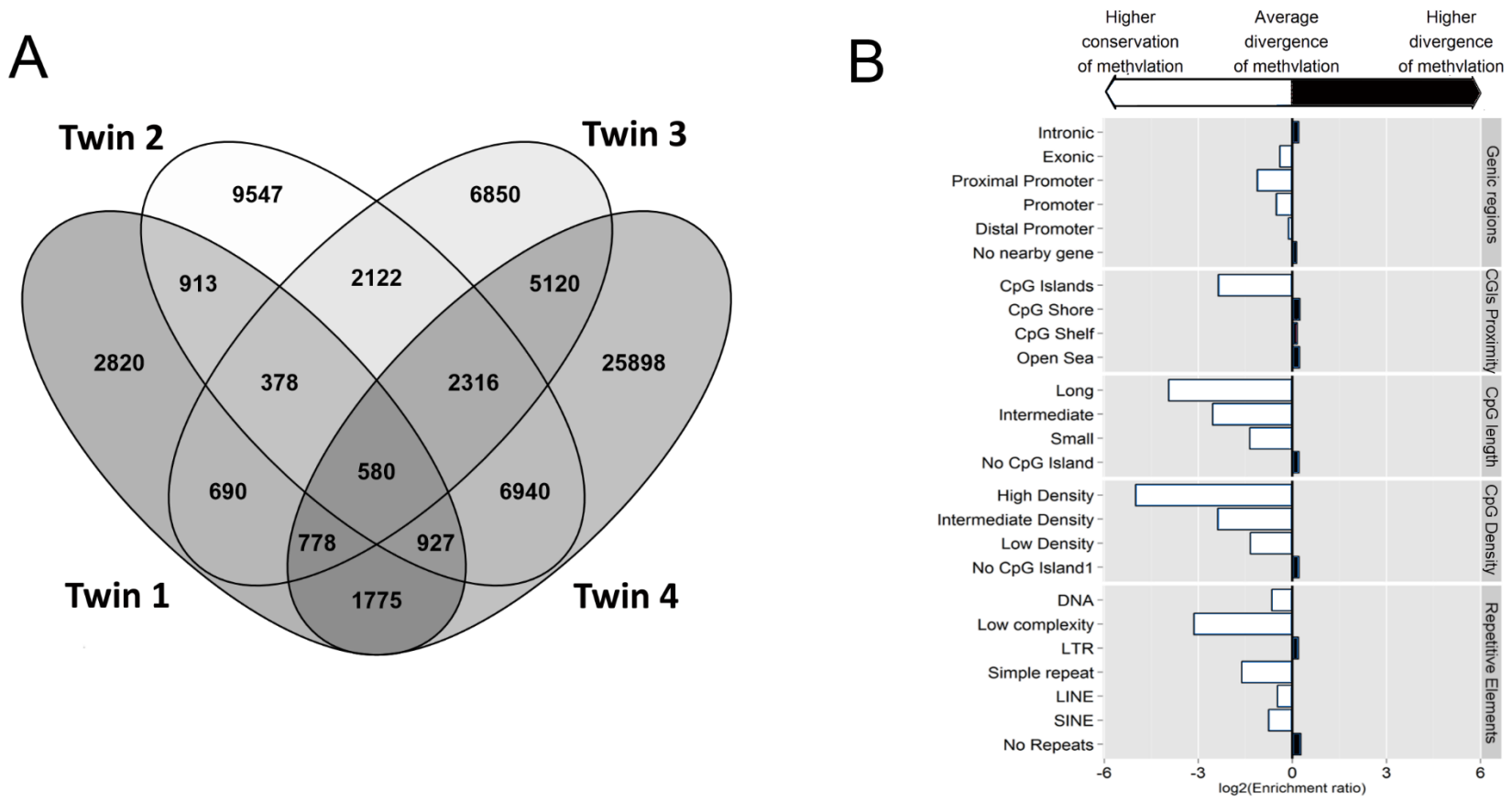

Figure 4-3 Distribution of differentially methylated probes in the semen of each pair of twins. (A) Venn diagram of identified differentially methylated probes between sperm of monozygotic twin bulls. **(B)** Comparative enrichment analysis of identified common probes between twin bulls' sperm. More conservation was observed in long, high-density CpG islands, as well as in low-complexity repetitive elements.

4.11. Supplementary figures

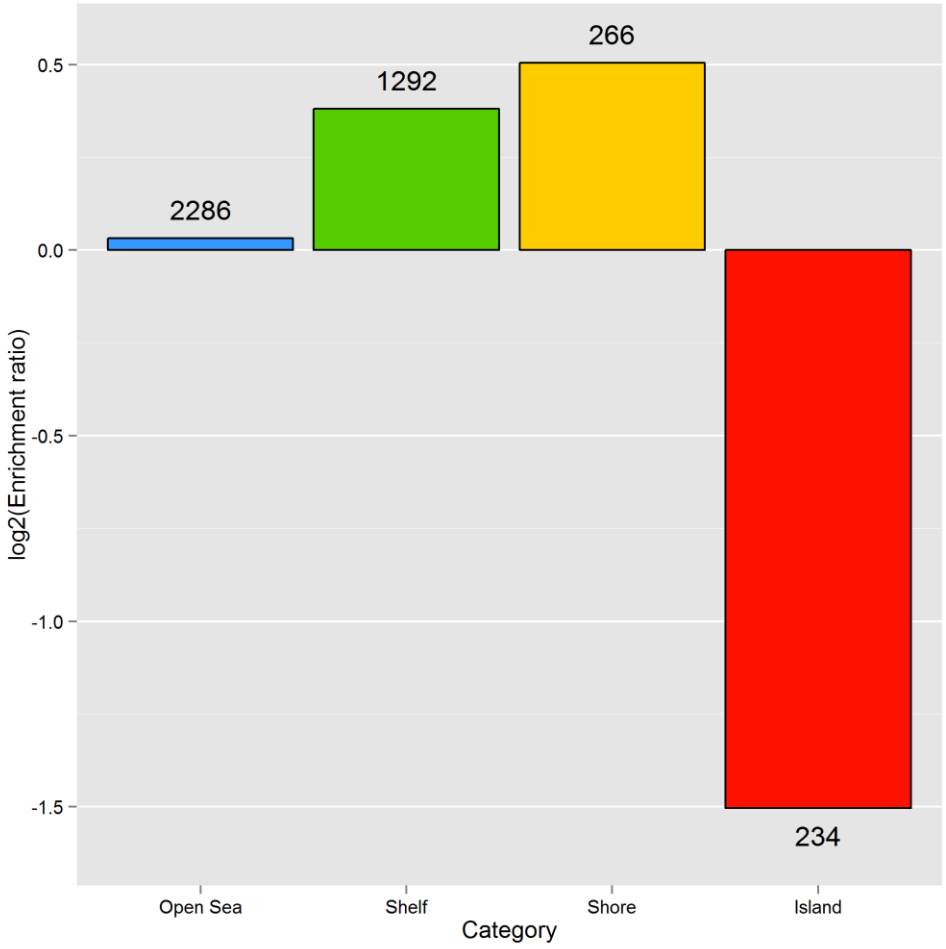

Figure 4-4 (supplementary) The proportion of the detected top 1% of the DMRs in MZ twin bulls sperm based on their distance from CpG islands. As can be seen more changes occurred in CpG shore and shelf regions of the bovine genome.

5. Responses of bovine early embryos to S-adenosyl methionine supplementation in culture

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Authors' contributions

HASS, MAS, and CR designed the study. HASS performed the IVF, phenotypic, transcriptome (RT-qPCR) and epigenetics (DNA methylation and pyrosequencing) experiments as well as data acquisition and analysis. DG performed the transcriptome microarray. EF performed bioinformatics analyses. LMBB and HASS performed the mitochondrial staining. CR supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

5.1. Résumé

Chez les embryons précoces, l'établissement des marques de méthylations sur l'ADN est un facteur clé pour la différenciation cellulaire et est un aspect important du développement. Le processus de méthylation nécessite la présence du S-adénosylméthionine (SAM) en tant que donneur de méthyle universel qui est généré par le métabolisme cellulaire monocarboné utilisant les folates alimentaires comme substrat. Nous avons évalué l'impact de soumettre les embryons précoces à une dose élevée de donneur universel de méthyle afin de déterminer s'il y aura un impact sur la méthylation de l'ADN, l'expression des gènes ainsi que le développement précoce.

Durant la production embryonnaire *in vitro* (PIV), les embryons bovins ont été traités avec une dose élevée de SAM à partir du stade de 8-cellules jusqu'au stade blastocyste. La supplémentation en SAM a augmenté de façon significative le taux d'embryons éclos de 20% à 80% sans toutefois affecter le taux de blastocyste. Au jour 7, un changement au niveau du ratio entre les sexes a été observé en faveur des mâles. De plus, la supplémentation en SAM a eu un impact sur la méthylation de l'ADN. Elle a causé une hyperméthylation pangénomique principalement dans des régions exoniques ainsi que dans les îlots CpG avec une densité intermédiaire et élevée en CpG indépendamment de leurs longueurs.

L'analyse comparative au niveau du transcriptome a révélé 247 ARNs codants et long non-codants différentiellement exprimés (DE). L'analyse d'enrichissessment en terme GO a

révélé une expression différentielle des gènes associés dans les réponses aux nutriments et les processus développementaux qui sont en accord avec notre traitement et ses effets sur l'embryon précoce. Toutefois, aucune correspondance n'a été trouvée entre les régions différentiellement méthylées (DMR) et des gènes DE suggérant que durant le développement embryonnaire précoce, les réponses cellulaires à un traitement SAM pourraient ne pas nécessiter des changements d'expression des gènes entraînés par méthylation de l'ADN et par conséquent, seraient modulés par d'autres mécanismes épigénétiques.

En conclusion, les embryons précoces bovins réagissent à des doses supraphysiologiques de donneur de méthyle universel (SAM). Par conséquent, il est prévu que la supplémentation à forte dose de substrats tels que l'acide folique pouvant entraîner une forte production de SAM pourrait avoir un impact important au cours du développement embryonnaire précoce.

Mots clés: S-Adenosyl méthionine (SAM), Épigenome, méthylation de l’ADN, transcriptome, embryon bovin précoce, Enrichisssement CpG, Ratio des sexes embryonnaire

5.2. Abstract

Aim: There is growing concern about the potential adverse effects of high dose folic acid (FA) supplementation before and during pregnancy. FA metabolism generates S-adenosyl methionine (SAM) which is an important cofactor of epigenetic programing. We sought to assess the impact of a large dose of SAM on early embryo development. **Materials & methods:** *In vitro* cultured bovine embryos were treated with SAM from the 8-cell stage to the blastocyst stage. In addition to the phenotype, the genome-wide epigenetic and transcription profiles were analyzed. **Results:** Treatment significantly improved embryo hatching and caused a shift in sex ratio in favor of males. SAM caused genome-wide hypermethylation mainly in exonic regions and in CpG islands. Although differentially expressed genes were associated with response to nutrients and developmental processes, no correspondence was found with the differentially methylated regions, suggesting that cellular responses to SAM treatment during early embryo development may not require DNA methylation-driven changes. **Conclusion:** Since bovine embryos were not indifferent to SAM, effects of large-dose folic acid supplements on early embryonic development in humans cannot be ruled out.

Keywords: S-adenosyl methionine (SAM), epigenome, DNA methylation, transcriptome, bovine early embryo, CpG enrichment, sex ratio

5.3. Introduction

The water-soluble vitamin known as folate (B9, folic acid) is an essential component of the human diet. Its principal source is green vegetables and fruits. Folate deficiency or low folate status is quite prevalent even in well-nourished populations and can become clinically significant when increased folate requirements and decreased availability occur simultaneously. It is well recognized that the requirement increases during pregnancy by 5–10 fold, due to rapid cell replication and growth of fetal, placental and maternal tissues. Any serious deficiency carries a high risk of adverse outcomes such as neural tube defects (NTD) in the child (McNulty *et al*., 2012). Monitoring of pregnant women for adequate dietary folate intake is therefore required in most health-care jurisdictions (Antony, 2007). Fortification of grain products with folic acid is currently mandatory in more than 70 countries and has been so in North America since 1998 (Pickell *et al*., 2011; Tamura and Picciano, 2006; Blom *et al*., 2006). Although the terms folate and folic acid (FA) are often used interchangeably, folate refers to the vitamin that occurs naturally in foods while FA usually refers to the synthetic compound, which is also the oxidized, more stable and more bioavailable form used widely for food fortification and in supplements, due to its low production cost (Bailey *et al*., 2010; Wright *et al*., 2007; Blom and Smulders, 2011; McNulty *et al*., 2012). Women are strongly advised to take FA supplement during pregnancy (Chanarin *et al*., 1959; Dunlap *et al*., 2011; Tamura and Picciano, 2006; Smithells *et al*., 1976) and even several months before planned pregnancy to ensure

needs are meet since the onset of NTD in humans begins at 25–29 days after conception (Medicine, 2015).

Despite the importance of prenatal (Scholl and Johnson, 2000) and postnatal (Kotsopoulos *et al*., 2008; Hervouet *et al*., 2009) folate intake, growing concerns about high folate concentrations resulting from grain fortification combined with FA supplementation have been expressed (Barua *et al*., 2014; Ulrich and Potter, 2006; O'Neill *et al*., 2014; Aarabi *et al*., 2015; Christensen *et al*., 2015; Allen, 2012). Recent studies suggest that such concentrations might have adverse effects on embryonic and fetal development (Mikael *et al*., 2013; Pickell *et al*., 2011). Alterations in methylation patterns and expression of several genes in the cerebrum of the offspring appear to affect post-natal brain development and function and ultimately the behavior of the individual (Barua *et al*., 2014; Sable *et al*., 2011; Sable *et al*., 2014; Junaid *et al*., 2011). High levels of FA supplementation have also been shown to increase the risk of asthma in infants and children, type-2 diabetes, metabolic dysfunction, and autism (Håberg *et al*., 2009; Whitrow *et al*., 2009; Yajnik and Deshmukh, 2008; Beard *et al*., 2011; Yang *et al*., 2015; Keating *et al*., 2015).

The only known function of folate in the body is as a co-enzyme in the transfer of one-carbon units in one-carbon metabolism, which utilizes folate, methionine, betaine, vitamin B12 and other substrates or cofactors (Choi and Mason, 2000; Steegers-Theunissen *et al*., 2013). A key output of one-carbon metabolism is S-adenosyl

methionine (SAM), which provides the methyl group for almost all methylation reactions, including epigenetic modifications (Smith Desirée *et al*., 2013; Lu and Mato, 2012). SAM is the most widely used source of methyl groups in living organisms and is the second most common enzyme substrate after ATP (Loenen, 2006; Fontecave *et al*., 2004; Klimasauskas *et al*., 2007). Synthesized from dietary L-methionine and ATP by the enzyme methionine adenosyl transferase (Anstee and Day), this universal methyl donor has a wide variety of acceptors (Kaelin Jr and McKnight, 2013; Lu and Mato, 2012; Fontecave *et al*., 2004; Klimasauskas *et al*., 2007). In mammals, more than 95 % of it is used in methyltransferase reactions that modify DNA, RNA, histones and other proteins and play a major role in ensuring the fidelity of replication, transcription and translation as well as in mismatch repair, chromatin modeling, epigenetic modifications and imprinting (Loenen, 2006; Brosnan and Brosnan, 2006). In early embryos, establishment of methylation marks on DNA (Smith *et al*., 2012; Guo *et al*., 2014; Smith and Meissner, 2013), histones (Vastenhouw and Schier, 2012; Serrano *et al*., 2013; Zhou and Dean, 2015; Bošković and Torres-Padilla, 2015) and RNA (Wang *et al*., 2014; Lin and Gregory, 2014; Yue *et al*., 2015; Geula *et al*., 2015; Batista *et al*.) is a key factor in cellular differentiation and therefore an important aspect of development. As the key factor in any methylation process, SAM is essential for embryonic development (Nishimura *et al*., 2002; Zhang *et al*., 2015; Kudo *et al*.) and its limitation appears to be involved in cell cycle arrest in somatic cells (Lin *et al*., 2014b) and stem cells (Shiraki *et al*., 2014) and in apoptosis leading

to epigenetic stability (Lin *et al*., 2014a). Furthermore, it is essential for the maintenance of pluripotent stem cells (Shyh-Chang *et al*., 2013; Shiraki *et al*., 2014). Forcing a drop in the intracellular concentration by methionine deprivation causes a reduction of Nanog expression in human induced pluripotent stem cells or embryonic stem cells (iPSC/ESCs), follow by potentiated differentiation into all three germ layers (Shiraki *et al*., 2014). In addition, SAM deficiency or depletion due to folate deficiency (appearing as low serum folate levels and high plasma homocysteine levels) or to a polymorphism in the methylene tetrahydrofolate reductase (MTHFR) is associated with a wide range of diseases in humans including different types of cancer (Wang *et al*., 2012; Clarke *et al*., 2012; de Batlle *et al*., 2015), chronic liver disease (Anstee and Day), pathogenic brain functions (Bottiglieri, 2013; Morris *et al*., 2003; Scala *et al*., 2006; Wang *et al*., 2001), fertility disorders (Lambrot *et al*., 2013; Gong *et al*., 2015; Ebisch *et al*., 2007) and embryo developmental defects (primarily NTD) (Mrc Vitamin Study Research, 1991).

This study was conducted to investigate whether high dose supplementation of SAM to the culture medium affects subsequent early development of cattle embryos. We hypothesized that it would cause aberrant DNA methylation, which would in turn affect gene expression, cell differentiation and hence early developmental rate. If embryos did not express any reaction to the treatment during the pre-implantation stages, then the reported adverse effects of high FA supplementation would need to be re-examined with focus on later stages of embryo development. The universal methyl donor was

supplemented directly to the culture medium for uptake by the embryonic cells rather than through maternal folate or methionine dietary supplementation to avoid the *in vivo* regulation of bio-available SAM known to be acting in the digestive system and liver ((NRC), 2001).

5.4. Materials and Methods:

5.4.1. Ethics statement

All animals used in this study were handled according to the guidelines provided by the Canadian Council on Animal Care. The local abattoir that provided all tissues and Alliance Boviteq, who provided semen samples for *in vitro* fertilization [IVF] procedures did so in strict compliance with these guidelines. No animals were handled on university premises in the course of this study.

5.4.2. *In vitro* embryo production

Bovine blastocyst production in synthetic oviduct fluid (SOF) was performed as described previously (Shojaei Saadi *et al*., 2014) with some modifications. Dairy cow ovaries were collected from a commercial slaughterhouse and were transported to the laboratory in saline (0.9 % NaCl) containing 1 % Antibiotic-Antimycotic 100 X agent (Gibco 15,240, Invitrogen, Burlington, ON, CAN). Cumulus oocyte complexes (COCs) were aspirated from 2–6 mm follicles within 2 h of reception. Only COCs with at least five layers of cumulus without fragmented cytoplasm, pyknotic cumulus, pale nuclei and abnormal morphology were selected for maturation. The collected COCs were washed three times in HEPES-buffered Tyrode's medium (TLH) containing 1.5 % bovine serum albumin (Fraction V, Sigma-Aldrich, Oakville, Canada), 200 µmol/mL pyruvate and 50 µg/mL of gentamycin. Pools of 10 COCs were placed in 50 µL droplets of maturation medium comprising TCM199 (Gibco 11150–059; Invitrogen, Burlington, ON, CAN), 0.6 % bovine serum albumin (Sigma fraction V), 200 µmol/mL pyruvate, 50 µg/mL of gentamycin, 0.1 µg/mL

of follicle stimulating hormone (FSH, Gonal-f, Serono Canada Inc., Mississauga, QC, CAN) and 1 µg/mL of estradiol (E2), covered with filtered mineral oil and incubated for 24 h at 38.5 °C in a humidified 5 % CO2/20 % O2 atmosphere. Matured COCs were washed three times in TLH, transferred (n = 5) to 50 µL drops of fertilization medium comprising modified Tyrode's lactate medium supplemented with 0.6 % bovine serum albumin (Sigma fraction V), 200 µmol/mL pyruvate and 50 µg/mL gentamycin, and covered with filtered mineral oil. Then 2 µL of a solution containing 1 mmol/L hypotaurine, 2 mmol/L penicillamine and 250 mmol/L epinephrine were added to the COC-containing fertilization droplets. The COCs were inseminated *in vitro* with 25 x 106 sperm cells/mL. Briefly, cryo-preserved Holstein bull semen obtained from Alliance Boviteq Inc. (Saint-Hyacinthe, QC, Canada) was thawed for 30 s at 37 °C in a water bath and washed for 30 min RT by centrifuging at 700 × g through a discontinuous Percoll gradient (45–90 %). After sperm motility assessment and adjustment to the appropriate concentration, the sperm cells were added to the COC-containing fertilization drops and incubated for 15–18 h at 38.5 °C in a humidified 5 % CO2/20 % O2 atmosphere.

