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**TESI DI LAUREA SPECIALISTICA**

**Effects of Methyl Cycle Substrate  
Availability on Epigenetic Stability of  
Human Embryonic Stem Cells**

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# TABLE OF CONTENTS

ABSTRACT.....	pag. 8
RIASSUNTO.....	pag. 9
ACKNOWLEDGEMENTS.....	pag. 11
LIST OF FIGURES.....	pag. 13
LIST OF TABLES.....	pag. 16
ABBREVIATION LIST.....	pag. 17
<b>1. Introduction.....</b>	<b>pag. 20</b>
1.1 hESCs: A General Introduction.....	pag. 20
1.1.1 Human embryonic stem cells.....	“ 20
1.1.2 hES cell lines.....	“ 23
1.1.3 hESC features.....	“ 24
1.1.4 hESC culture.....	“ 28
1.1.5 hES cell applications.....	“ 29
1.2 hESCs as a Model for Early Human Development.....	pag. 30
1.2.1 A model for the human embryo.....	“ 30
1.2.2 A model for human gastrulation.....	“ 31
1.2.3 hEB formation: mimicking the human gastrula.....	“ 31
1.2.4 Ectodermal differentiation and associated markers.....	“ 32
1.2.5 Mesodermal differentiation and associated markers.....	“ 35
1.2.6 Endodermal differentiation and associated markers.....	“ 35
1.3 hESCs: a Model to Study Foetal Origin of Adult Diseases.....	pag. 36

1.3.1 Developmental origin of adult diseases.....“	36
<b>1.4 DNA Methylation and Nutritional Programming...pag.</b>	<b>38</b>
1.4.1 DNA methylation.....“	38
1.4.2 DNA methylation pattern establishment.....“	40
1.4.3 Epigenetic reprogramming in the developing embryo...“	41
1.4.4 Methyl/folate cycle.....“	44
1.4.5 Epigenetic consequences of early..... nutritional programming.....“	46
1.4.6 Nutritional programming and genomic imprinting.....“	48
<b>2. Aim of the Project.....pag.</b>	<b>49</b>
<b>3. Materials and Methods.....pag.</b>	<b>50</b>
<b>3.1 Media.....pag.</b>	<b>50</b>
I. Feeder culture media.....“	50
3.1.1 MEF medium.....“	50
II. hESC culture media.....“	50
3.1.2 BGK medium.....“	50
3.1.3 KOH medium.....“	51
3.1.4 MSLH medium.....“	51
3.1.5 Differentiation medium.....“	51
<b>3.2 Cell Culture.....pag</b>	<b>52</b>
I. Feeder culture.....“	52
3.2.1 MEF isolation and storage.....“	52
3.2.2 MEF culture.....“	53
3.2.3 MMC treatment of MEFs.....“	54
3.2.4 Media conditioning by MEFs.....“	54
II. hESC culture.....“	55
3.2.5 HUES-7 feeder-free culture and passage with trypsin...“	55
3.2.6 HUES-7 feeder-free culture in KOH and passage with collagenase IV.....“	56
3.2.7 HUES-7 feeder-free and conditioned medium-free culture in KOH and passage with collagenase IV.....“	56

3.2.8 HUES-7 culture in methyl donor deficient media and passage with collagenase IV.....	“ 57
3.2.9 HUES-7 treatment with methyl cycle inhibitors.....	“ 57
3.2.10 hEB formation.....	“ 58
3.2.11 hEB fixation.....	“ 58
<b>3.3 Molecular Biology.....</b>	<b>pag. 59</b>
DNA Analysis.....	“ 59
3.3.1 DNA extraction and quantification.....	“ 59
3.3.2 Bisulfite primer design .....	“ 60
3.3.3 Bisulfite treatment.....	“ 61
3.3.4 PCR.....	“ 63
3.3.5 Gel electrophoresis.....	“ 65
3.3.6 Gel extraction and purification.....	“ 65
3.3.7 DNA sequencing.....	“ 65
3.3.8 Imprinted gene analysis.....	“ 66
RNA Analysis.....	“ 67
3.3.9 RNA extraction.....	“ 67
3.3.10 cDNA synthesis.....	“ 67
3.3.11 RT-PCR.....	“ 68
3.3.12 Gel electrophoresis.....	“ 68
3.3.13 Gel extraction and purification.....	“ 68
3.3.14 Allelic expression analysis by RFLP.....	“ 68
Protein Analysis.....	“ 69
3.3.15 Nuclear protein extraction.....	“ 69
3.3.16 DNMT activity test.....	“ 70
<b>3.4 Immunohistochemistry.....</b>	<b>pag 70</b>
3.4.1 Tissue preparation and embedding.....	“ 70
3.4.2 Tissue sectioning.....	“ 71
3.4.3 Antigen retrieval and slide staining.....	“ 71
<b>4. Results.....</b>	<b>pag 73</b>
4.1 Altering Methyl/Folate Cycle Substrate Availability: Medium Design.....	pag 73