Zygotes and unfertilized COCs were denuded mechanically by repeated pipetting and washed three times in TLH to ensure complete removal of cumulus cells and sperm. For standard culture conditions, groups of 10 embryos were placed in 10 µL droplets of modified SOF under filtered mineral oil. SOF media were used sequentially as follows: the embryos were placed first in SOF#1 (6 mM lactate, 0.2 mM glucose) and the culture dishes

were incubated at 38.5 °C in a humidified 6.5 % CO2/5 % O2 atmosphere. Embryos were then transferred to 10 µL droplets of SOF#2 (1 mM lactate, 0.5 mM glucose) for 96 h post-fertilization (hpf) and finally to 10 µL droplets of SOF#3 (1 mM lactate, 2.5 mM glucose) for an additional 144 hpf.

5.4.3. S-Adenosyl Methionine treatment of the bovine early embryos

The 10 µmol/L solution of S-(5'-adenosyl)-L-methionine chloride dihydrochloride (product # A7007, Sigma-Aldrich, Oakville, Canada) was prepared freshly in SOF medium for each IVC trial, divided immediately into aliquots of 100 µL and kept frozen at -80 °C until use. For controls, SOF medium without SAM was prepared freshly and frozen in aliquots of 100 µL at -80 °C until use. The treatment was initiated 72 h after *in vitro* fertilization (8-cell stage). Embryos were collected and divided randomly and equally among the SAM-treated and control groups. The SAM treatment was carried out by adding 2 µL of SAM aliquot thawed at 38.5 °C to each 8 µL SOF droplet to obtain a SAM concentration of 2 µmol/L (Ikeda *et al*., 2012b). Control embryos were treated in the same manner with 2 µL of thawed aliquot of SOF. Since SAM is highly unstable (Lu and Mato, 2012; Mato and Lu, 2007; Yokochi and Robertson, 2004), the dosage procedure was renewed four times per day (at 3 h intervals) until blastocyst collection, by transferring the embryo to a new SOF droplet (8 µL) and then adding 2 µL of SAM or SOF accordingly (Supplementary Figure S3). For each IVC trial, both groups were transferred and treated simultaneously. For the DNA methylome and transcriptome parallel study, only expanded-blastocyst-stage embryos

were collected, in four pools of ten (n = 40) per group. To minimize the impact of phenotypic divergence (different blastocyst sizes) on the results, only expanded blastocysts of similar size were collected. Due to technical challenges (McGraw *et al*., 2013; Shojaei Saadi *et al*., 2014), molecular (transcriptome and epigenome) analysis based on sexed embryos was not feasible. Four additional pools of 10 expanded blastocysts (n = 40 replicates) per group were collected for validation by quantitative polymerase chain reaction (qPCR). Additional expanded blastocysts were produced and collected for mitochondrial activity analysis and trophectoderm/inner cell mass differential staining. In addition, individual d7 expanded blastocysts were collected for pyro-sequencing to avoid the impact of subjectivity from embryo staging. Except for those used for staining purposes, all embryos collected for analysis were washed three times with RNAse-free phosphate-buffered saline (PBS) prior to snap-freezing and storage at –80 °C.

5.4.4. Sex ratio determination

Embryos (45 SAM-treated and 37 control) collected at the morula, expanded (day 7) and hatching blastocyst (day 8-9) stages were used for sex determination. A transcriptomics technique was applied to single blastocysts to avoid contamination with sperm cell gDNA which may remain attached to blastocyst since the fertilization. Total RNA from individual embryos was extracted and purified using a PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA, USA). The extracted RNA was reverse-transcribed immediately

using a q-Script Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with oligo-dT (20) and random primers according to the manufacturer's recommendations. Sexing was performed using a proprietary bovine-specific sexing assay developed at Alliance Boviteq inc. (St-Hyacinthe, QC, Canada). Briefly, a paralog gene carried on both sex chromosomes (X and Y) was targeted. The transcripts are similar but not identical, differing by a few single nucleotide polymorphisms (SNPs), which translate into a shift in the peak during the high-resolution melting curve analysis. The sexing fluorescent probe detects both transcripts, but only one peak for female and two peaks for male embryos.

5.4.5. Differential staining

Expanded blastocysts were transferred immediately to fresh SOF#3 culture drops and incubated for 1 h at 38.5 °C with 100 µg/mL RNase A (Qiagen Canada Inc., Mississauga, ON, Canada). They were then transferred quickly for 20 s to fresh medium containing 0.2 % Triton X-100 and 100 µg/mL propidium iodide (Sigma-Aldrich, Oakville, Canada) to render trophectorderm cells permeable to the stain, then washed three times in PBS with 2 % bovine serum albumin (BSA) and fixed with 4 % paraformaldehyde containing with 0.2 % Hoechst 33,342 dye (Sigma-Aldrich, Oakville, Canada) for 30 min to stain the blastomer nuclei. The fixed and stained embryos were then washed three times in 2 % PBS-BSA and mounted on a slide with a drop of Prolong Antifade Gold (Molecular Probes, Invitrogen Canada, Burlington, ON, Canada). The slides were examined using a LSM 740 confocal fluorescence microscope (Carl Zeiss Canada, Kirkland, QC, Canada) with the 40X

objective and the images were captured by Zeiss 2011 (Blue edition) software in CZI format and analyzed using ImageJ 1.48V software.

5.4.6. Evaluation of mitochondrial activity in expanded blastocysts

Mitochondria in expanded blastocysts were stained as described previously (Baldoceda-Baldeon *et al*., 2014). Briefly, blastocysts were placed in SOF droplets containing 300 nM of the active dye CMX-rosamine (Mitotracker Red, Molecular Probes, Eugene, OR, USA) as an active mitochondrial dye and incubated for 40 min at 38.5 °C in a humidified 5 % CO2 atmosphere, then in SOF containing 1 µg/mL Hoechst blue dye 33342 (Sigma-Aldrich, Oakville, Canada) for 10 min at room temperature to stain nuclei, followed by three washings in SOF and observation on a slide with a coverslip using a Nikon TE2000 confocal microscope (Nikon, Mississauga, ON, Canada) with a water-immersion objective at an optical magnification of 60 X. Mitochondrial activity was recorded as an epifluorescence image of CMX-rosamine dye in grey scale. All measurement parameters were considered and the mean fluorescence intensity of all samples within a group was calculated in arbitrary units (AU) by ImageJ IMAGE J software 1.48V. The stained blastocysts were also imaged using an LSM 740 confocal microscope (Carl Zeiss Canada, Kirkland, QC, Canada). Images were taken as orthogonal projections using the Lambda Mode scanning procedure at a resolution of 1,024 x 1,024 pixels with the same settings for all samples.

5.4.7. Genome-wide parallel DNA methylome and transcriptome analysis

5.4.7.1. Simultaneous DNA and total RNA extraction from expanded blastocysts

This was carried out using the AllPrep DNA/RNA Mini Kit (Qiagen Canada Inc., Mississauga, ON, Canada) according to the manufacture's instructions. Samples were eluted in 37 µL for extracted DNA and 30 µL for RNA. The quantity and quality of each pool of RNA were verified on a 2100 Bioanalyzer (Agilent Technologies, Mississauga, On, Canada). The extracted DNA and total RNA were snap frozen and kept at –80 °C until use.

5.4.7.2. Genome-wide DNA methylation analysis

In order to identify differentially methylated regions (DMRs) on a genomic scale, expanded blastocysts were processed using our genome-wide DNA methylation analysis platform (Shojaei Saadi *et al*., 2014). Briefly, DNA digested to *MseI -MseI* fragments by restriction endonuclease *MseI* (T/TAA) were ligated with specific *MseI* adaptors. Samples then underwent tiny fragment enrichment by ligation-mediated PCR (HELP) cocktail cleavage using sequential digestion by three methyl-sensitive restriction endonucleases: *HpaII* (C/CGG), *HinP1I* (GC/GC) and *Aci1I* (C/CGC) (Fermentas Thermo Fisher Scientific). Under these conditions, only *MseI -MseI* fragments that are methylated at the endonuclease recognition sites remain intact. After a quality control step to ensure cleavage, the samples underwent ligation-mediated PCR (LM-PCR) to amplify the methylated fragments. The adaptors were removed and the fragment amplicons were labeled with either Cy3 or Cy5 using the Universal Linkage System (ULS) labeling kit (Kreatech Biotechnology). The four pools (n = 40, 10 embryos/pool, biological replicates)

of expanded blastocysts were hybridized on a custom-designed Agilent SurePrint 2x400K array slide (Agilent Technologies) using a dye-swap design (technical replicates) for a total of eight arrays. After hybridization for 40 h, the slide was washed and scanned with the PowerScanner (Tecan) and analyzed with Array-Pro Analyzer 6.3 software (MediaCybernetics).

5.4.7.3. Transcriptome analysis

Transcriptome analysis was performed using a microarray as described previously (Robert *et al*., 2011). Briefly, RNA extracted from each sample (n = 10 embryos, four replicates per treatment) was amplified *in vitro* by T7 RNA transcription using the RiboAmp HS Plus RNA Amplification Kit (Molecular Devices) to produce anti-sense RNA (aRNA). The quantity of aRNA was measured using a NanoDrop ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA) and 2 µg of aRNA were labeled (Cy3 and Cy5) using the ULS Fluorescent Labelling Kit for Agilent arrays (Kreatech Diagnostics, Amsterdam, The Netherlands). Following purification using a Pico-Pure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA, USA), the labeling efficiency was evaluated using the Nano-Drop ND-1000. Four biological replicates (10 embryos each) were hybridized on EmbryoGENE bovine transcriptome 44k Agilent microarray slides (V2, Agilent Technologies) using a dye-swap design (technical replicates) for a total of eight arrays.

5.4.8. Analysis of microarray and bioinformatics data

Microarray data (EmbryoGENE (http://embryogene.ca) DNA Methylation Analysis (EDMA) transcriptome) were analyzed using the LIMMA package from Bioconductor. Loess intra-array normalization was applied first, followed by quantile inter-array scale normalization. Normalized data were then fitted to a linear model, and differential expression was tested using Bayesian statistics. DNA methylome data analysis was carried out using our comprehensive EDMA bioinformatic data analysis pipeline to identify alterations of DNA methylation in genomic regions under various enrichment outputs in parallel with transcription of the loci of interest (Shojaei Saadi *et al*., 2014). Analyses by QC check points, microarray (LIMMA), enrichment and concordance were performed and plotted. Differences in DNA methylation (using EDMA) were considered significant when the P value was < 0.05 and the absolute log2 change was ≥ 0.5 fold.

Transcriptomic data were analyzed as described previously (Robert *et al*., 2011) using the Flex Array version 1.6 (Genome Quebec, genomequebec.mcgill.ca/FlexArray). Genes were considered expressed differentially at a change > 0.5 fold with a P-value < 0.05. All analyses were performed using R version 2.12.1 and LIMMA package version 3.6.9. Array information and raw data were filed in the NCBI Gene Expression Omnibus for transcriptome (GSE75430) and DNA methylome (GSE74081) analysis. Pathway analysis was performed using DAVID and ingenuity pathway analysis (IPA; QIAGEN) respectively for the transcriptome and the DNA methylome.

5.4.9. Data validation

5.4.9.1. DNA methylome

Pyro-sequencing was performed to validate the identified DMRs of interest. Three biological replicates (single blastocysts) and four technical replicates (pyro-sequences) were considered for each candidate. Individual blastocysts were first bisulfite-converted using an EZ DNA direct methylation KIT (ZYMO Research) according to the manufacturer's instructions with some modifications. The digested sample (20 µL) was bisulfite-converted by adding 130 µL of CT conversion reagent (bisulfate) followed by 8 min at 98 °C then 8 h at 64 °C in a thermal cycler. The bisulfite-converted gDNA was eluted in 42 µL of pre-heated (50 °C) M-elution buffer and the loci of interest were then amplified by PCR. For all pyro-sequencing assays, PyroMark Assay Design software (Qiagen) was used to design three oligonucleotide primers (forward, reverse and sequencing), synthesized by Integrated DNA technologies (Coralville). All reverse primers were biotinylated at the 5' terminus and purified by HPLC. PCR amplification was carried out using ZymoTaq™ DNA polymerase (ZYMO Research) with minor modifications. The PCR reaction was performed in 50 µL containing 0.2 µmol/L of each forward and reverse 5'-biotinylated primer, 2 U of ZymoTaq™ DNA Polymerase, 1X reaction buffer, 0.25 mM dNTPs, 2 mM MgCl2, 5 µL of bisulfite-convertrd DNA and 16.1 µL of nuclease-free water. The following three-step program was used: 95 °C (10 min) for 1 cycle, 95 °C (30 sec), various annealing/acquisition temperatures of interest (Supplementary Table S2) (30 sec) and 72 °C (30 sec) for 40 cycles, and finally 72 °C (7 min). The specificity of the amplification was verified by

electrophoresis on 2 % w/v agarose gel at 90 V for 45 min. Templates were purified by adding 2 µL of streptavidin-coated Sepharose beads (GE Healthcare) in the presence of 40 µL of binding buffer (Qiagen Canada Inc., Mississauga, ON, Canada) and then pyro-sequenced.

5.4.9.2. Transcriptome

q- RT-PCR was used to measure transcription levels. Four biological replicates (pool of 10 expanded blastocysts) were considered for each transcript of interest. Prior to RNA extraction using a Pico-Pure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA, USA), the External RNA Controls Consortium (ERCC) RNA Spike-in Control Mix (Ambion, 4456740) was added (1: 1,000) to the lysis buffer mixture. Extracted RNA was reverse-transcribed using q-Script Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) priming the reaction with both oligo-dT(20) (2µL of supplied 10X solution) and random primers (2µL of supplied 10X solution) according to manufacturer's recommendations. The bovine genomic sequences were obtained from the UMD3.1/bosTau5 assembled version of the bovine genome and microarray analysis results. All primers were designed using Primer3 software (version 0.4.0) (http://frodo.wi.mit.edu/primer3/) and their quality (dimers, hairpin formation probability, etc.) was examined in silico using the OligoAnalyzer 3.1 tool (available at Integrated DNA Technologies website). Primer pair specificity was determined ultimately from a run of the amplified fragment of interest on standard 1 % agarose gel

electrophoresis and sequencing. Complementary DNA quantification was achieved using RT-qPCR and a LightCycler machine (Roche Diagnostics, Laval, QC, Canada) with LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics). The following RT-qPCR conditions were used for the selected ERCC spikes and genes: denaturing cycle for 10 min at 95 °C, 50 PCR cycles (denaturation at 95 °C for 1 s, annealing (Supplementary Table S4) for 5 s and extension at 72 °C for 5 s), melting curve of 94 °C for 5 s, 72 °C for 30 s. In order to confirm the specificity of each primer pair, each amplicon was further sequenced. Quantification was performed using LightCycler Software Version 4.1 (Roche) by comparison with the standard curves. Analysis of gene expression was normalized using subgroup A Ambion ERCC RNA Spike-In Mix plus the housekeeping gene (GAPDH) according to normalization factors obtained from a stable gene analyzed using the BioGazelle qBase Plus program (version 1.3; (Biogazelle NV, Zwijnaarde, Belgium). Data were log transformed and significant differences ($p < 0.05$) were calculated using Student's T-test (Graphpad prism IV software, La Jolla, CA, USA).

5.4.10. Statistical Analyses

For developmental trophectoderm (TE) and inner cell mass (ICM) (TE/ICM) differential staining and early embryo mitochondrial activity, all analyses were conducted using Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA). The P values of these parameters were calculated using an unpaired two-tailed Student's T-test with $P < 0.05$ considered significant. Data were reported as mean ± SD. The DAVID bioinformatics resource (see

http:// david.abcc.ncifcrf.gov/) (Huang *et al*., 2008) was used to identify common biological processes (GOTERM_BP_ALL), pathways, molecular functions and cellular components, as defined by Gene Ontology (GO) criteria. Only the ‘‘high stringency’’ setting of the DAVID instrument with enrichment thresholds (EASE) of 0.05 was selected, which identified functional annotation clusters with enrichment scores above 1.3 (which corresponds to $p < 0.05$). A list of 156 *Bos taurus* genes annotated in the UMD 3.1 assembly was uploaded to the DAVID resources.

5.5. Results

5.5.1. Phenotypic observations

5.5.1.1. Developmental speed, cell numbers and mitochondrial activities

SAM is highly unstable and has a short half-life (Lu and Mato, 2012; Mato and Lu, 2007; Yokochi and Robertson, 2004). In order to account for degradation and to ensure constant exposure, the treatment was repeated four times over a 12 h period (see method). To minimize the impact of phenotypic divergence (different blastocyst sizes) on the results, only expanded blastocysts of similar size were collected, for both phenotypic and molecular analyses. The concentration of SAM used in this study (2 µmol/L) was the highest non-lethal concentration reported previously for bovine embryos (Ikeda *et al*., 2012b). Based on blastocyst counts (Table 1), there was no loss of embryos at this concentration. However, the hatching percentage was noticeably higher among SAM-treated embryos: 82 % ± 5 versus 33 % ± 3 for the control embryos (Figure 1). The proliferation of blastocyst cells is one of the main factors involved in embryo hatching. It was therefore hypothesized that cell numbers and allocation to the inner cell mass (ICM) and

trophectoderm (TE) might differ between non-treated embryos and SAM-treated embryos exhibiting higher hatching percentages. It was also expected that SAM in high doses would influence early embryo differentiation. However, based on differential staining of expanded blastocysts to quantify the ratio of putative ICM to TE, no significant different was found between the SAM- treated and non- treated embryos (Figure 2A). Since SAM is essential for mitochondrial metabolism and methylation and is present at relatively high concentrations in mitochondria (Chandel, 2014; Stover, 2009; Shaughnessy *et al*., 2014), it was hypothesized that a higher hatching percentage among SAM-treated embryos would require greater mitochondrial activity. In addition, it was expected that in the presence of a high concentration of SAM, mitochondrial activity might be increased. However, no significant difference between the groups was observed in day 7 expanded blastocysts (Figure 2B).

5.5.1.2. Sex ratio

Male and female early embryos differ in their metabolism and developmental rates (Mao *et al*., 2010; Shang *et al*., 2015; Gallou-Kabani *et al*., 2010; Gabory *et al*., 2013; Guti *et al*., 2001; Jiménez *et al*., 2003; Pérez-Crespo *et al*., 2005; Ricart *et al*., 2009; Rosenfeld and Trainor, 2014; Sinclair *et al*., 2007), transcripts and epigenetic events such as X chromosome inactivation. We therefore hypothesized that SAM might affect male and female embryo development differently and thereby alter sex ratio. To investigate this, the sex of 313 embryos was determined at different stages of early development including the 8-cell stage (before SAM treatment; n = 50), morula (n = 80), day 7 expanded

blastocysts, and blastocysts collected on or after day 8 (n = 183 in total). The SAM treatment was found to skew the sex ratio to 64 % male vs. 36 % female compared to the non-treated group (51% males, 49% females) and to increase the male embryo population by 13 % among day 7 expanded blastocysts but not at the other stages examined (Figure 3A). We hypothesized that SAM treatment would cause a delay in female embryo development and that more females therefore would be found on or after day 8. However, this was not observed among blastocysts, whether early, hatching or hatched. At least 80 % of these embryos in both the non-treated and treated groups were male. Considering therefore that 60 % of the embryos at the initiation of SAM treatment, at the 8-cell stage and at morula stage 60% in the untreated group compared to 54% in SAM-treated group were female, it was possible that the d7 female embryos might be found among the non-developed and/or dead embryos. To investigate this, arrested d7 embryos in culture (n = 50) were collected and sexed. The results showed that while the sex ratio was respectively 60% vs. 40% for male and female in d7 expanded blastocyst, it was conversely 40% vs. 60% for male and female in d7 dead and/or non-expanded blastocysts (Figure 3B). This indicated that high dose SAM treatment had a sex-specific impact on development of a portion (20%) of all embryos during transition from the morula to the d7 EB stage. It appears that SAM treatment arrested development from the morula to the d7 EB stage in 20 % of the all female embryos, in contrast with an increase by the same degree among the corresponding all male embryos (Figure 3B). Therefore, such equal but

opposite impact of high dose SAM treatment on embryos based on their sex (20% decline in all female versus 20% increase in all male) during transition from the morula to the d7 EB stage resulted in no changes in the "number" of d7 EB (blastocyst rate) when compared with non-treated d7 EB (Table 1).

5.5.2. Genome-wide DNA methylome profile

5.5.2.1. Genome-wide DNA methylation and genomic localization

Genome-wide analysis was performed to investigate the impact of SAM on DNA methylation patterns in d7 expanded blastocysts early embryos. A total of 4,056 differentially methylated regions (DMRs) were found distributed throughout the entire bovine genome and all of the chromosomes (Figure 4A; Supplementary Figure S1). The SAM treatment caused global hypermethylation as produced by hypermethylation in 2,884 DMRs, and hypomethylation in 1,172 DMRs. Hypermethylation was more frequent in intragenic regions (in exons specifically) than in promoter regions, and also in CpG islands with intermediate and high CpG density, regardless of the CpG islands length. Treatment-associated hypermethylation also appeared partial to repetitive elements categorized as low complexity and simple repeat. LINE, SINE and long-terminal-repeat (LTR) retro repetitive elements were either indifferent to the SAM treatment or responded with hypomethylation (Figure 4B).

5.5.2.2. Functional annotation clustering of genes with differentially methylated regions

Cluster analysis of the differentially methylated genes with similar and common function might reveal which cellular and molecular functions are affected most by the SAM treatment. Gene pathway enrichment analysis pointed to the base excision repair (BER) pathway and embryonic stem cell pluripotency. Currently, it is now known that DNMT3B, which is the *de novo* DNA methyl transferase enzyme, contributes in DNA re-methylation during blastocyst formation (Petrussa *et al*., 2014), and the BER pathway involves in active DNA methylation removal. To support these results, quantitative measurement of DNA methylation of BER pathway genes (Figure 5A) was done by pyrosequencing. This revealed significant ($p < 0.05$) hypomethylation of three BER-related genes (PARP1, PNKP, XRCC1) in SAM-treated EB embryos (Figure 5B). Since the BER pathway is involved in active DNA demethylation in conjunction with Ten-Eleven Translocation (TET) enzymes (Gu *et al*., 2011; Koh and Rao, 2013; Müller *et al*., 2014; Rivera and Ross, 2013; Seisenberger *et al*., 2013) (Figure 5A), pyrosequencing was used to determine the extent of DNA methylation of the TET3 gene. Similar to BER pathway genes, notable significant ($p < 0.01$; $p < 0.0001$) hypomethylation was confirmed in TET3 gene loci in SAM-treated EB embryos (Figure 5C). The significant hypomethylation of genes involved in active DNA demethylation (*i.e.* BER pathway and TET3) following the treatment suggested the presence of an epigenetic compensatory mechanism in early embryos. To provide additional evidence for this, we hypothesized that the exogenous methyl donor affected one or more genes involved in *de novo* DNA methylation at the blastocyst stage (*i.e.*

DNMT3B), and that in contrast with genes involved in active DNA demethylation, the effect was hypermethylation. This was confirmed using pyro-sequencing (Figure 5D). In summary, in cultured embryos subjected to the SAM treatment, genes involved in active DNA demethylation pathways (BER and TET3) were hypomethylated whereas at least one gene involved in *de novo* DNA methylation (DNMT3B) was hypermethylated. Of the embryonic stem cell pluripotency genes affected upstream, only one (BMP4) was chosen for confirmation of DNA methylation status by pyro-sequencing. Hypomethylation was thus found significant ($p < 0.01$; $p < 0.0001$) in SAM-treated EB embryos (Figure 6). However, none of these DNA methylation effects were reflected in gene transcription levels (Figure 7).

5.5.3. Parallel analysis of the DNA methylome and transcriptome of SAM-treated expanded blastocysts

Evidence is strong that DNA methylation does not necessarily lead to suppression of transcription. The platform used in this study allows parallel analysis of the DNA methylome and the transcriptome (Shojaei Saadi *et al.*, 2014) and hence investigation of a possible association between the two in SAM-treated EB. Genes that are "concordant" for changes in both DNA methylation and RNA transcription may thus be identified. Although no such genes were identified when strict criteria were applied (change > 1.5 fold and P-value < 0.05), 13 "potentially concordant" genes were identified using relaxed criteria (change > 1.2 fold and P-value ≤ 0.1). Among these, two were selected, namely

the genes encoding the proteins CARS (cysteinyl-tRNA synthetase) and BRD9 (bromodomain containing 9), since they are reportedly involved in Beckwith Wiedemann syndrome (Massah *et al*., 2014; Engemann *et al*., 2000) and chromatin remodeling (Hargreaves and Crabtree, 2011) respectively. The differential methylation of these loci was found to be insignificant (Supplementary Figure S2), suggesting that in the current study correlation between differential DNA methylation and differential RNA expression is weak and that other factors such as histone methylation might be involved in regulating transcription at the EB stage of development.

5.5.4. Transcriptome profile

Of 247 RNA transcripts (coding and non-coding) expressed differentially in SAM-treated embryos, 127 were overexpressed versus 115 under-expressed (the criteria for declaring differential expression was change > 1.5 fold and P-value < 0.05). From this list, GO terms enrichment analysis identified two annotation clusters. The upper cluster, with an enrichment score of 2.81, was centered on multicellular organism development processes (GO: 0007275), and the second cluster, with an enrichment score of 1.7, corresponded to response to vitamins (GO: 0033273) and nutrients (GO: 0007584). Data analysis revealed 48 long-non coding RNAs (lncRNAs) expressed differentially relative to the un-treated condition, of which 33 were overexpressed and 15 were under-expressed (Figure 8). Nineteen of the 33 (*i.e.* about 60 %) were associated with bovine repetitive elements

belonging mainly to the LINE family. However, in non-treated EB embryos, only three (20 %) of the differentially expressed lncRNAs identified were associated with bovine repetitive elements, and the largest group (n = 6) within the 15 corresponded to short and long subunits of ribosomal RNA (Supplementary Table S1).

5.6. Discussion

Many studies have shown that folate deficiency in combination with a deficiency in one or more one-carbon metabolism nutrients leads to a decline in SAM production, either by insufficient nutritional intake or by dysregulated metabolism (Crider *et al*., 2012; Bailey *et al*., 2015; Duthie *et al*., 2002; Nazki *et al*., 2014). This may cause several cellular and molecular dysfunctions such as long and short telomeres (Bull *et al*., 2014), chromosomal instability (Fenech, 2012), and impaired DNA synthesis and repair, in addition to subsequent pathophysiological diseases and developmental disorders. This has led to widespread supplementation of foodstuffs with folic acid and recommendations to use supplements specifically during pregnancy. However, recent studies suggest that consumption of folic acid in high or even moderate doses during gestation may have adverse effects on embryonic and fetal development (Mikael *et al*., 2013; Pickell *et al*., 2011; Allen, 2012) and ultimately on the offspring and the adult (Aarabi *et al*., 2015; Keating *et al*., 2015), Because of these controversies (O'Neill *et al*., 2014; Shorter *et al*., 2015) SAM was found as a valuable nutrient to investigate its high dose supplementation on IVC bovine embryos.

The bovine embryo was chosen for the present study because it is a good model for human pre-implantation in terms of timing of genome activation, intermediate metabolism, interactions with the culture medium (Wrenzycki *et al*., 2001; Kues *et al*., 2008; Xie *et al*., 2010; Iqbal *et al*., 2011), amino acid metabolism and SAM synthesis (Ménézo and Hérubel, 2002). However, the implications of the findings for human nutrition are limited, since ruminants are poor models of human nutrition and folate metabolism. The symptoms of folate deficiency in humans have not been observed in cattle ((NRC), 2001), since rumen bacteria can synthetize B vitamins, including folic acid and vitamin B12 (Duplessis *et al*.). We therefore turned our attention to SAM, the principal intracellular substrate of metabolic reactions for FA, to investigate indirectly the effects of high levels of folate on early development in cultured cattle embryos.

Practically all enzymes known to participate in one-carbon metabolism are expressed in mammalian early embryos, meaning that the embryos are capable of metabolizing all one-carbon metabolism nutrients independently (Ikeda *et al*., 2012a). *In vitro* inhibition of SAM production in bovine early embryos has been shown to suppress development beyond the 8-cell stage (Ikeda *et al*., 2012b) concurrent with *de novo* DNA methylation in bovine early embryos (Dean *et al*., 2001). We therefore chose to initiate the SAM treatment at the 8-cell stage. The concentration used (2 µmol/L) is reportedly the highest non-lethal dose for bovine early embryos (Ikeda *et al*., 2012b).

The folate cycle is linked to the methionine cycle via 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), which methylates homocysteine to methionine, one of the substrates used to produce SAM (Ikeda *et al*., 2012a). In a previous study of cultured cattle embryos, it was found that methionine concentration did not affect cleavage rate, total cell number, cell allocation or apoptosis but had a positive impact on blastocyst development, in particular expansion, hatching and subsequent steps. The effect reached a plateau at a methionine concentration of 21 µmol/L, at which about 73 % of d7 embryos developed into expanded, hatching or hatched blastocysts, while the percentage was only 27 % for the control condition (Bonilla *et al*., 2010). The authors also noted increased blastocoel cavity volume, and suggested that the effect is due to increased SAM production and hence methylation of membrane lipids as well as higher Na/K ATPase activity (Muriel, 1993). Our results were similar (no impact on blastocyst percentage, total cell number or allocation, but higher percentage of hatching), although in our conditions the SAM treatment also led to a shift in sex ratio in favor of male embryos.

Since mitochondria hold approximately 40 % of the total intracellular folate (Stover, 2009) and generate much SAM, they have profound effects on epigenetic status (Chandel, 2014). The impact of SAM on mitochondrial proteins and functions has been demonstrated in somatic tissues (Andringa *et al*., 2010; Jin *et al*., 2007; Bailey *et al*., 2006). Folic acid deficiency and subsequent SAM deficiency during the peri-conception and early

pre-implantation periods of mammalian development are believed to affect mitochondrial function in the offspring (Maloney *et al*., 2013). We did not observe any significant change in early embryo mitochondrial activity in association with the SAM treatment in day 7 expanded blastocysts, which implies either that SAM does not have the same type of effect in embryonic cells as in somatic tissues or that the methods of detecting the effect (quantification of mitochondrial DNA density versus staining) are not equivalent.

It has been demonstrated that the morula-to-blastocyst transition is the period most dependent on methionine metabolism and SAM in bovine early embryo development (Ikeda *et al*., 2012b). Based on embryo sexing, we observed similar sensitivity to SAM during this period. Such gender-biased sensitivity to methyl group metabolism has been observed in male offspring of sheep (Sinclair *et al*., 2007) and rats (Maloney *et al*., 2011). In a more recent study, it was shown that over-supplementation with folic acid during pregnancy can induce early sex-specific responses in the placenta (Penailillo *et al*., 2015). In cultured embryos, shifts in sex ratio generally favor males, which develop faster than females (Rubessa *et al*.), although this is not absolute (Rubessa *et al*.; Zhu *et al*., 2015). Moreover, gender-specific sensitivity is not limited to one-carbon nutrients, and gender-specific chromosome-led hormone-independent sexually dimorphic sensitivity to perturbations in maternal nutrition is known to affect early embryo development (Fernandez-Gonzalez *et al*., 2004; Gallou-Kabani *et al*., 2010; Guti *et al*., 2001; Jiménez *et*

al., 2003; Pérez-Crespo *et al*., 2005; Ricart *et al*., 2009; Rosenfeld and Trainor, 2014; Sinclair *et al*., 2007; Sjöblom *et al*., 2005) and the placenta (Mao *et al*., 2010; Shang *et al*., 2015; Gallou-Kabani *et al*., 2010; Gabory *et al*., 2013) in male (Jiménez *et al*., 2003; Sjöblom *et al*., 2005; Sinclair *et al*., 2007; Ricart *et al*., 2009; Rosenfeld and Trainor, 2014; Pérez-Crespo *et al*., 2005) or female embryos (Guti *et al*., 2001; Fernández-Gonzalez *et al*., 2004; Gallou-Kabani *et al*., 2010). The mechanisms underlying this gender-specific sensitivity remain unclear (Laguna-Barraza *et al*., 2012), but appear to involve environmental stresses and insults (Rosenfeld and Trainor, 2014; DiPietro and Voegtline).

One possible mechanism suggested is related to differences in sex chromosome activity prior to X chromosome inactivation. The presence of a Y-chromosome and only one X chromosome in male embryos during this period determines different transcriptional levels of sex chromosome-encoded genes and expression of autosomal genes under regulation by sex chromosomes (Bermejo-Alvarez *et al*., 2011; Bermejo-Alvarez *et al*., 2010). This affects genes involved in translation, proteolysis and protein transport (Bermejo-Alvarez *et al*., 2010), epigenetic regulation, particularly DNA methylation (Bermejo-Álvarez *et al*., 2008), protein metabolism (Sturmey *et al*., 2010) and mitochondrial activity, causing gender-associated metabolic differences (Mittwoch, 2004; Bermejo-Alvarez *et al*., 2010; Bermejo-Álvarez *et al*., 2008) and hence notable differences in molecular pathways (Hansen *et al*., 2015) that influence environmental epigenetic programming (Gabory *et al*., 2009), metabolism, cell number and survival of pre-

implantation embryos under environmental stress and resulting ultimately in gender-specific mortality rates or long-term effects in the offspring (Bermejo-Alvarez *et al*., 2011).

Based on our observations in the present study, we hypothesize that the mainly gender-specific differences in early embryo regulation of DNA methylation (Gebert *et al*., 2009; Bermejo-Álvarez *et al*., 2008) play important roles in favoring males and arresting the development of females at the d7 EB stage. In fact, *de novo* expression of DNA methyltransferases (DNMT3 A, B) was weaker in female embryos, which were therefore more hypomethylated than male embryos (Bermejo-Álvarez *et al*., 2008). Such differences might explain the gender-specific sensitivity to SAM during early development, although other mechanisms such as unbalanced expression of X-linked genes (Rubessa *et al*.) and metabolism could play roles. It should be considered that changes in sex ratio do not necessarily mean gender-specific altered survival rate and embryo loss (Bermejo-Alvarez *et al*., 2011). It is therefore not possible to draw any conclusions regarding the outcome of SAM-treated embryo gestation until further investigation has been carried out. Since the SAM treatment was initiated at the 8-cell stage, factors such as oocyte maturation, sperm, fertilization and zygotic genome activation may be ruled out as effectors of the sex ratio skew observed in this study.

Methylation of DNA repetitive elements is dynamic during human early development (Guo *et al*., 2014; Smith *et al*., 2014), and identification of lncRNA expressed at higher levels following SAM treatment could shed light on the function of this process. Although

we did not observe any direct association between gene methylation and expression in this study, we suspect that epigenetic and transcriptomic events are part of a cascade that includes other factors such as histone modification (Shyh-Chang *et al*., 2013) and that this needs to be investigated. Our observation corroborates growing evidence that DNA methylation does not necessarily lead to suppressed transcription, especially when it occurs in non-promoter inter-genic and intra-genic regions (Moore *et al*., 2013; Jones, 2012).

Recent studies have shown that methylation occurs mainly in gene bodies rather than in promoters (Maunakea *et al*., 2010; Liang *et al*., 2011; Illingworth *et al*., 2008; Deaton *et al*., 2011) and more in exonic than in intronic regions (Anastasiadou *et al*., 2011; Chodavarapu *et al*., 2010; Choi, 2010; Feng *et al*., 2010; Jones, 2012), and that it might play a role in modulating alternative splicing (Maunakea *et al*., 2013) and position-dependent and tissue-specific transcriptional regulation (Brenet *et al*., 2011; Chuang *et al*., 2012; Moore *et al*., 2013). We observed that SAM targeted mainly intragenic regions and exonic genomic regions in particular. Although CpG Islands are generally un-methylated (Deaton and Bird, 2011) and CpG-poor regions of the genome (*i.e.* shore and shelf) are expected to be more susceptible than CpG islands to environmental stimuli and stochastic factors (van Dongen *et al*., 2014), as seen in human sperm cells in response to high doses of folic acid (Aarabi *et al*., 2015), in contrast we observed more hypermethylation in CpG islands in SAM-treated embryos. Excess SAM might thus have

an effect related to that of overexpression of DNA methyltransferase (Vertino *et al*., 1996), both resulting in *de novo* methylation at susceptible CpG island loci.

Recently, Houde *et al*., reported the presence of an in utero "epigenetic compensatory" signature for maternal metabolic status in placental and cord blood (Houde *et al*., 2013). In this study, we observed hypo-methylation of BER pathway genes and the TET3 enzyme, both involved in active DNA methylation removal (Seisenberger *et al*., 2013; Koh and Rao, 2013; Müller *et al*., 2014; Gu *et al*., 2011; Rivera and Ross, 2013), and hypermethylation in the DNMT3B gene in response to the SAM treatment, suggesting the existence of such mechanisms. It is possible that in response to general hypermethylation following such a treatment, hypomethylation of genes involved in active DNA demethylation (BER pathway and TET) and hypermethylation of DNA methyl transferase genes both occur in an attempt to compensate. So far, the only DNA methyl transferase found to play a role in re-methylation during human blastocyst formation is DNMT3B, which is the principal *de novo* methylation enzyme at the blastocyst stage (Petrussa *et al*., 2014). Furthermore, the impact of folate deficiency or over-supplementation is mainly on DNMT3B expression in embryonic stem cells (Shiraki *et al*., 2014) but often DNMT1 and DNMT3A expression in somatic cells (Ghoshal *et al*., 2006; Piyathilake *et al*.; Ding *et al*., 2012). It is therefore not surprising that we observed the compensatory changes only to DNMT3B and not other DNMTs as well. To our knowledge, this is the first report of a possible epigenetic compensatory response in early embryos.

5.7. Conclusion

It was demonstrated that exposing cultured bovine embryos to a high concentration of S-adenosyl methionine affects phenotypic (higher hatching rate and shift in sex ratio in favor of male embryos), transcriptomics (differentially expressed (DE) coding and long non-coding RNAs) and epigenomics (global DNA hypermethylation methylome, mainly in exonic regions and in CpG islands). However, it remains unclear whether or not this has a positive or negative impact on gestation and the health of the offspring. Further studies are needed to answer this and other questions. This study also reiterates that visual appreciation of embryos is an inadequate metric for evaluating the soundness of culture media supplementation, since treated embryos look healthier due to the more rapid expansion. The results suggest that the morphological healthiness of early embryos is not indicative of epigenetic status.

5.8. Competing interests

The authors declare that they have no competing interests.

5.9. Authors' contributions

HASS, MAS, and CR designed the study. HASS performed the IVF, phenotypic, transcriptome (RT-qPCR) and epigenetics (DNA methylation and pyrosequencing) experiments as well as data acquisition and analysis. DG performed the transcriptome microarray. EF performed bioinformatics analyses. LMBB and HASS performed the mitochondrial staining. CR supervised the work. HASS and CR drafted the final manuscript. All authors read and approved the final manuscript.

5.10. Acknowledgements

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5.11. Executive Summary

Background

- Recently there is growing concern about high folate concentrations resulting from grain fortification combined with folic acid supplementation on pre- and post-natal development.
- S-adenosyl methionine (SAM) is the universal methyl donor, essential for methylation process such as DNA methylation and is generated through cellular one-carbon metabolism using dietary folates as substrate.
- The impact of a large dose of SAM as principal intracellular substrate of metabolic reactions for folic acid were investigated on bovine early embryo development, genome-wide DNA methylation and transcription.

Results

- The SAM treatment did not affect the percentage of embryos reaching the blastocyst stage but remarkably increased blastocyst hatching rate.
- Notably, there was a shift in sex ratio in favor of day 7 expanded blastocysts male embryos following SAM treatment.

- Excessive SAM concentration had an impact on DNA methylation, causing genome-wide hypermethylation mainly in exonic regions and in CpG islands with intermediate and high CpG density regardless of CpG island length.
- Transcriptomic analysis revealed 247 differentially expressed coding and long noncoding RNA sequences.
- GO term enrichment analyses revealed differential gene expression in response to nutrients and developmental processes that were in agreement with the impact of our treatment on early embryos.

Conclusion:

- It was demonstrated that exposing cultured bovine embryos to a high concentration of SAM affects phenotype, transcriptome and epigenome.
- Since bovine embryos were not indifferent to SAM, effects of large-dose folic acid supplements on early embryonic development in humans cannot be ruled out.