4.1.1 Medium design and methyl substrate availability.....“	73
4.2 Establishing hESC Culture with Collagenase IV Passaging.....pag	76
4.2.1 Aims.....“	76
4.2.2 MEF culture establishment.....“	76
4.2.3 Conditioned medium induced changes on MEFs.....“	77
4.2.4 HUES-7 trypsin culture in BGK medium.....“	80
4.2.5 Adapting HUES-7 to collagenase IV passage using conditioned BGK medium.....“	82
4.2.6 Adapting HUES-7 to conditioned KOH medium.....“	83
4.2.7 Adapting HUES-7 to conditioned MSLH medium.....“	85
4.2.8 Adapting HUES-7 to feeder-free and conditioned medium- free conditions.....“	86
4.2.9 3-DZA methyl cycle inhibition test on HUES-7.....“	89
4.3 Effects of Methyl/Folate Cycle Substrate Availability on DNA Methylation.....pag	92
4.3.1 Imprinted gene DMR methylation analysis.....“	92
4.3.2 Bisulfite conversion of gDNA.....“	92
4.3.3 Amplification of DMRs of interest.....“	92
4.3.4 DMR sequencing.....“	93
4.3.5 Alignment and methylation analysis.....“	94
4.3.6 <i>PEG3</i> DMR.....“	95
4.3.7 <i>IGF2</i> DMR 2.....“	97
4.3.8 <i>IGF2R</i> DMR 1* and 2.....“	99
4.3.9 <i>SNRPN</i> DMR.....“	102
4.3.10 <i>GRB10</i> DMR.....“	103
4.3.11 <i>H19</i> DMR.....“	105
4.4 Effects of Methyl/Folate Cycle Substrate Availability on <i>H19</i> Expression.....pag	108
4.4.1 Aims.....“	108
4.4.2 Methyl group availability induced changes on <i>H19</i> expression.....“	109
4.5 Effects of Methyl/Folate Cycle Substrate Availability on DNMTs' activity.....pag	113

4.5.1 Aims.....“	113
4.5.2 Methyl deficiency induced changes on DNMTs’ activity.....“	113
<b>4.6 Methyl/Folate Cycle Substrate Availability Consequence on Differentiation.....pag.</b>	<b>117</b>
4.6.1 Aims.....“	117
4.6.2 HUES-7 BGK/trypsin culture derived hEBs: morphology and differentiation.....“	117
4.6.3 The effect of methyl deficient conditioned medium on HUES-7 derived hEBs.....“	120
4.6.4 The effect of methyl deficient medium on HUES-7 derived hEBs in high FGF cultures.....“	121
4.6.5 Effects of methyl deficiency on pluripotency and germ layer-specific marker expression in hEBs.....“	123
<b>5. Discussion.....pag.</b>	<b>130</b>
<b>5.1 Effects of Methyl Deficient Media on hESCs...pag.</b>	<b>130</b>
5.1.1 Environmental and culture condition consequences on hESC.....“	130
5.1.2 Effects of lower methyl group availability on HUES-7 stem cell cultures .....	“ 131
<b>5.2 Effects of Lower Methyl Group Availability on Imprinted Genes .....</b>	<b>pag. 132</b>
5.2.1 Imprinting disruption and foetal origin of adult diseases“	132
5.2.2 Methylation status at analysed imprinted gene loci... “	133
5.2.3 Methyl-deficient medium-induced demethylation on imprinted gene DMRs.....“	134
5.2.4 Methyl group deficiency dependent alteration of <i>H19</i> monoallelic expression.....“	142
<b>5.3 Methyl Deficient Medium Effects on DNMT Activity.....pag.</b>	<b>145</b>