5.12. References

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5.13. Figures

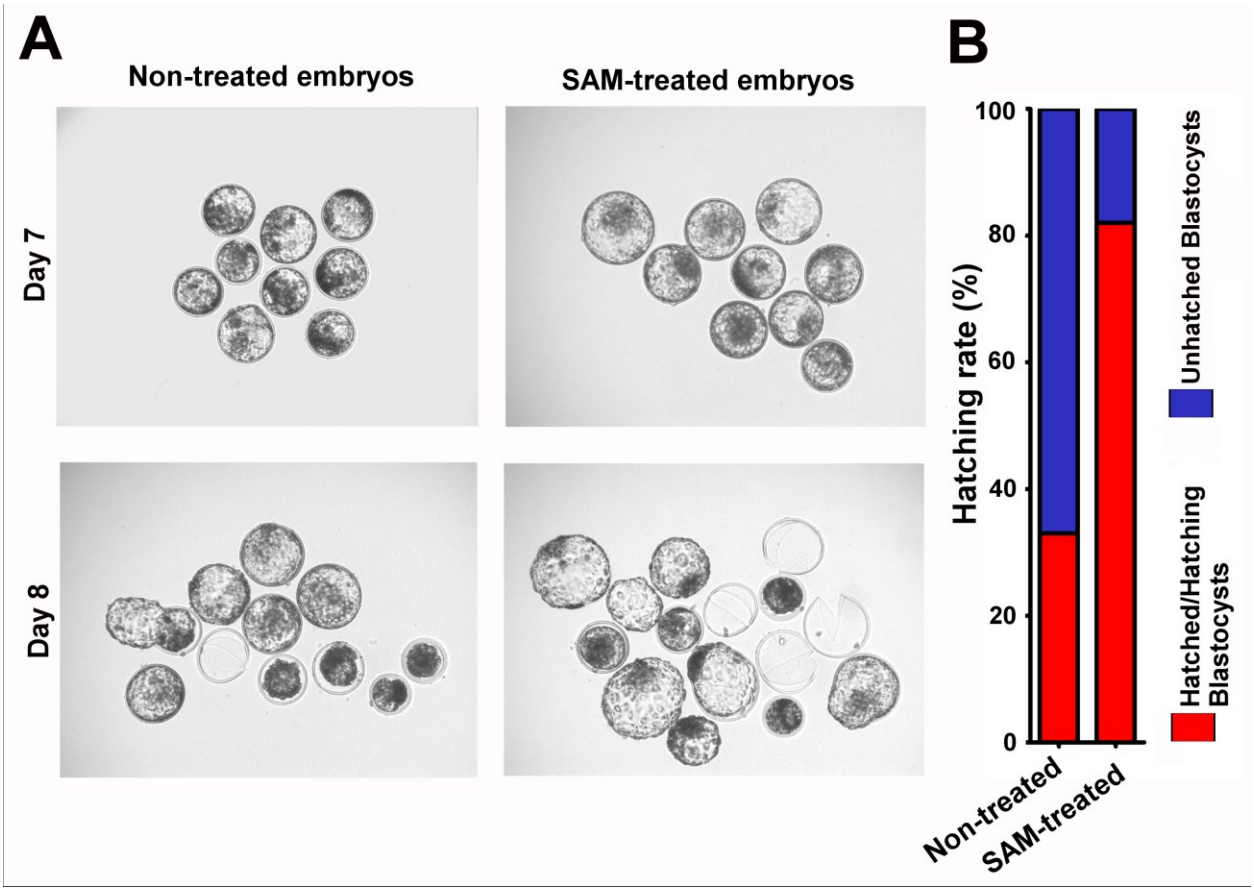


Figure 5-1 Hatching of cultured bovine blastocyst embryos. (A) Appearance of embryos in the absence and presence of S-adenosyl methionine (SAM, 2 μmol/L) in the culture medium. There is essentially no difference on day 7**. (B)** On day 8, more SAM-treated embryos are hatched or hatching.

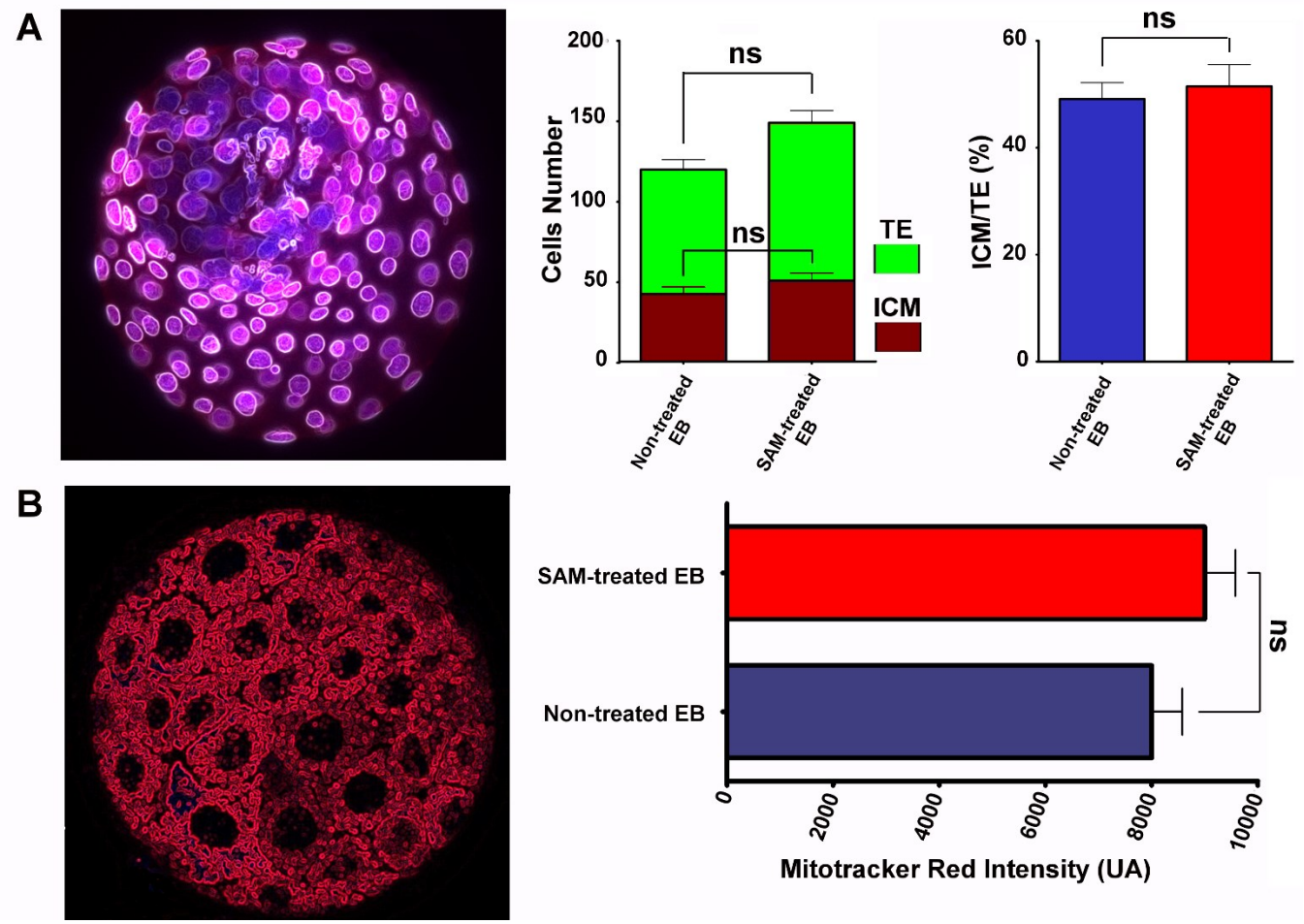
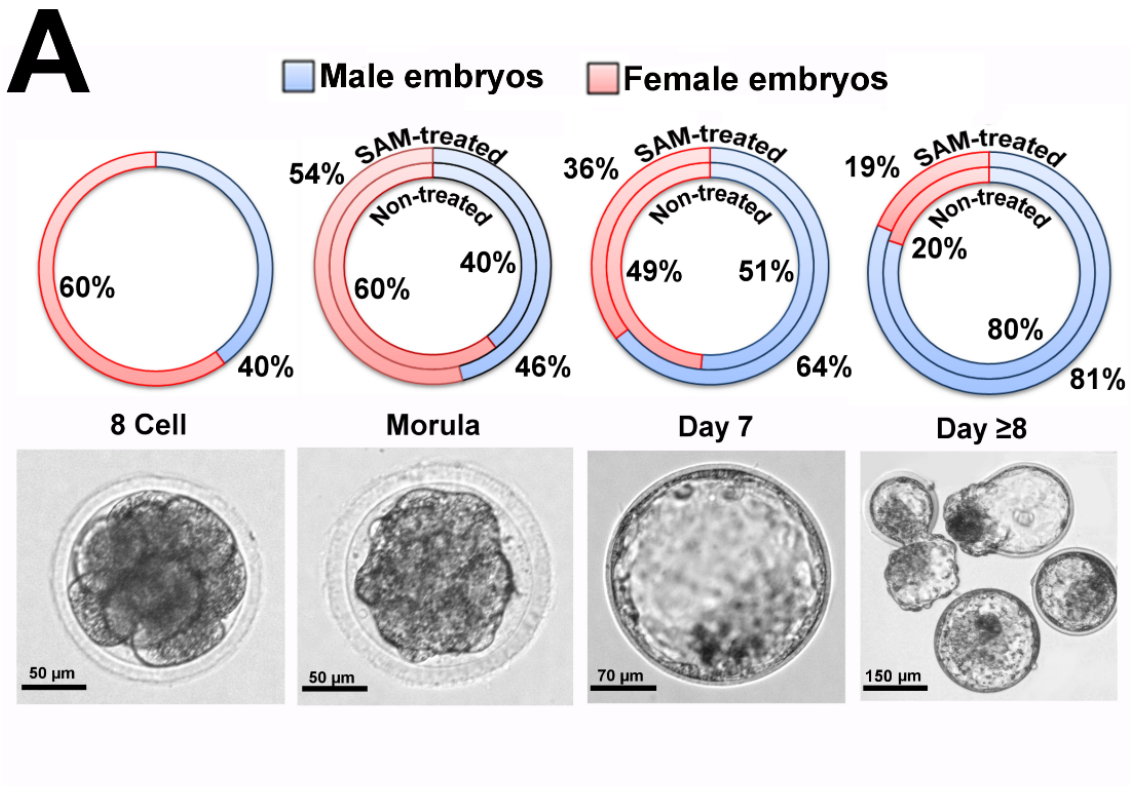


Figure 5-2 Cell number, TE/ICM allocation and mitochondrial activity of cultured bovine blastocysts. (A) No significant difference between non-treated and SAM-treated expanded blastocysts in terms of cell number or allocation. **(B)** No difference in terms of mitochondrial activity measured using Mitotracker Red staining (ns: statistically non-significant).


A
Male embryos
Female embryos
60%
40%
SAM-treated
Non-treated
54%
60%
40%
46%
36%
49%
51%
64%
19%
20%
80%
81%
8 Cell
Morula
Day 7
Day ≥8
50 µm
50 µm
70 µm
150 µm

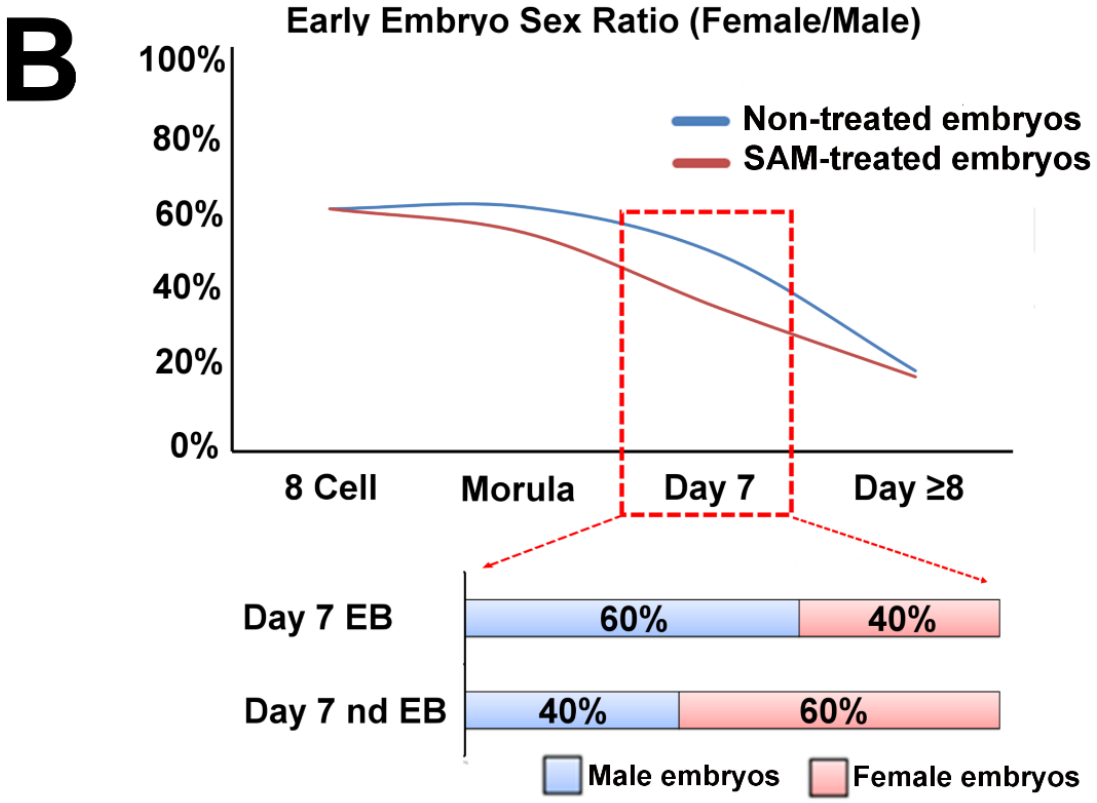
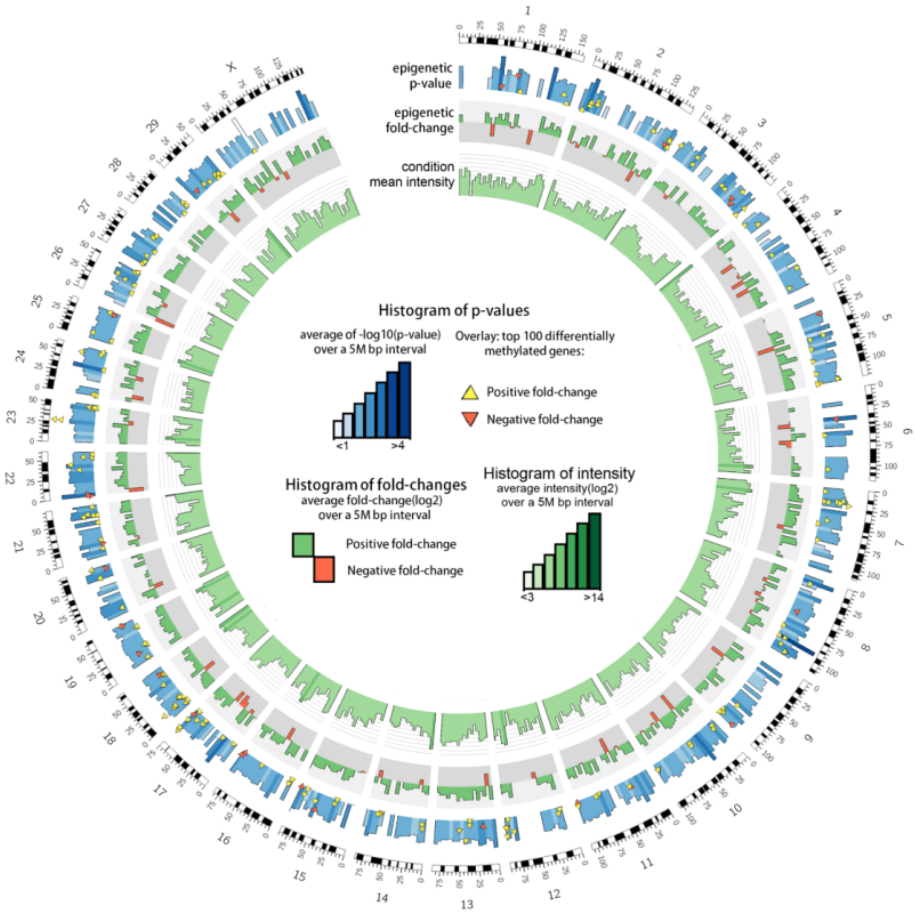

B
Early Embryo Sex Ratio (Female/Male)
100%
80%
60%
40%
20%
0%
Non-treated embryos
SAM-treated embryos
8 Cell
Morula
Day 7
Day ≥8
Day 7 EB
60%
40%
Day 7 nd EB
40%
60%
Male embryos
Female embryos

Figure 5-3 Impact of S-adenosyl methionine on the sex ratio of cultured bovine embryos. (A) Forty percent of 8-cell embryos before the experimental treatment began were male. This number remained unchanged at the morula stage in the absence of SAM but increased to 46 % in its presence. On day 7, 64 % of SAM-treated expanded blastocysts were male, as were 51 % of the un-treated. Of the late (hatched/hatching) blastocysts, 80–81 % were male regardless of SAM treatment. **(B)** Female: male sex ratio of non-treated and SAM-treated embryos at the four studied stages of early embryo development in culture. The percentage of female embryos declines throughout, regardless of SAM treatment, but faster among SAM-treated embryos, especially day 7 expanded blastocysts (dotted rectangle). The decline (20 %) corresponded to the number of non-expanded/dead embryos sexed female.

A


epigenetic p-value
epigenetic fold-change
condition mean intensity
Histogram of p-values
average of -log10(p-value) over a 5M bp interval
<1
>4
Overlay: top 100 differentially methylated genes:
Positive fold-change
Negative fold-change
Histogram of fold-changes
average fold-change(log2) over a 5M bp interval
Positive fold-change
Negative fold-change
Histogram of intensity
average intensity(log2) over a 5M bp interval
<3
>14

B

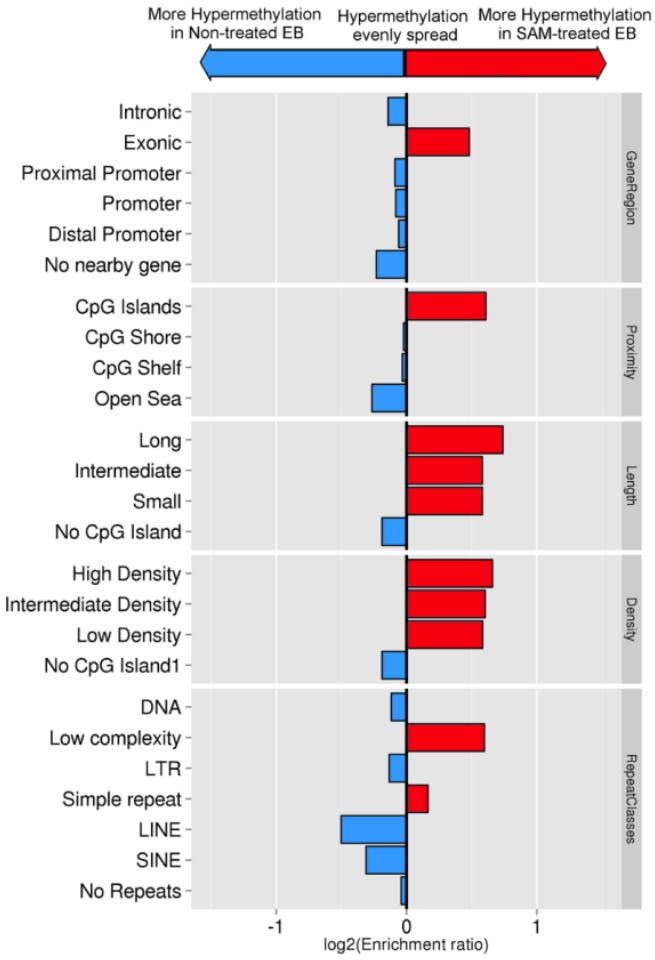

More Hypermethylation in Non-treated EB
Hypermethylation evenly spread
More Hypermethylation in SAM-treated EB
Intronic
Exonic
Proximal Promoter
Promoter
Distal Promoter
No nearby gene
GeneRegion
CpG Islands
CpG Shore
CpG Shelf
Open Sea
Proximity
Long
Intermediate
Small
No CpG Island
Length
High Density
Intermediate Density
Low Density
No CpG Island1
Density
DNA
Low complexity
LTR
Simple repeat
LINE
SINE
No Repeats
RepeatClasses
-1
0
1
log2(Enrichment ratio)

Figure 5-4 Distribution of differentially methylated regions of the genome of cultured bovine embryos. (A) The circos plot shows the average epigenetic change due to the SAM treatment as determined at intervals of 5 million base pairs (using *HinP1I*) in expanded blastocysts. Outside to inside: chromosome number, epigenetic p-value, epigenetic change (relative to the control) and condition mean intensity. **(B)** The calculated log2 methylation ratio for various genomic region types. Blue indicates hypomethylation and red indicates hypermethylation (change due to the SAM treatment, relative to the control).

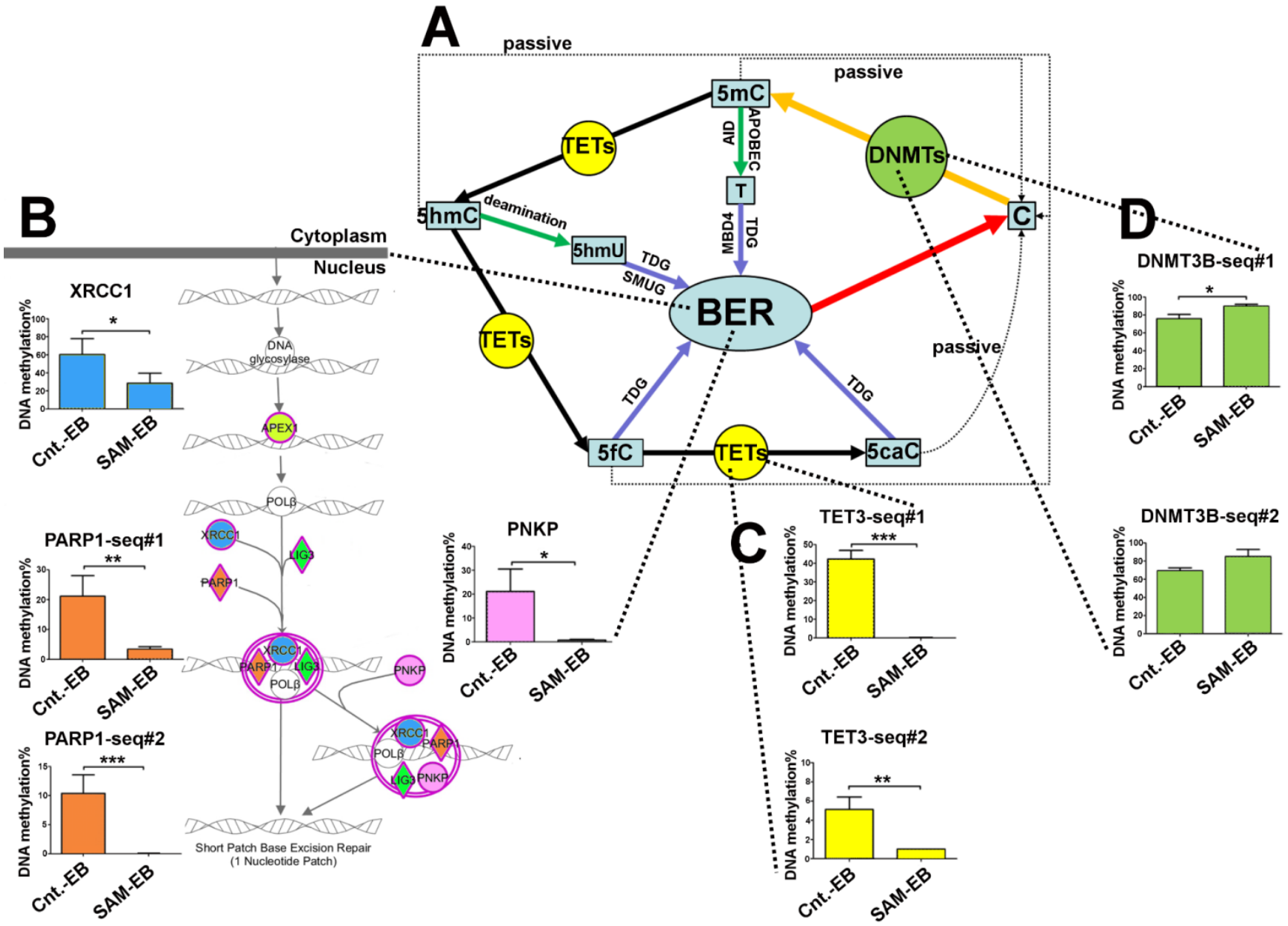

A
passive
passive
5mC
TETs
AID
APOBEC
DNMTs
T
5hmC
deamination
C
5hmU
TDG
MBD4
TDG
SMUG
BER
TETs
passive
TDG
TDG
5fC
TETs
5caC
B
Cytoplasm
Nucleus
XRCC1
DNA methylation%
*
Cnt.-EB
SAM-EB
DNA glycosylase
APEX1
POLβ
XRCC1
LIG3
PARP1
XRCC1
PARP1
LIG3
POLβ
PNKP
XRCC1
PARP1
POLβ
LIG3
PNKP
Short Patch Base Excision Repair
(1 Nucleotide Patch)
PARP1-seq#1
**
PARP1-seq#2
***
PNKP
*
C
TET3-seq#1
***
TET3-seq#2
**
D
DNMT3B-seq#1
*
DNMT3B-seq#2

Figure 5-5 Hypomethylation of genes involved in active DNA demethylation and hypermethylation of *de novo* DNA methylation genes in bovine embryos cultured in a medium supplemented with S-adenosyl methionine. (A) Illustration of the pathways involved in DNA methylation and active DNA demethylation. Whereas DNA methyl transferases are responsible for *de novo* or maintenance methylation, TET enzymes in collaboration with the BER pathway are involved in active DNA de-methylation (modified from Stefanie Seisenberger *et al.*, Phil. Trans. R. Soc. B 2013; 368:20110330). **(B)** BER pathway genes identified by ingenuity pathway analysis, and genes (in color) found differentially methylated in this study. Pyro-sequencing of three genes (XRCC1, PAPRP1 and PNKP) confirms significant hypomethylation in SAM-treated expanded blastocysts. **(C)** Similar findings for the TET3 gene. **(D)** In contrast, significant hypermethylation of DNMT3B was detected. **5caC:** 5-carboxylcytosine, **5fC:** 5-formylcytosine, **5hmC:** 5-hydroxymethylcytosine, **5mC:** 5-methylcytosine, **AID:** activation-induced deaminase, **APOBEC:** apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like, **BER:** Base excision repair pathway, **C:** non-methylated cytosine, **Cnt.-EB:** Non-treated expanded blastocysts, **DNMTs:** DNA methyltransferase enzymes (DNMT1, 3A & 3B), **MBD4:** and methyl-CpG-binding domain protein 4, **SAM-EB:** SAM-treated expanded blastocysts, **SMUG1:** Single strand-selective monofunctional uracil DNA glycosylase 1, **T:** Thymine, **TETs:** Ten eleven translocase enzymes including TET1-3. **TDG:** Thymine DNA glycosylase, *****, ******, *******: means are significantly different, p value < 0.05, < 0.01 and < 0.001, respectively.

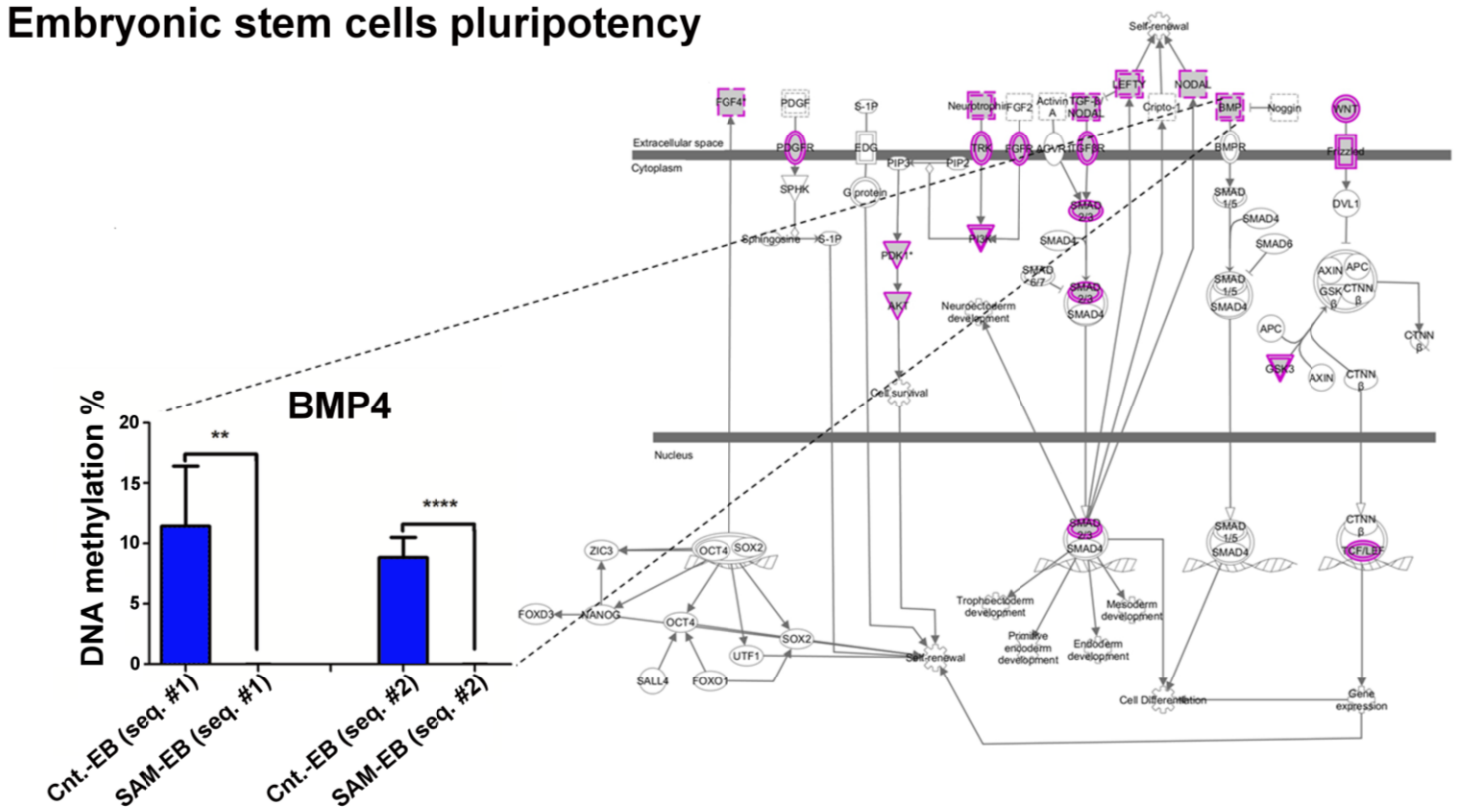


Figure 5-6 Embryonic stem cell pluripotency (ESC) genes (in color) found differentially methylated in SAM-treated day 7 expanded blastocysts. Pyro-sequencing confirmed the remarkable hypomethylation of the BMP gene.

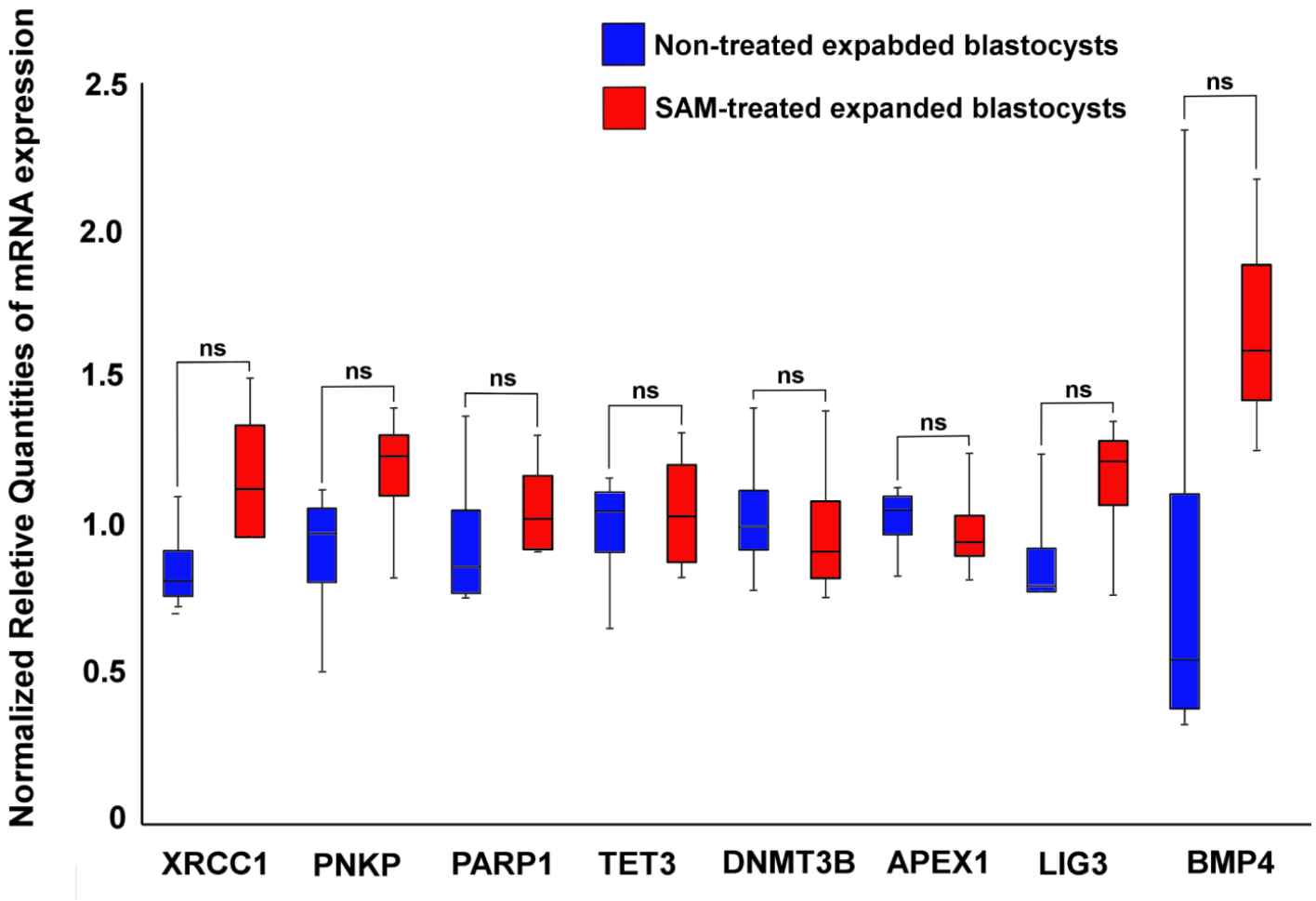


Figure 5-7 The relative expression differentially methylated genes of interest. The box-and-whisker plot of RT-qPCR analyses confirm the absence of differential expression of the differentially methylated genes of interest, as revealed earlier by array-based transcriptome analysis (ns: statistically non-significant; Error bar: SD).

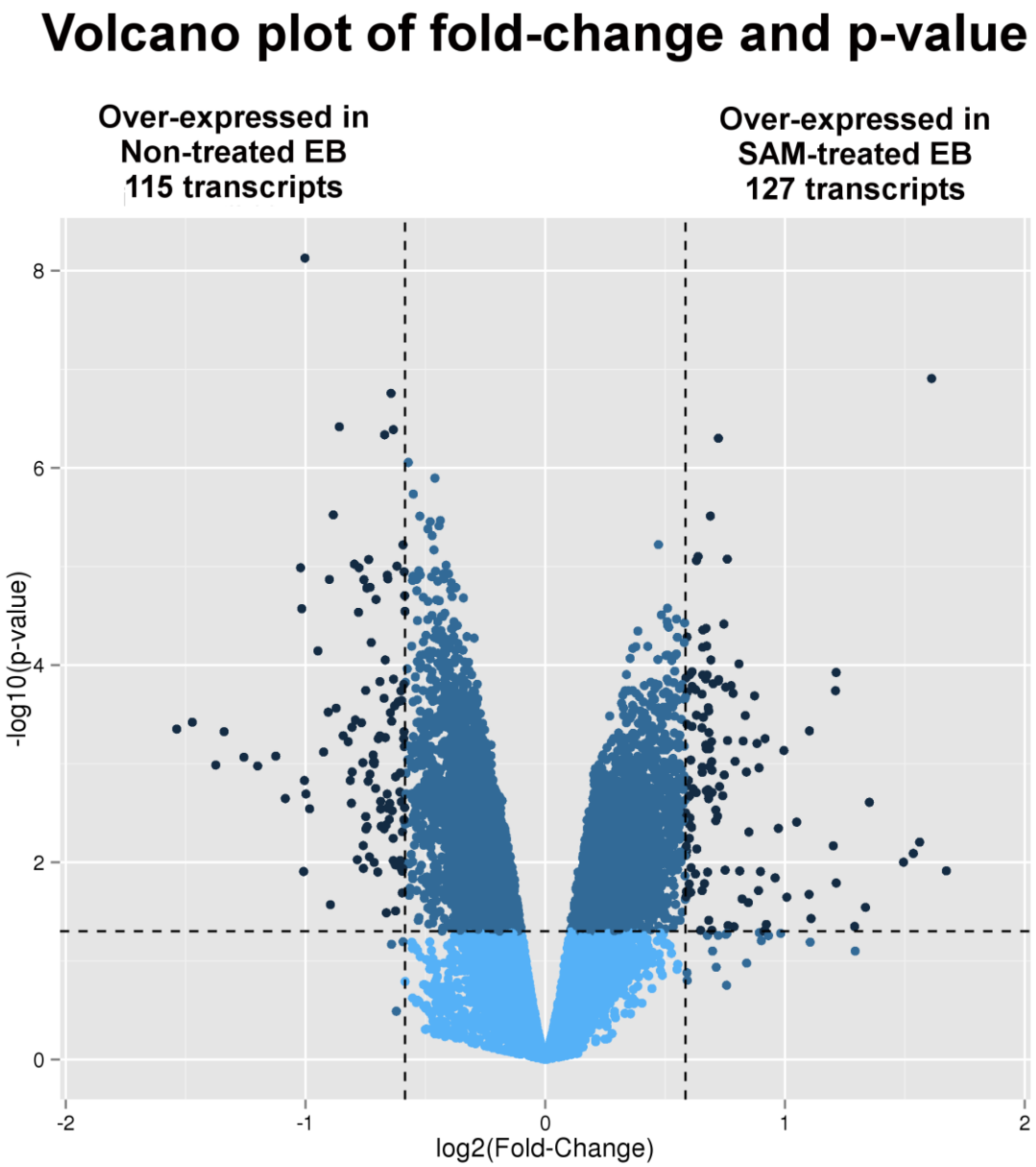


Figure 5-8 Volcano plot of transcripts expressed differentially in non-treated and SAM-treated day 7 expanded bovine blastocysts in culture.

5.14. Tables

Table 5-1 Information relating to the production of bovine embryos *in vitro*. Adding S-adenosyl methionine to the culture medium did not have any significant impact on the percent reaching the blastocyst stage.

Oocytes (n)	Cleavage	8-cell embryos		Reaching blastocyst	Hatching
1646	79% ± 04%	45% ± 06%	**SAM-treated**	24% ±04%	82% ± 05%
			Control	25%± 03%	33%± 03%

5.15. Supplementary Figures

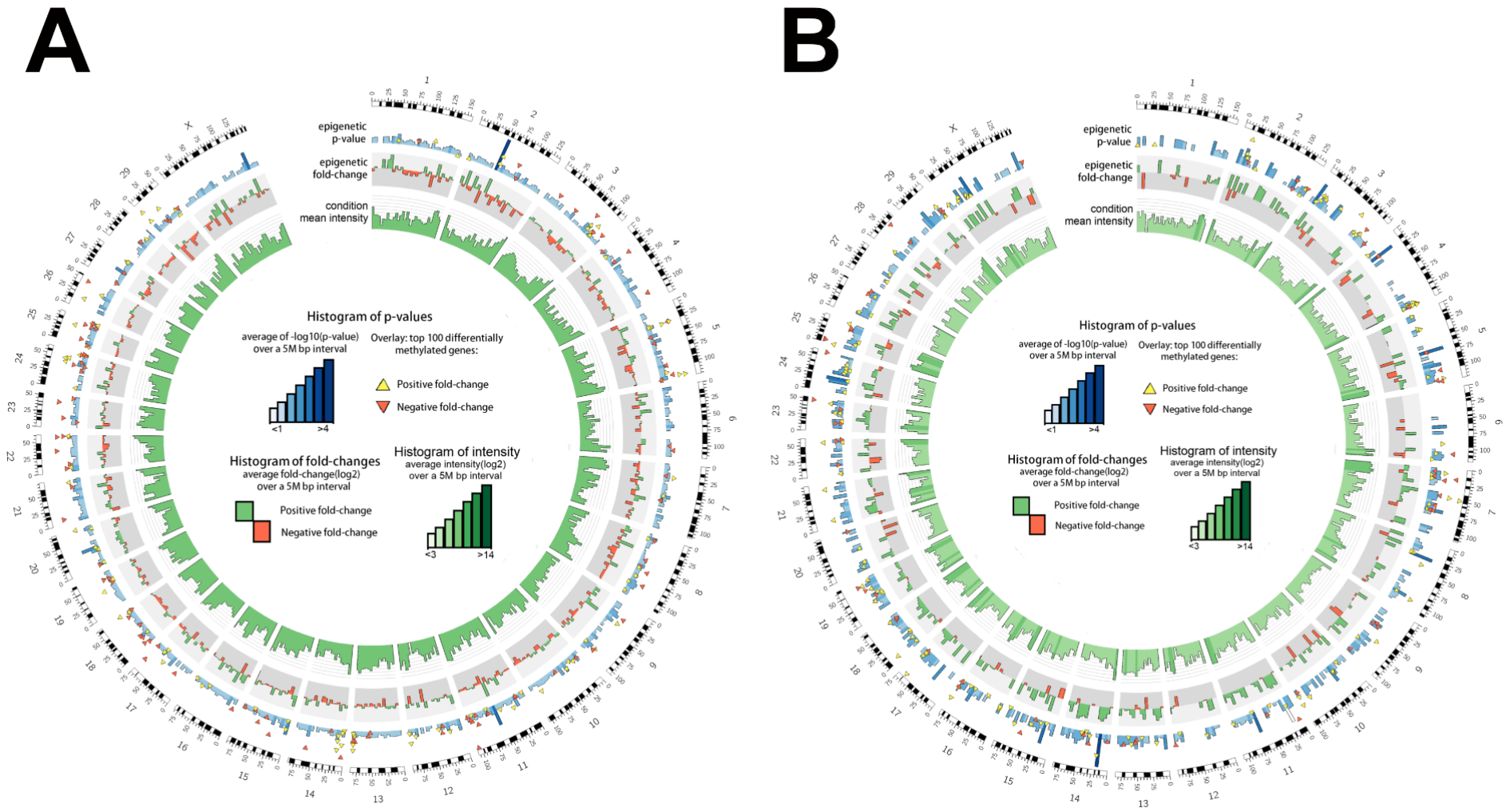


Figure 5-9 (Supplementary) Genome-wide distribution of differentially methylated regions identified in cultured bovine embryos. The circos plot shows the average epigenetic change in SAM-treated expanded blastocysts as determined using *HpaII* (**A)** and *Aci1I* (**B)** at intervals of 5 million base pairs across all chromosomes. From outside to inside: chromosome number, epigenetic p-value, epigenetic relative change and condition mean intensity.