5.3.1 DNMT activity alterations and related consequences on early mammalian development.....	“ 145
5.4 Methyl Deficiency Consequences on Differentiation.....	pag. 148
5.4.1 Methyl substrate availability induced changes in pluripotency markers' expression.....	“ 148
5.4.2 Germ layer-specific marker expression in methyl deficient culture-derived hEBs.....	“ 151
5.5 Future Work.....	pag. 153
<b>6. References.....</b>	<b>pag. 155</b>

# ABSTRACT

A link has been hypothesised to occur between suboptimal maternal nutrition and impaired foetal development leading to a predisposition to a range of adult pathologies. As a clear connection between dietary intake of methyl group donors and epigenetic defects has been demonstrated both *in vivo* and *in vitro*, this project had the purpose of generating a disruption into the methyl/folate cycle to investigate DNA methylation alterations during human preimplantation embryo development, using human embryonic stem cells (hESCs) as an *in vitro* model. In particular, HUES-7 stem cells were employed and cultured using either standard or methyl deficient media to test this hypothesis. After the treatments, that included an inhibitor of a key enzyme of the cycle, Differentially Methylated Regions (DMRs) of six imprinted genes were analysed and assessed for their methylation status at Cytosine-phospho-Guanosine (CpG) sites. As a consistent decrease of methylation was observed for the gene *H19* in treated cultures, its allelic expression was then investigated and an initial process of Loss Of Imprinting (LOI) was found. Additionally, global DNA MethylTransferase (DNMT) activity was examined and a statistically significant decrease in treated samples was detected. Finally, hESCs were differentiated into Embryoid Bodies (hEBs), which were compared and stained for pluripotency and germ-layer specific markers. Consistently different expression of *OCT-4* and *NANOG* was noticed for treated-culture derived hEBs.

## RIASSUNTO

Negli ultimi anni è stata ipotizzata l'esistenza di un rapporto tra livelli sub-ottimali di composti donatori di metile nell'alimentazione materna e un aberrante sviluppo embrionale, spesso causa di predisposizione a varie patologie nell'età adulta. Poiché è stata dimostrata sia *in vitro* che *in vivo* una chiara relazione tra apporto, con la dieta, di molecole donatrici di gruppi metile e alterazioni epigenetiche, questo progetto ha avuto lo scopo di generare e indurre scompensi nei cicli congiunti del metile e del folato per studiare alterazioni nel dinamico processo di metilazione del DNA che avviene durante lo sviluppo embrionale pre-impianto, con l'utilizzo come modello di cellule staminali embrionali umane (hESCs). In particolare, si è fatto uso di una linea cellulare, HUES-7, coltivata usando sia terreni standard sia terreni a bassa concentrazione di donatori di gruppi metile. Successivamente ai trattamenti, che hanno incluso anche un inibitore di uno degli enzimi chiave del ciclo del metile, specifiche Differentially Methylated Regions (DMRs) di sei geni imprinted sono state analizzate annotandone lo stato di metilazione dei siti Citosina-fosfo-Guanina (CpG) che le compongono. Poiché un rilevante fenomeno di demetilazione è stato osservato per il gene *H19*, ne è stata esaminata l'espressione allelica portando alla luce un processo iniziale di perdita di imprinting (LOI). Inoltre, è stata misurata l'attività globale degli enzimi DNA MetilTransferasi (DNMTs) e un decremento statisticamente significativo è stato individuato nei campioni trattati. Infine, le cellule sono state indotte al differenziamento in corpi embrioidi (hEBs) che sono stati successivamente comparati tra loro e saggiati per marcatori di pluripotenza e foglietto germinativo specifici tramite tecniche immunostochimiche. Un diverso livello di espressione è

stato individuato per *OCT-4* e *NANOG* negli hEBs derivati da colture trattate con terreni a bassa concentrazione di donatori di gruppi metile.