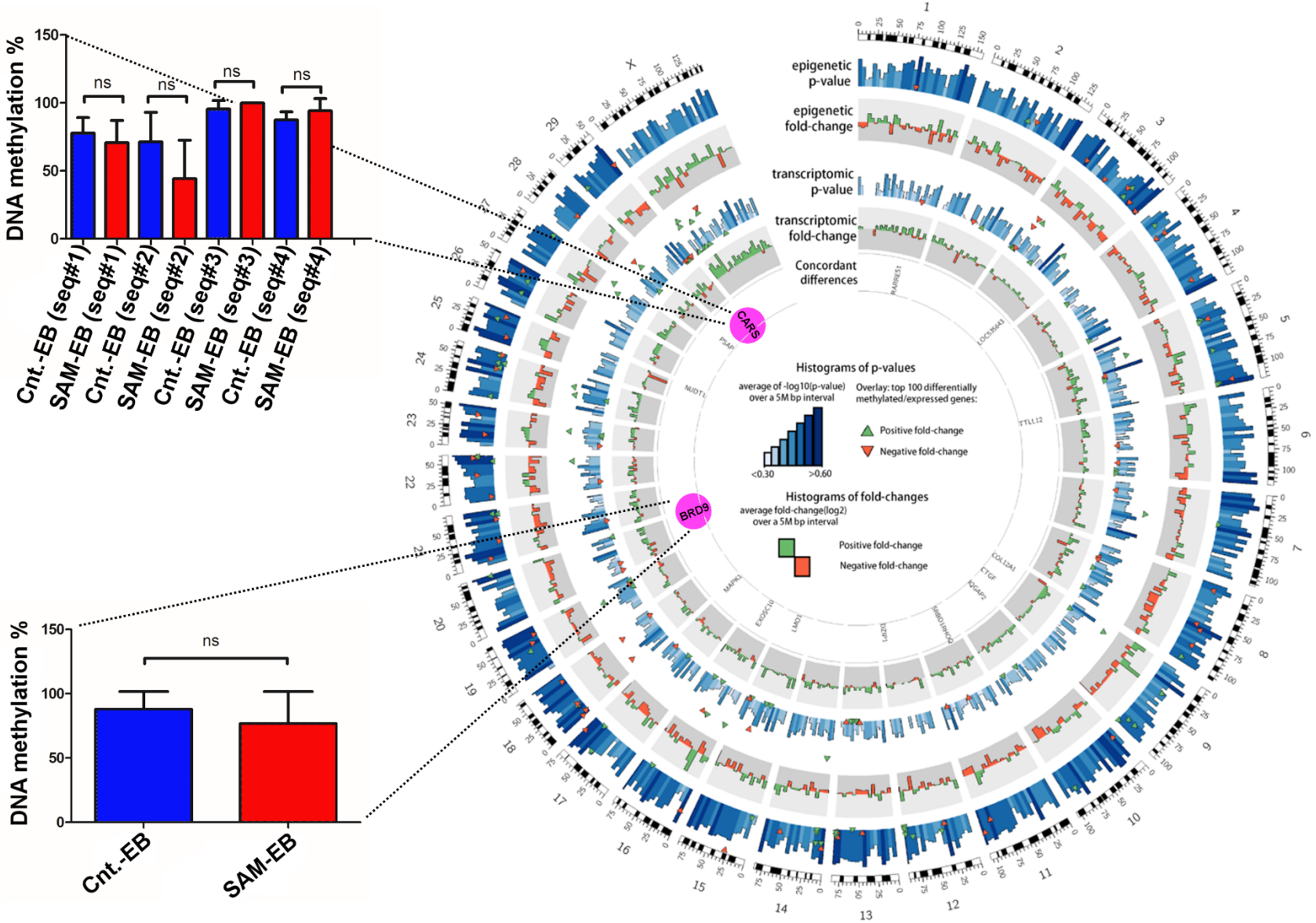

DNA methylation %
150
100
50
0
ns
ns
ns
ns
Cnt.-EB (seq#1)
SAM-EB (seq#1)
Cnt.-EB (seq#2)
SAM-EB (seq#2)
Cnt.-EB (seq#3)
SAM-EB (seq#3)
Cnt.-EB (seq#4)
SAM-EB (seq#4)
CARS
BRD9
epigenetic p-value
epigenetic fold-change
transcriptomic p-value
transcriptomic fold-change
Concordant differences
Histograms of p-values
average of -log10(p-value) over a 5M bp interval
<0,30
>0,60
Overlay: top 100 differentially methylated/expressed genes:
Positive fold-change
Negative fold-change
Histograms of fold-changes
average fold-change(log2) over a 5M bp interval
Positive fold-change
Negative fold-change
DNA methylation %
150
100
50
0
ns
Cnt.-EB
SAM-EB

Figure 5-10 (Supplementary) Parallel analysis of DNA methylation and gene expression in cultured bovine embryos. The circos plot represents changes in DNA methylation and gene expression in parallel in all bovine chromosomes. Transcriptomic and DNA methylation data from the same pools of embryos allowed us to search for correlations (concordant genes) suggesting that DNA methylation drives gene expression. None were found. From outside to inside: chromosome number, epigenetic change, transcriptomic P-value, relative change and concordant genes (genes with significant changes in both expression and methylation) **ns:** statistically non-significant.

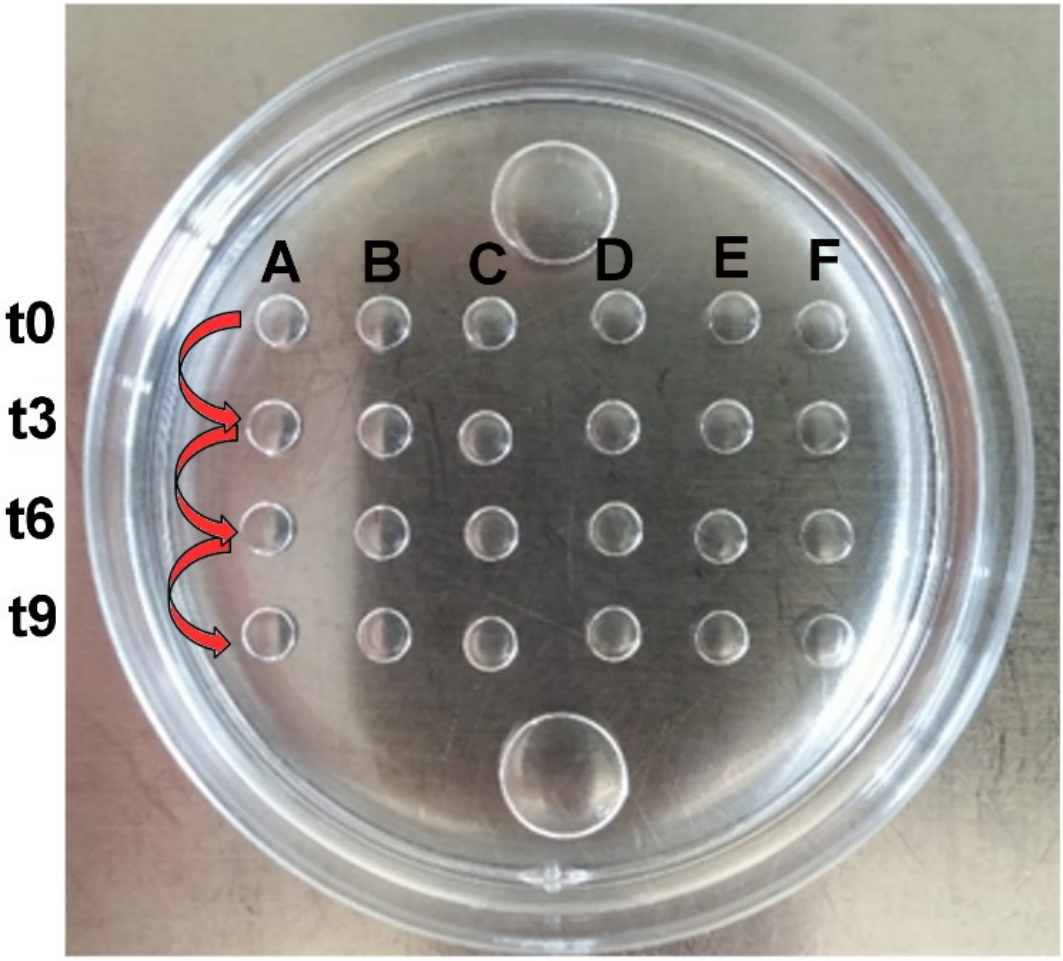


Figure 5-11 (Supplementary) IVC culture dish used for the treatment of bovine embryos with S-adenosyl methionine. The order in which embryos suspended in SOF droplets (8 µL) were treated is shown. Pools of 10 embryos per droplet were placed in each well (A-F) of the first horizontal line at t 0, and 2 µL of SAM solution (10 mM) and/or SOF (control) were added to each droplet after 3 h of incubation (t 3). For each renewal of the treatment (after 6 h, 9 h, etc.) the embryos were transferred to the droplets immediately below. This procedure was repeated until transfer to the next culture dish.

5.16. Supplementary Tables

Table 5-2 (Supplementary) The GO terms of genes analyzed by DAVID for all levels of biological process ontology (GOTERM_BP_ALL)

Annotation Cluster 1	Enrichment Score: 2.8094611592552248						
Category	Term	Count	%	PValue	Genes	Fold Enrichment	FDR
GOTERM_BP_ALL	GO:0007275~multicellular organismal development	19	14.72868	7.51E-04	RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, UCHL1, CDH2, ENPEP, YWHAE, CSRP3, CKB, NOTCH2, PSMC3, CTGF, CASP8, PRM1, SPAST, SPP1	2.334158	1.139721
GOTERM_BP_ALL	GO:0048856~anatomical structure development	17	13.17829	0.00121	RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, UCHL1, CDH2, ENPEP, YWHAE, CSRP3, CKB, PSMC3, CTGF, CASP8, SPAST, SPP1	2.407925	1.83161
GOTERM_BP_ALL	GO:0048731~system development	16	12.4031	0.001393	RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, UCHL1, CDH2, ENPEP, YWHAE, CSRP3, CKB, CTGF, CASP8, SPAST, SPP1	2.47539	2.105655
GOTERM_BP_ALL	GO:0048513~organ development	14	10.85271	0.002023	RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, CDH2, ENPEP, YWHAE, CSRP3, CKB, CTGF, CASP8, SPP1	2.616122	3.043634
GOTERM_BP_ALL	GO:0032501~multicellular organismal process	22	17.05426	0.002205	LYZ3, RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, UCHL1, MST1, CDH2, ENPEP, CSRP3, YWHAE, CKB, NOTCH2, PSMC3, CTGF, SERPINA5, CASP8, PRM1, SPAST, SPP1	1.95961	3.313917
GOTERM_BP_ALL	GO:0032502~developmental process	19	14.72868	0.002463	RBP4, RPSA, NANOG, PDPN, NDP, COL3A1, UCHL1, CDH2, ENPEP, YWHAE, CSRP3, CKB, NOTCH2, PSMC3, CTGF, CASP8, PRM1, SPAST, SPP1	2.104911	3.694936
Annotation Cluster 2	Enrichment Score: 1.6985015587291024						
Category	Term	Count	%	PValue	Genes	Fold Enrichment	FDR
GOTERM_BP_ALL	GO:0033273~response to vitamin	3	2.325581	0.011886	RBP4, NANOG, SPP1	17.72556	16.6844
GOTERM_BP_ALL	GO:0007584~response to nutrient	3	2.325581	0.014175	RBP4, NANOG, SPP1	16.18421	19.58129
GOTERM_BP_ALL	GO:0031667~response to nutrient levels	3	2.325581	0.047636	RBP4, NANOG, SPP1	8.459928	52.52925

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics.

	ID	Coef	PVal	Chromosome	Gene_Type	Gene_Symbol
1	EMBV3_17133	1.673063	0.012212	13	GENE	VIM
2	EMBV3_29572	1.611714	1.24E-07	9	NOVEL	NULL
3	EMBV3_08186	1.561526	0.006253	16	GENE	XCL2
4	EMBV3_02163	1.535193	0.008134	21	GENE	ISG12(A)
5	EMBV3_36695	1.494471	0.009979	21	GENE	SERPINA5
6	EMBV3_33422	1.351887	0.002465	6	GENE	IGFBP7
7	EMBV3_36151	1.33561	0.028657	5	GENE	LUM
8	EMBV3_03650	1.290352	0.0447	5	GENE	OLR1
9	EMBV3_08523	1.213819	0.016202	X	GENE	NDP
10	EMBV3_17805	1.213458	0.000119	X	GENE	LOC783362
11	EMBV3_02417	1.210686	0.000182	X	GENE	CT47B1
12	EMBV3_06148	1.201471	0.006815	24	GENE	CDH2
13	EMBV3_13727	1.109058	0.037175	16	GENE	FMOD
14	EMBV3_34100	1.10187	0.000465	5	GENE	LYZ3
15	EMBV3_20430	1.100363	0.021169	26	GENE	IFIT5
16	EMBV3_27187	1.048894	0.003901	9	GENE	SMOC2
17	EMBV3_19609	1.007696	0.022584	X	GENE	NDP
18	EMBV3_05142	0.995505	0.000736	X	GENE	LOC786942
19	EMBV3_08029	0.972618	0.004534	6	GENE	UCHL1
20	EMBV3_25612	0.959007	0.014377	9	GENE	MGC148328
21	EMBV3_01090	0.922085	0.042654	11	GENE	IFITM2
22	EMBV3_17889	0.916989	0.000558	2	GENE	COL3A1
23	EMBV3_12804	0.916983	0.04953	2	NOVEL	NULL
24	EMBV3_16519	0.912215	0.049949	16	GENE	MXRA8
25	EMBV3_42229	0.897675	0.012405	13	NOVEL	NULL
26	EMBV3_36574	0.891625	0.001107	6	GENE	LOC782977
27	EMBV3_04609	0.889486	0.01937	2	NOVEL	NULL
28	EMBV3_17723	0.883234	0.000622	24	GENE	SERPINB4
29	EMBV3_12256	0.873071	0.000205	19	GENE	MXRA7
30	EMBV3_38409	0.849255	0.00493	5	GENE	KCNMB4
31	EMBV3_20674	0.846799	0.025623	11	GENE	LOC613747
32	EMBV3_06496	0.839017	0.001213	2	NOVEL	NULL
33	EMBV3_08914	0.834702	0.000325	9	GENE	CTGF
34	EMBV3_14649	0.825776	0.000589	14	NOVEL	NULL
35	EMBV3_43421	0.821139	0.023516	7	GENE	FSTL3
36	EMBV3_13104	0.811304	0.012257	1	GENE	RARRES1
37	EMBV3_39515	0.80795	9.74E-05	4	GENE	PRKAG2
38	EMBV3_36658	0.791723	0.00095	9	GENE	NCOA7

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
39	EMBV3_30451	0.787578	0.044971	13	GENE	BMP2
40	EMBV3_38142	0.783625	0.000193	28	NOVEL	NULL
41	EMBV3_42584	0.774918	0.000162	23	NOVEL	NULL
42	EMBV3_11100	0.76232	0.043812	7	GENE	PAM
43	EMBV3_30553	0.760931	0.000579	29	NOVEL	NULL
44	EMBV3_34329	0.759212	8.42E-06	3	GENE	GBP4
45	EMBV3_13587	0.752869	0.000167	6	NOVEL	NULL
46	EMBV3_43410	0.750431	0.011986	29	GENE	CSRP3
47	EMBV3_21795	0.746521	0.001302	3	GENE	NOTCH2
48	EMBV3_18382	0.745057	3.84E-05	23	GENE	BOLA
49	EMBV3_37249	0.740203	0.002112	10	NOVEL	NULL
50	EMBV3_27365	0.727043	0.001704	26	GENE	RBP4
51	EMBV3_08270	0.723187	0.000141	8	GENE	ALDH1A1
52	EMBV3_06210	0.72223	5.02E-07	29	NOVEL	NULL
53	EMBV3_07255	0.721899	0.000145	5	GENE	NR4A1
54	EMBV3_40169	0.718863	0.003413	X	GENE	LOC781486
55	EMBV3_21535	0.711858	0.002934	6	GENE	SPP1
56	EMBV3_05238	0.711492	0.003794	5	GENE	MSRB3
57	EMBV3_07957	0.700655	0.000154	14	GENE	CPSF1
58	EMBV3_35334	0.695827	0.001959	28	GENE	PLAC9
59	EMBV3_16943	0.695566	0.001168	7	GENE	MGC137030
60	EMBV3_37583	0.695421	0.048896	10	GENE	LOC100125763
61	EMBV3_25242	0.693694	0.000958	2	GENE	CASP8
62	EMBV3_25252	0.69347	0.000598	X	GENE	WBP5
63	EMBV3_13421	0.69097	8.88E-05	24	GENE	KCTD1
64	EMBV3_14010	0.688722	3.08E-06	14	NOVEL	NULL
65	EMBV3_04070	0.685904	0.001072	3	GENE	CYP4A22
66	EMBV3_14949	0.68503	0.000707	8	GENE	TOPORS
67	EMBV3_04787	0.682661	0.000484	8	GENE	NXNL2
68	EMBV3_25622	0.68251	0.002103	29	GENE	ATG2A
69	EMBV3_25324	0.682462	0.000292	25	GENE	ZNF394
70	EMBV3_34430	0.68221	0.038737	19	GENE	C1QTNF1
71	EMBV3_25814	0.681403	0.002263	27	NOVEL	NULL
72	EMBV3_15309	0.679536	0.000271	29	GENE	LOC100296410
73	EMBV3_38269	0.678653	0.001835	12	GENE	DZIP1
74	EMBV3_42048	0.677204	0.012588	25	GENE	GP2
75	EMBV3_19433	0.675268	0.000126	2	GENE	SERPINE2
76	EMBV3_22567	0.673297	6.41E-05	X	GENE	TSPAN6
77	EMBV3_36359	0.672099	0.000704	17	NOVEL	NULL
78	EMBV3_07938	0.671687	0.001888	6	GENE	ENPEP

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
79	EMBV3_31781	0.670366	4.24E-05	15	NOVEL	NULL
80	EMBV3_37713	0.669678	0.000646	26	GENE	LOXL4
81	EMBV3_26105	0.669337	0.000138	15	NOVEL	NULL
82	EMBV3_27384	0.664003	0.016447	22	GENE	LOC788112
83	EMBV3_09661	0.659444	0.000339	13	GENE	TKDP1
84	EMBV3_00431	0.65752	4.40E-05	13	GENE	LOC616297
85	EMBV3_30638	0.657342	0.000126	8	GENE	LOC100336882
86	EMBV3_42650	0.656509	6.60E-05	15	NOVEL	NULL
87	EMBV3_04434	0.654777	0.001226	21	NOVEL	NULL
88	EMBV3_37707	0.654727	0.001083	20	GENE	MYO10
89	EMBV3_22395	0.654292	0.019398	10	GENE	GATM
90	EMBV3_21545	0.653849	0.000198	3	GENE	MFSD2A
91	EMBV3_19528	0.653585	0.000433	X	GENE	AP1S2
92	EMBV3_27312	0.647523	0.049085	7	GENE	IL27RA
93	EMBV3_13990	0.637387	7.92E-06	3	NOVEL	NULL
94	EMBV3_16606	0.632138	0.007331	23	NOVEL	NULL
95	EMBV3_40489	0.630793	0.000323	22	NOVEL	NULL
96	EMBV3_37628	0.630617	8.71E-06	2	NOVEL	NULL
97	EMBV3_42511	0.630158	0.001963	17	GENE	TRIM2
98	EMBV3_29144	0.627118	0.000178	11	GENE	ZAP70
99	EMBV3_42392	0.625873	0.013164	17	GENE	OAS1
100	EMBV3_11141	0.616988	0.001795	28	GENE	SIPA1L2
101	EMBV3_08701	0.612287	0.000165	15	GENE	TNKS1BP1
102	EMBV3_08430	0.612166	0.000419	10	GENE	LIN52
103	EMBV3_06518	0.612085	0.000117	13	GENE	TGIF2
104	EMBV3_37725	0.610632	0.004414	9	NOVEL	NULL
105	EMBV3_17818	0.608364	0.011302	21	NOVEL	NULL
106	EMBV3_07474	0.607178	0.00092	4	GENE	CD36
107	EMBV3_35502	0.606907	0.020168	3	GENE	MLLT11
108	EMBV3_00816	0.603886	0.003544	16	GENE	NAV1
109	EMBV3_09749	0.602955	0.000125	13	PSEUDO	LOC618696
110	EMBV3_37500	0.60232	0.002113	13	GENE	VAPB
111	EMBV3_27852	0.601451	0.005717	22	GENE	MST1
112	EMBV3_26065	0.600545	0.004089	X	GENE	PRDX4
113	EMBV3_23347	0.60007	0.01663	25	GENE	PRM1
114	EMBV3_07932	0.59795	0.00216	3	NOVEL	NULL
115	EMBV3_28166	0.597061	0.00148	19	GENE	MED13
116	EMBV3_01674	0.595392	0.005822	6	NOVEL	NULL
117	EMBV3_19978	0.591649	0.000189	5	GENE	MICALL1
118	EMBV3_19843	0.591184	5.19E-05	20	GENE	BRD9

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
119	EMBV3_29310	0.590835	0.000398	11	GENE	SPAST
120	EMBV3_18358	0.590727	0.00167	3	PSEUDO	LOC535643
121	EMBV3_43123	0.589611	0.00766	20	NOVEL	NULL
122	EMBV3_17585	0.588953	0.019849	6	GENE	C6H4ORF31
123	EMBV3_35377	0.588681	0.006893	23	NOVEL	NULL
124	EMBV3_18931	0.587466	0.022351	10	PSEUDO	FLRT2
125	EMBV3_28960	0.586979	0.008472	6	GENE	AFF1
126	EMBV3_03860	0.586529	0.000135	10	GENE	ZFYVE26
127	EMBV3_07328	0.585324	0.003158	2	NOVEL	NULL
128	EMBV3_37167	-0.58509	2.85E-05	23	GENE	PFDN6
129	EMBV3_39726	-0.58625	0.002787	NULL	NULL	NULL
130	EMBV3_28746	-0.58625	1.98E-05	NULL	NULL	NULL
131	EMBV3_04660	-0.58748	0.002068	NULL	NULL	NULL
132	EMBV3_42453	-0.58792	0.000672	NULL	NULL	NULL
133	EMBV3_17023	-0.5884	1.13E-05	22	GENE	RPSA
134	EMBV3_07077	-0.58907	0.000477	17	GENE	RPLP0
135	EMBV3_30115	-0.58908	0.000473	NULL	NULL	NULL
136	EMBV3_16610	-0.5895	0.003702	17	GENE	SLC25A1
137	EMBV3_11589	-0.59028	0.000157	25	GENE	NUDT1
138	EMBV3_26581	-0.59124	0.000551	NULL	NULL	NULL
139	EMBV3_00880	-0.59316	6.00E-06	NULL	NULL	NULL
140	EMBV3_40909	-0.59433	0.01157	10	GENE	FERMT2
141	EMBV3_15436	-0.59561	0.004904	18	GENE	DERPC
142	EMBV3_20588	-0.59653	0.020427	16	GENE	PDPN
143	EMBV3_20993	-0.59655	0.01233	29	NOVEL	NULL
144	EMBV3_07008	-0.59853	0.000229	13	GENE	SNRPB
145	EMBV3_36971	-0.60052	0.002431	24	GENE	NARS
146	EMBV3_35211	-0.60074	0.011303	7	NOVEL	NULL
147	EMBV3_26774	-0.60215	0.000183	17	GENE	ARPC3
148	EMBV3_23969	-0.60304	0.011328	12	GENE	ZAR1L
149	EMBV3_05540	-0.60494	0.001242	7	PSEUDO	LOC784642
150	EMBV3_05539	-0.60509	0.009556	NULL	NULL	NULL
151	EMBV3_17283	-0.60634	0.001934	NULL	NULL	NULL
152	EMBV3_42976	-0.61307	0.000235	11	PSEUDO	LOC785416
153	EMBV3_13397	-0.61858	9.91E-06	29	GENE	TMEM45B
154	EMBV3_19918	-0.62393	0.031139	24	GENE	EMILIN2
155	EMBV3_15785	-0.62437	0.001358	NULL	NULL	NULL
156	EMBV3_11182	-0.62485	0.01062	25	GENE	SLC12A9
157	EMBV3_22586	-0.62783	0.000258	17	GENE	LOC100336744
158	EMBV3_34427	-0.63284	0.000139	NULL	NULL	NULL

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
159	EMBV3_37322	-0.63367	4.08E-07	29	GENE	CARS
160	EMBV3_38532	-0.63386	0.009614	10	GENE	CALM1
161	EMBV3_41489	-0.63434	0.005719	28	GENE	PSAP
162	EMBV3_36234	-0.63856	0.002997	9	GENE	ACAT2
163	EMBV3_28695	-0.64063	0.000372	9	GENE	LOC100299681
164	EMBV3_29583	-0.64322	1.75E-07	NULL	NULL	NULL
165	EMBV3_36126	-0.64599	0.000304	19	GENE	ARHGDIA
166	EMBV3_33117	-0.6483	0.002528	NULL	NULL	NULL
167	EMBV3_41267	-0.65079	0.003683	NULL	NULL	NULL
168	EMBV3_15502	-0.65659	1.33E-05	21	GENE	MRPL46
169	EMBV3_13842	-0.65872	1.23E-05	20	GENE	SMN1
170	EMBV3_33260	-0.65882	0.002825	10	GENE	LOC524749
171	EMBV3_15225	-0.65944	0.002032	15	NOVEL	NULL
172	EMBV3_14295	-0.66259	0.032552	NULL	NULL	NULL
173	EMBV3_39172	-0.66355	0.004194	NULL	NULL	NULL
174	EMBV3_43681	-0.66689	0.000544	5	GENE	MYL6
175	EMBV3_16404	-0.66834	8.81E-05	NULL	NULL	NULL
176	EMBV3_37310	-0.67003	4.61E-07	20	GENE	BAG1
177	EMBV3_43343	-0.6719	0.004576	NULL	NULL	NULL
178	EMBV3_35126	-0.67242	0.000217	17	NOVEL	NULL
179	EMBV3_19771	-0.68422	0.004312	10	NOVEL	NULL
180	EMBV3_00276	-0.68625	0.00288	19	GENE	YWHAE
181	EMBV3_35024	-0.6865	0.002412	2	GENE	LOC100337018
182	EMBV3_28124	-0.68962	0.000147	4	NOVEL	NULL
183	EMBV3_29350	-0.68984	0.000522	7	GENE	TPM4
184	EMBV3_10343	-0.69638	0.000559	12	GENE	LOC100336086
185	EMBV3_13383	-0.69855	0.012554	NULL	NULL	NULL
186	EMBV3_02692	-0.70586	2.16E-05	8	GENE	SLC1A1
187	EMBV3_37840	-0.7076	0.001782	16	GENE	VAMP3
188	EMBV3_23121	-0.71298	0.00096	1	GENE	PDXK
189	EMBV3_03894	-0.7133	0.001004	9	GENE	EEF1A1
190	EMBV3_36191	-0.71374	0.010057	NULL	NULL	NULL
191	EMBV3_16546	-0.71718	0.000968	15	GENE	PSMC3
192	EMBV3_38859	-0.71777	0.000817	NULL	NULL	NULL
193	EMBV3_39570	-0.72554	5.93E-05	17	GENE	MAPK1
194	EMBV3_25457	-0.73056	1.62E-05	NULL	NULL	NULL
195	EMBV3_01945	-0.73098	0.001279	NULL	NULL	NULL
196	EMBV3_27556	-0.73348	0.008818	21	GENE	CKB
197	EMBV3_19530	-0.73625	8.47E-06	29	GENE	LOC100298939
198	EMBV3_22591	-0.73847	0.001509	18	PSEUDO	LOC100337420

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
199	EMBV3_36886	-0.74312	0.004347	5	GENE	LOC784294
200	EMBV3_03199	-0.74432	1.66E-05	NULL	NULL	NULL
201	EMBV3_01657	-0.74803	0.004595	15	GENE	LMO1
202	EMBV3_40324	-0.74893	0.003439	NULL	NULL	NULL
203	EMBV3_19570	-0.74907	0.000181	10	PSEUDO	LOC781059
204	EMBV3_38928	-0.75643	1.36E-05	1	PSEUDO	LOC781565
205	EMBV3_01949	-0.75846	0.011534	2	GENE	LOC528262
206	EMBV3_42262	-0.75919	0.006771	27	NOVEL	NULL
207	EMBV3_24050	-0.76071	0.000977	5	GENE	MYL6
208	EMBV3_07860	-0.76661	0.000385	7	GENE	canx
209	EMBV3_26295	-0.77661	1.03E-05	NULL	NULL	NULL
210	EMBV3_36067	-0.77922	2.91E-05	NULL	NULL	NULL
211	EMBV3_09705	-0.78374	0.009425	NULL	NULL	NULL
212	EMBV3_06088	-0.79212	0.00036	NULL	NULL	NULL
213	EMBV3_06455	-0.79636	9.47E-06	5	GENE	MYL6
214	EMBV3_00154	-0.8054	0.001213	NULL	NULL	NULL
215	EMBV3_14514	-0.80608	0.000427	8	GENE	SLC31A1
216	EMBV3_37946	-0.8086	0.002515	NULL	NULL	NULL
217	EMBV3_03684	-0.81331	0.00148	NULL	NULL	NULL
218	EMBV3_09181	-0.82204	0.000599	7	GENE	CNN2
219	EMBV3_19872	-0.84276	0.000521	17	GENE	SC4MOL
220	EMBV3_33390	-0.85889	3.84E-07	18	PSEUDO	LOC100337434
221	EMBV3_25171	-0.87223	0.000274	NULL	NULL	NULL
222	EMBV3_00068	-0.88406	2.99E-06	NULL	NULL	NULL
223	EMBV3_07344	-0.89602	0.026934	5	GENE	NANOG
224	EMBV3_36451	-0.90051	1.35E-05	NULL	NULL	NULL
225	EMBV3_02305	-0.90534	0.0003	NULL	NULL	NULL
226	EMBV3_35371	-0.92477	0.000762	25	NOVEL	NULL
227	EMBV3_23729	-0.94826	7.19E-05	27	NOVEL	NULL
228	EMBV3_27101	-0.98276	0.002871	8	GENE	LOC100337180
229	EMBV3_32081	-0.99816	0.002029	25	GENE	LOC100336700
230	EMBV3_18869	-1.00231	7.46E-09	NULL	NULL	NULL
231	EMBV3_41490	-1.00436	0.001481	25	GENE	LOC100336663
232	EMBV3_33870	-1.00789	0.012403	25	GENE	LOC100337210
233	EMBV3_41184	-1.01563	2.68E-05	NULL	NULL	NULL
234	EMBV3_09892	-1.02058	1.03E-05	6	GENE	RPL7A
235	EMBV3_14884	-1.08434	0.002257	2	GENE	LOC100337018
236	EMBV3_19967	-1.12388	0.000838	25	GENE	LOC100336625
237	EMBV3_32083	-1.19957	0.001056	25	GENE	LOC100336512
238	EMBV3_16319	-1.25679	0.000859	25	GENE	LOC100337406

Table 5-3 (Supplementary) The list of identified differentially expressed transcripts in the bovine embryo genome and their characteristics (*continued*).						
239	EMBV3_20623	-1.33929	0.000473	25	GENE	LOC100336470
240	EMBV3_28462	-1.37443	0.001033	25	GENE	LOC100337255
241	EMBV3_17731	-1.47237	0.00038	25	GENE	LOC100337423
242	EMBV3_38059	-1.53688	0.000446	25	GENE	LOC100337385

Null: There is no information.

Novel: Novel transcript mainly represents long non-coding RNA.

Table 5-4 (Supplementary) Properties of the selected differentially methylated regions of the bovine embryo genome and the primers designed for their pyro-sequencing.

Gene	Bovine genomic sequence number	Pyrosequencing primers	Sequence	*Temp.*
XRCC	chr18:52086188-52087342	Forward Reverse (5'- Biotinated) Sequencing	5'- GGTGGGTTGTGTTGAGGTTTTTAG-3' 5'- CATCCTAAATCCACTCTCCC-3' 5'- GTTGTTTGTTGTTTTGGAGG -3'	52 ^{0}C
PNKP	chr18:56668624-56669787	Forward Reverse (5'- Biotinated) Sequencing	5'- GGAATTTGGAGGAAGTTAGTTAGAATTTGG-3' 5'- CCCATCCACAAACAAACCTAACC-3' 5'- GGAGGAAGTTAGTTAGAATTTGG-3'	53 ^{0}C
PARP-seq#1,2	chr16:30159834-30159904	Forward Reverse (5'- Biotinated) Sequencing	5'- GGTAGAGTTTGTTTGAAG-3' 5'- GACAATAACAACAAC-3' 5'- GGTAGAGTTTGTTTGAAG-3'	43 ^{0}C
TET3-seq#1	chr11:10594945-10595628	Forward Reverse (5'- Biotinated) Sequencing	5'- AGGGTGGGGTTTAAGAGTTTG-3' 5'- GTCTCCAAACAACCTAAAAAC-3' 5'- TTAATAAGTGTTTAGGTGTC-3'	50 ^{0}C
TET3-seq#2		Forward Reverse (5'- Biotinated) Sequencing	5'- GTTTTTAGGTTGTTTGGAGAC-3' 5'- CTACAACCTCCTATACTAAC-3' 5'- GGTTTTAGTTGTTTTATTGTTTTG-3'	46 ^{0}C
DNMT3B- seq#1	chr13:62711472-62712998	Forward Reverse (5'- Biotinated) Sequencing	5'- GTTGGATTTGGTTGGTTGTGTTC-3' 5'- GTATTACTACACAAAAACAACTC-3' 5'- GTATTGTATC/TGTGTGTTGC/TGAGGG-3'	48 ^{0}C
DNMT3B- seq#2		Forward Reverse (5'- Biotinated) Sequencing	5'- GGTTTGTAGATTTAGGTTAGTGG-3' 5'- GAACACAACCAACCAAATCC-3' 5'- GTTTTTTTTTGGTTGTTAGG-3'	49 ^{0}C

Temp. (^{0}C): *Annealing temperature.*

Table 5-5 (Supplementary) Properties of the selected differentially methylated regions of the bovine embryo genome and the primers designed for their pyro-sequencing (*continued*).

Gene	Bovine genomic sequence number	Pyrosequencing primers	Sequence	*Temp.*
BMP	chr10:66754123-66755450	Forward Reverse (5'- Biotinated) Sequencing	5'- GTTTC/TGAGC/TGTAGTGGTTG-3' 5'- GCAACTCCTCTAAACCTTTC-3' 5'- GGAGGGTAGAGTGAATT-3'	49 ^{0}C
CARS	chr29:49230867-49231380	Forward Reverse (5'- Biotinated) Sequencing	5'-GGGGTTGATTGTTTTTTTAAGGGTC-3' 5'- CAAAAC/TGCTCTAAAACTTTCC-3' 5'- TTTTTAAGGGTC/TGGGTTGGG-3' 5'-GGTC/TGGGTTGGATTTTTG-3' 5'- GGC/TGTTTTGTTGAGTGAGC-3' 5'- TTAC/TGGGGTAGTAGGGAG-3'	49 ^{0}C 49 ^{0}C
BRD9	chr20:71523333-71524254	Forward Reverse (5'- Biotinated) Sequencing	5'- GGGC/TGTGAGTTTTAGTAGTC-3' 5'- TCCCTAAAAAATAACC/TGACC-3' 5'- TAGGGAGTGTGTGTGAGGTG-3'	48 ^{0}C

***Temp.* (^{0}C):** *Annealing temperature.*

Table 5-6 (Supplementary) Properties of the selected genes and the primers designed for their amplification by RT-qPCR.

Gene	primers	Sequence	*Product length (nt)*	*Ann. Temp. (^{0}C)*	*Acq. Temp. (^{0}C)*
XRCC	Forward/reverse	5'- CGAGCAGAAGGAAGAAAGGC-3' 5'- ATGTAGTCCTCAAGCTCCCC-3'	243	57	82
PNKP	*Forward/reverse*	5- 'GATCGACAACACAAACCCCG-3' 5'- GCCTCGAACTGCTTCCTGTA-3'	204	57	88
PARP	*Forward/reverse*	5'- GTGCCAACTACTGCCACAC-3' 5'- TGAAATCCCTGTCCCGAGAG-3'	218	57	84
Tet3	Forward/reverse	5'-TGCAGACTGCCATTGCCATTGA -3' 5'-AAAGGATGGGTGCTCTTCAGG -3'	203	57	84
Dnmt3b	Forward/reverse	5'-CCTGTGATAGCATCCAAGAAT -3' 5'-GAAAGCCGAAGATCCTTTCT -3'	205	57	76
APEX	Forward/reverse	5' - GCTCTGTGACAACCTCTTCC-3' 5'- ACCCCTACACAAACACCCTT-3'	201	57	76
LIG3	Forward/reverse	5'- AGCAAAGGTGGCATGATGTC-3' 5'- TGGAACGATGAAGTCAGGGT-3'	216	57	85
BMP4	*Forward/reverse*	5'- GTGTGTGTGTGTCCCCAAAG-3' 5'-CACCTCAACTCAACCACCCA -3'	202	57	83
ERCC 130	*Forward/reverse*	5'- ACAAACGCAGGGAAATCAAC-3' 5'-AATGTCGTCGGAAGATCCTG-3'	173	57	82

Temp. (^{0}C): *Annealing temp; Acquisition temperature (C)*

6. General Conclusion

The overall objective of this thesis was to develop a streamlined and sensitive platform, compatible with low gDNA input, to study genome-wide DNA methylation and transcription profiles of ART-treated bovine early embryos and gametes. The general hypothesis of this doctoral thesis was that ART treatments not only lead to transcriptomics effects in early embryos but also impact the maintenance of DNA methylation patterns in both gametes and early embryos. In addition, such deviation in DNA methylation patterns is correlated with response to the stimuli and have short- and long-term impacts on development.

Research carried out in this project allowed us to test our hypothesis. Initially a bovine-specific genome-wide DNA methylation analysis platform was developed which enabled us to perform parallel analysis of global DNA methylation and transcription of the given sample with minimum requirement of gDNA input (*i.e.* 10 ng gDNA; corresponding to 10 expanded bovine blastocysts). Such a platform is therefore compatible with early embryo DNA methylome analysis, given the limited amount of DNA in single early embryos. As a proof of concept, global DNA methylation profiles between bovine blastocysts and sperm were determined. The general results of this study demonstrated that similar to other studied species such as human, mouse and zebra fish, bovine sperm is notably hypermethylated compared to blastocysts (Smallwood *et al.,* 2011; Smith *et al.,* 2012a; Andersen *et al*., 2012). It was also revealed that for monozygotic twin bulls, that were generated via embryo splitting (one of the

ART methods) and exhibited different progeny performances, there was a variation between the genome-wide sperm DNA methylation profiles, despite the fact that they had identical genetic backgrounds. Such epigenetic variation was suggested may contribute to incongruous diverging performances of the daughters sired by bulls that are monozygotic twins. Furthermore, it was demonstrated that high dose supplementation of SAM as global methyl donor, which is the major product of folate-dependent one-carbon metabolism, can cause deviations in the normal phenotype, transcriptome and epigenome (DNA methylation) of the treated embryos. It was shown that a high dose supplementation of SAM led to higher blastocyst hatching rates at d8 blastocysts stages and greater number of male embryo development at d7 blastocysts stages. Furthermore, it led to global DNA hypermethylation and over/under expression of transcripts (coding and long non-coding RNAs) in the treated early embryos with no correspondence between the differentially methylated regions (DMRs) and differentially expressed genes (DEGs). Overall, this project can contribute to the field of animal and human reproductive biology and can be used as basic knowledge for more in-depth future investigations as discussed following:

6.1. Achievements and original contributions

During the course of this thesis project, the achievements of the three major objectives permitted to demonstrate some key points. The developed platform, in addition to the advantage of parallel genome-wide study of bovine DNA methylation and transcription, included a complete pipeline for data analysis and visualization. Furthermore, using this platform, genome-wide DNA methylation profile of the bovine sperm and blastocysts were compared and revealed notable differentially hypermethylated regions in bovine sperm in comparison with bovine blastocysts. This was the first evidence of non-fluorescent staining study demonstrating the low levels of global DNA methylation at bovine blastocyst as well as revealing the extent of global high DNA methylation level in bovine sperm. Indeed, a unified and reliable approach (*i.e.* HELP cocktails) which was employed to analyze small amounts of genomic DNA in the developed epigenetic platform of this project, offering a good balance between genomic coverage and data turnaround time which has been already successfully applied in different studies by different groups (Caballero *et al*., 2014; Dobbs *et al*., 2014; Salilew-Wondim *et al*., 2015; O'Doherty *et al*., unpublished). Furthermore, implementing of a microarray approach into the platform as well as the built-in data analysis pipeline provide efficient means for data interpretation even for researchers with limited bioinformatics resources and knowledge. Moreover, similar approach used for this objective could be adapted easily to any species which the entire genome is known.

The second objective, presented in the article “Genome-wide analysis of sperm DNA methylation from monozygotic twin bulls” allowed us to reveal the presence of sperm global DNA methylation deviation between the monozygotic twin bulls with different progeny performances (daughters’ performance records) in spite of their identical genetic materials. Initially, the semen end points of the monozygotic twin bulls were profiled and showed some extent of divergent semen quality including sperm morphology, motility and viability between the monozygotic twin brothers. Such a difference in sperm motility within monozygotic twin bulls (Galli *et al*., 1997) or genetically identical quadruplet bulls (Lessard *et al*., 2003) has been already reported. However, it is also controversial as in some study no significant difference between the motility and velocity of fresh and frozen semen of identical monozygotic twin bulls were found (Braun *et al* 1990). Moreover, it appears that such a gamete variation can be observed solely in male identical twins (Machado *et al*., 2006). In addition, demonstrating that up to 10% of all probes (400 K) in the arrays were found to be differentially methylated between the MZ twin pairs allowed us to suggest that differences in the sperm epigenome may contribute to incongruous diverging performances of the daughters sired by bulls that are monozygotic twins. This report reveals the presence of sperm epigenetic (global DNA methylation) difference between the monozygotic twin bulls. However, the origin of spermatic epigenetic discordance between the monozygotic twin bulls remains unknown. Considering the fact that these monozygotic twin bulls were generated through an ART

method called embryo splitting and then transferred to different recipient cows (*i.e.* in utero environments) allowed us to hypothesize that different pre-natal conditions resulted from developing in different uterine environments or other post-natal effects from environmental exposures and conditions may induce the observed epigenetic differences. As discussed later (6.2. research perspective), the results of this study can open the door to use monozygotic twin bulls for further explorations of critical in utero and post-natal environments effects on epigenetic variation and subsequently development and health as well as potential long-term impacts on offspring.

The results of the third objective were presented in the written (unpublished) manuscript entitled “Responses of bovine early embryos to S-adenosyl methionine supplementation in culture”. Currently, there are growing concerns regarding the potential adverse effects of high dose supplementation of folic acid (FA) to pregnant women during pre- and post-natal development (Barua *et al*., 2014; Ulrich *et al*., 2006; O'Neill *et al*., 2014; Aarabi *et al*., 2015; Christensen *et al*., 2015; Allen *et al*., 2012). In this study, through investigating the direct supplementation of S-adenosyl methionine, the final substrate of folic acid for methylation process, on IVC bovine early embryos, it was aimed to address how early embryos cope with excess global methyl donor and if early embryos would not react to supraphysiological SAM concentration, the observed consequences of high FA would need to be investigated in later stages of development. However, based on the results of this study, it was shown that SAM at high dose led to notable changes at phenotype (sex ratio, hatching rate) and molecular

levels (transcription and DNA methylation) of early embryos. Hence, it can be suggested that at high concentration, SAM can have an impact on early embryo development through deviation in sex ratio, transcription (coding and non-coding RNAs) and DNA methylation. In addition, through this study an evidence of compensatory epigenetic responses to external stimuli in early embryo was suggested which has been already proposed (Barker *et al*., 2002; Vyssotski 2011; Crews & Gore, 2012; Gambini *et al*., 2012; Youngson & Morris, 2013, Dalton *et al*., 2014; Tait *et al*., 2015; Weyrich *et al*., 2015).

Together, the results of the three objectives provide support the research project hypothesis that ART treatments and manipulations of early embryo and its environment can lead to deviations in transcription of early embryo and changes in establishment of DNA methylation patterns in gametes and early embryos. Furthermore, the results suggested that these effects could be associated with response to the stimuli and can cause potential short- and long-term impacts on development (Denomme and Mann, 2012; van Montfoort *et al*., 2012; El Haji & Haaf, 2013; Uyar & Seli, 2014; Tarín *et al*., 2014; Urrego *et al*., 2014; Hoeijmakers *et al*., 2015). Furthermore, the outcomes of this project resulted in development of a platform to study genome-wide DNA methylation profile of bovine early embryos and gametes and subsequently generating several lines of novel information and knowledge regarding the bovine sperm and early embryo methylome and their deviations following ART manipulations (Anckaert & Fair, 2015). Thus, current PhD project provided new information about ART-treated bovine early embryo and sperm DNA methylome which can advance our current

knowledge about bovine sperm and early embryo epigenetics. These new information include the potential epigenetics (DNA methylation) deviations in the mature germ cells (sperm) of monozygotic twin bulls produced by embryo splitting despite their identical genetic information. And the notable phenotypic (higher hatching rate, expanded blastocysts sex ratio deviation in favor of male embryos), transcriptomics (DE coding and long non-coding RNAs) and epigenomics (genome-wide DNA hypermethylation mainly at exonic regions and CpG islands) impacts of excess supplementation of universal methyl donor (SAM) on *in vitro* culture bovine early embryos.

6.2. Research perspectives

In light of the results reported in this thesis, certain perspectives can be suggested for each objective and identified results for more in depth comprehension of the proposed hypothesis. The following suggestions can be made for every each of the three objectives in this research project:

6.2.1. The first objective

Currently, 5hmC has been receiving growing attentions because of its possible and mainly unknown roles in early embryo development (Wossidlo *et al*., 2011; Inoue & Zhang, 2011; Zhao & Chen, 2013; Hill *et al*., 2014; Xu & Wong, 2015; Amouroux *et al*., 2016) and differentiations (Ficz *et al*., 2011; Koh *et al*., 2011; Koh & Rao, 2013; Shen & Zhang, 2013). Several technologies developed for analysis of 5hmC (Booth *et al*., 2012b; Yu *et al*., 2012; Song *et al*., 2012a; Yuan and Feng, 2014). However, they all require high gDNA as starting materials

(Yu *et al*., 2012), therefore due to requirement for large number of samples sizes, currently it is challenging to apply them for study 5hmC in early embryos.

- Considering the specific features of the developed platform in this project, it is possible to use specific MSRE such as BfaI (5 U in 10× NEB4, New England Biolabs at 37C 12h) to discriminate between 5mC and 5hmC (de Montrea *et al*., 2013). Doing so, one can investigate the potential impacts of ART-treated embryos on 5hmC as an important and new epigenetic mark modifications to understand its generation and genomic distribution with critical roles in epigenetics (Yoshizawa *et al*., 2010; Zhu *et al*., 2014). Currently, such information is missing and further investigation are required to be carried out.

Non-cytosine methylation such as N (6)-methyladenine (6mA) has been recently suggested to possibly play role as new carrier of heritable epigenetic information in eukaryotes (Greer *et al*., 2015; Sun *et al*., 2015; Luo *et al*., 2015). Such information regarding the roles of 6mA obtained from several invertebrate model systems (Fu *et al*., 2015; Greer *et al*., 2015; Zhang *et al*., 2015), however, exploring the presence of 6mA modification in vertebrates, especially in mammals remains to be investigated. It has been also suggested that 6mA might have important roles in a developmental stage-dependent and/or cell lineage-dependent manner despite expected their extremely low levels in mouse and human (Sun *et al*., 2015). Currently, several approaches are available to detect 6mA. For instance, methyladenine is detectable by chromatography-based technologies, such as the Ultrahigh performance liquid chromatography-triple quadrupole mass spectrometry coupled with multiple-reaction

monitoring (UHPLC-MRM-MS/ MS) method or sequencing approaches. For the specific quantification of methyladenine, next generation sequencing (NGS)-based strategies which are coupled with immunoprecipitation of 6mA (6mA-IPseq) or restriction enzyme guidance (6mA-REseq) can be used (Heyn & Esteller, 2015; Luo *et al*., 2015).

- Considering our developed platform, it is possible to use MSRE that recognize and target adenine in specific recognition sequence and can discriminate between the methylated and non-methylated adenine. Fortunately, there are isoschizomer MSRE enzymes DpnI and DpnII which discriminate methylated and non-methylated adenine, respectively in GATC sequence (Dyachenko *et al*., 2013). The digestion (DpnI & DpnII restriction enzyme; 5 U in 10× NEB1.1 & 3.1, respectively New England Biolabs at 37C overnight) is compatible with the developed epigenetic method in this project. However, this study could be challenging due to several reasons such as extremely low frequency of the 6mA in mammals, lack of knowledge of the frequency of the DpnI and DpnII recognition sites in bovine genome as well as in the designed probes of our developed array which both can be determined through in silico analysis. In addition, validation can carry out through restriction-enzyme-digestion assay followed by quantitative PCR (6mA-RE-qPCR) to quantitatively evaluate the methylation status on specific sequences of interest (Fu *et al*., 2015). Altogether, it appears to be feasible to use this approach to investigate the presence of 6mA in bovine early embryo for the first time.

6.2.2. The second objective

There are few important limitations associated with the study for the second objective. Although, the study provided valuable information with respect to the variations of DNA methylation between the MZ twin bulls and the association of the common differentially methylated loci with fertility quantitative trait loci (P=0.033) but due to the low sample size, the statistical and bioinformatics analysis were restricted. In fact, the power of discordant MZ twin studies to detect DMRs will depend on several factors including sample size, the effect size of the epigenetic change on the phenotype, the similarity of methylation profiles between MZ twins, and the sensitivity and coverage of the methylation assay (Bell & Spector, 2011). Hence, in our case, specifically due to limited sample size a number of important information such as the correlation of the sperm characteristics with DNA methylation profile (Montjean *et al*., 2015) and the important question, if the observed DNA methylation difference between MZ twin bulls sperm were random effects or due to environmental exposures remained to be addressed. Indeed, for the latter, it is necessary to perform comparison between the twin bull pairs (genetically related) and random non-twin bulls (genetically non-related bulls). Although such a comparison was already performed in this study but due to the low sample sizes, it was not feasible to reveal the causation of the detected DNA methylation difference in MZ twin bulls sperm. Therefore, to address these important missing information, there is a necessity to perform the similar experiments with a larger sample size and subsequently proper analysis design (Tan *et al*., 2015; Bell & Spector, 2011) to comprehensively investigate the DNA methylation profile of MZ twin bulls sperm.

Although share a common genotype, most monozygotic twin pairs are not identical in terms of several types of phenotypic discordance such as differences in susceptibilities to disease and a wide range of anthropomorphic features mainly due to epigenetics effects and differences (Fraga *et al*., 2005; Petronis 2006; Kaminsky *et al*., 2009; Bell & Spector, 2011). Therefore, twins are useful model to study the genetic and environmental contributions to epigenetic regulation of gene activity during developmental, ageing and disease processes (Bell & Saffery, 2012 Van Dongen *et al*., 2014). However, monozygotic (identical) twins, in particular are becoming a popular and powerful tool for epigenome-wide association study to link environmental exposure to differential epigenetic regulation and to disease status while controlling for individual genetic make-up (Singh *et al*., 2002; Kaminsky *et al*., 2009; Zwijnenburg *et al*., 2010; Van Dongen *et al*., 2014; Tan *et al*., 2015). Regardless of several but limited epigenetic studies on human MZ twins (Bell & Spector, 2011; Tan *et al*., 2015), currently there is not any laboratory animal models to use for (MZ) twin studies including epigenetics analysis.

It appears that production of MZ twin through embryo splitting in cattle (Illmensee & Levanduski, 2010; Rodriguez-Martinez, 2012; Seidel, 2015) can serve as a valuable tool to produce MZ animal model for epigenetics studies especially those related with complex traits (Tan *et al*., 2015). Therefore, at the global level various hypothesis and experiments can be proposed to use MZ twin cattle in order to investigate several important questions in the field of reproductive biology, nutrition & metabolism, diseases susceptibility and etc. For instance,

in terms of the reproductive biology, using MZ twin cattle a number of studies can be designed and performed which following are some examples:

1) It is noteworthy to investigate under fully control conditions (identical maternal diet, age, breed, maintenance and etc.) if there would be any and if so, in what extent, epigenetic (DNA methylation) variations between the generated demi-embryos following in utero transfer; at different levels such as placenta and developing fetus during pregnancy (first trimester and etc.). Since demi-embryos are transferred to different recipient cows, this study will address a current important question whether in utero environments lead to epigenetic variations in developing embryo or fetus? (Teh *et al*., 2014).

2) Barker proposed the concept of fetal programming by explaining incidence and relationship among poor maternal physique and health, poor fetal growth, and high death rates from cardiovascular disease in adult humans (Barker, 1992). Currently, it is now clear that maternal nutrient status can cause epigenetic alterations to the genome of the developing fetus with potential impact on future generations (Rhind, 2004; Zeisel, 2009; Ashworth *et al*., 2009; Canani *et al*., 2011; McKay & Mathers, 2011). There are numerous studies in cattle showing that maternal nutrition or early postnatal nutrition have permanent and profound effects on physiology and reproduction performances in both sexes (Greenwood & Café, 2007; Ashworth *et al*., 2009; Funston *et al*., 2010a; Funston *et al*., 2010b; Long *et al*., 2010). Monozygotic twin model can serve as an

excellent mean to investigate the impact of maternal nutrition on fetus epigenome (DNA methylation). Having identical genetic background, it feasible to use bovine MZ twin to investigate the extent of maternal nutrition effects on fetus DNA methylation profile. This serves as excellent model because it is devoid of potential DNA sequence SNP effects on DNA methylation profile; therefore, increasing the power of detection and interpretations to examine which variation is related to individual-specific environmental influences and stochastic events or to familial factors (*e.g.* DNA sequence) (Kaminsky *et al*., 2009; McRae *et al*., 2014; Van Dongen *et al*., 2014). The developed platform in this study can be used to investigate genome-wide DNA methylation profile between MZ twin under different maternal nutrition diets and then through GO and pathway analysis of identified DMRs, one can detect which set of genes with similar annotations are affected and how it can be translated to adult physiology and reproductive performances.

6.2.3. The third objective

According to the results of the third objective study, it was revealed that early embryo reacted to excess *in vitro* SAM (key output of the folate one-carbon metabolism) supplementation in its microenvironment. Now, due to the current growing concerns regarding the high dose folic acid supplementation pre- and during gestation and potential adverse effects on embryo (Picke *et al*., 2011; Mikael *et al*., 2013), the exciting question is whether *in vivo* supplementation of high dose folic acid pre- and during gestation leads to higher folate concentration in microenvironment of developing early embryos such oviductal fluid? If so, would higher folate concentrations cause similar observed results to the third objective of this project? And if not, it would suggest that high folate supplementation prior gestation may either impacts its effects on endogenous concentration of folate in ovulated oocyte (Boxmeer *et al*., 2008; Twigt *et al*., 2015) as an important source of one-carbon metabolites required for early preimplantation development (Steegers-Theunissen *et al*., 2013) and/or induces its impacts post implantation through higher folate transition from maternal blood to placenta and fetus (Laanpere *et al*., 2010) or even combination of both. Hence, several experiments are required to be carried out to address these fundamental questions.

- In this regards, due to ruminal digestion ((NRC), 2001), pre-gestational (three months) folic acid supplementation in treated group of cows can be performed via intramuscular injection of 320 mg of folic acid (pteroylmonoglutamic acid, ICN Biochemicals Inc., Cleveland) which has already demonstrated to result in high folate

concentration in blood serum (Gagnon *et al*., 2015). However, the control cows will receive only saline injection. Then, both groups will be synchronized and inseminated with identical semen and ultimately the *in vivo* produced embryos can be recovered (Salilew-Wondim *et al*., 2015) at d7 blastocyst stage for phenotypic, transcriptome and epigenome analysis using the developed platform in this project. Doing so, one can investigate if high folic acid supplementation leads to similar observation with the results of the study (third objective) in this project. Furthermore, it is noteworthy to collect and measure the folate concentration in ovuductal fluid in treated cows to determine whether high folate in the blood serum will result in higher folate in the microenvironments of early embryo in oviducts.

- Based on the results of the third objective study, another exciting question which remain to be answer is whether *in vitro* SAM-treated d7 expanded blastocysts transferred to synchronized recipient cows for in utero and *in vivo* studies of the post hatching embryos leads to long-term effects on embryos, fetuses and offspring. Doing so, it will possible to study phenotype, transcriptome and DNA methylome of the ovoid and/or elongated conceptus at d13 and d16, respectively; implantation and placenta formation as well as the potential impacts on fetus and the produced calves and adults such as impacts on their sex ratio, health and etc.

- Regardless of sex chromosomes, male and female early embryos differ in their metabolism transcripts and epigenetics patterns (Mao *et al*., 2010; Shang *et al*., 2015;

Gallou-Kabani *et al*., 2010; Gabory *et al*., 2013; Guti *et al*., 2001; Jiménez *et al*., 2003, Pérez-Crespo, *et al*., 2005; Ricart *et al*., 2009; Rosenfeld *et al*., 2014; Sinclair *et al*., 2007). Therefore, generally there is a sex-specific chromosome-led hormone-independent sexual dimorphism sensitivities to maternal nutritional perturbations for early embryo development (Fernandez-Gonzalez *et al* 200; Gallou-Kabani et al., 2010; Guti *et al*., 2001; Jiménez *et al*., 2003; Pérez-Crespo *et al*., 2005; Ricart *et al*., 2009; Rosenfeld *et al*., 2014; Sjöblom *et al*., 2005). Considering these facts, it is noteworthy to analysis the extent of the confirmed DNA methylation for the candidate genes in this objective to address the important question whether the observed sex ratio deviation in this study may impact on the epigenome results. Male and female embryos can be *in vitro* produced by sexed semen following similar *in vitro* SAM concentration and then collection at expanded d7 blastocysts stage for individual pyrosequencing confirmation of the interested genes in the third objective study. If it is determined that sex can impact the DNA methylation results, then it would be quite interesting to investigate the genome-wide DNA methylation analysis on the pool of discriminated male and female embryos.

- In addition to the studying the impact of high dose SAM on the global DNA methylation, investigating the very likely impacts of high dose SAM on histone modifications at genome-wide level (Shyh-Chang *et al*., 2013) would be very informative. Thanks to the current developed ultra-low-input micrococcal nuclease-based native ChIP (ULI-NChIP)

method which is compatible with early embryos histone modifications analysis (Brind'Amour *et al*., 2015), it is now feasible to investigate impacts of excess SAM on histone modifications in early embryos.

- Since very recently it has been shown that high folic acid consumption reduces MTHFR protein and activity levels in mouse liver (Christensen *et al*., 2015), it would be noteworthy to investigate the level of MTHFR protein expression in SAM-treated expanded blastocysts to see if high dose of SAM can impact on MTHFR protein expression and activity in early embryos.

6.3. Final statement

Currently animal production is facing many challenges such as global competitiveness, insufficient animal-source foods for the global population and environmental challenges, including worldwide climate changes and pollution, which necessitate to introduce and apply new and innovative strategy for animal production for the next generation. Epigenetics as a growing research field has a great potential to address this demand to study the environmental effects on livestock performances and productivity. Epigenetics is the study of heritable changes in gene expression potential that do not involve a change in DNA sequence and includes several types of modifications to the genome which can result from environmental factors (internal or external stimuli). It is expected that in future, study of livestock epigenetics will shed light on what practices through which underlying mechanisms will be associated to favorable epigenetic patterns that can affect disease resistance and other economically important traits in livestock such as fetal development, milk yield, growth and carcass traits, and fat and meat deposition. In addition, it would be feasible to incorporate epigenetic information in genomic evaluations improve the accuracy of prediction of breeding value through discrimination of epigenetic from genetic effects. Currently, it is well known that certain chronic, non-communicable diseases in adulthood are influenced by environmental factors acting in early life. Hence, making this specific period of life as one the most susceptible windows in development. Therefore, several important factors and stimuli

such as maternal nutrition and assisted reproductive technologies which are widely used in cattle industry can cause epigenetic modifications which required further studies.

Nevertheless, it is not expected that epigenetic research will yield immediate benefits to livestock industry, but future studies will certainly advance our current knowledge about livestock epigenetic processes and will help us to understand how the environment affects animal performance and productivity. We can then use these information to modulate gene expression in favor of our interest to optimize the breeding programs and even livestock susceptibility and/or responses to diseases. Considering the rapid advances in future next-generation sequencing (NGS) technologies, it is also expected that future NGS technologies will permit researchers to perform streamlined epigenome-wide association analyses in livestock at a rapid pace and affordable cost.

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