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# LIST OF FIGURES

<b>Figure 1-1.</b> Derivation of hESC lines.....	pag. 22
<b>Figure 1-2.</b> hESC signalling pathways.....	“ 27
<b>Figure 1-3.</b> Ectodermal differentiation and associated gene expression.....	“ 34
<b>Figure 1-4.</b> DNA methylation establishment during mammalian development.....	“ 39
<b>Figure 1-5.</b> Epigenetic reprogramming in the mammalian embryo.....	“ 43
<b>Figure 1-6.</b> Methyl/folate cycle in mammalian cells.....	“ 45
<b>Figure 1-7.</b> Methyl/folate cycle influence on DOHaD.....	“ 47
<b>Figure 4-1.</b> MEF cultures reaching confluency.....	“ 77
<b>Figure 4-2.</b> MMC-treated MEF cultures during BGK-CM collection.....	“ 79
<b>Figure 4-3.</b> MEFs' morphology in different media.....	“ 80
<b>Figure 4-4.</b> HUES-7 stem cell culture in BGK medium using a trypsin passage method.....	“ 81
<b>Figure 4-5.</b> HUES-7 stem cell culture using a trypsin (A) or a collagenase IV passage method (B).....	“ 83
<b>Figure 4-6.</b> HUES-7 stem cell culture in KOH-CM.....	“ 85
<b>Figure 4-7.</b> HUES-7 stem cell culture in MSLH-CM.....	“ 86
<b>Figure 4-8.</b> HUES-7 stem cell culture adaptation to high FGF culture method.....	“ 88

<b>Figure 4-9.</b> HUES-7 stem cell high FGF cultures in different media.....	“ 89
<b>Figure 4-10.</b> HUES-7 stem cell cultures treated with scalar concentrations of 3-DZA.....	“ 91
<b>Figure 4-11.</b> Agarose gel electrophoresis of PCR amplified <i>H19</i> DMR.....	“ 93
<b>Figure 4-12.</b> Direct sequencing of <i>H19</i> DMR in MSLH 2x-FGF sample.....	“ 94
<b>Figure 4-13.</b> Alignment of direct sequenced MSLH 2x-FGF sample with bisulfite converted <i>H19</i> DMR. ....	“ 95
<b>Figure 4-14.</b> Methylation analysis of <i>PEG3</i> DMR.....	“ 97
<b>Figure 4-15.</b> Methylation analysis of <i>IGF2</i> DMR.....	“ 99
<b>Figure 4-16.</b> Methylation analysis of <i>IGF2R</i> DMR 1*.....	“ 101
<b>Figure 4-17.</b> Methylation analysis of <i>IGF2R</i> DMR 2.....	“ 102
<b>Figure 4-18.</b> Methylation analysis of <i>SNRPN</i> DMR.....	“ 103
<b>Figure 4-19.</b> Methylation analysis of <i>GRB10</i> DMR.....	“ 105
<b>Figure 4-20.</b> Methylation analysis of <i>H19</i> CTCF6 DMR.....	“ 107
<b>Figure 4-21.</b> Genotypization of HUES-7 stem cell line for a <i>RsaI</i> -sensitive SNP on <i>H19</i> .....	“ 109
<b>Figure 4-22.</b> <i>H19</i> Allelic expression analysis through RFLP assay.....	“ 111
<b>Figure 4-23.</b> DNMT activity test by ELISA. ....	“ 115
<b>Figure 4-24.</b> d4 BGK/trypsin hEBs in suspension cultures.....	“ 118

<b>Figure 4-25.</b> Differentiated cells from attached hEBs.....	“ 119
<b>Figure 4-26.</b> hEBs from HUES-7 stem cells cultured in KOH- and MSLH- CM.....	“ 120
<b>Figure 4-27.</b> d4 hEBs obtained from HUES-7 stem cells cultured in high FGF.....	“ 121
<b>Figure 4-28.</b> hEBs’ dimensions.....	“ 122.
<b>Figure 4-29.</b> OCT-4 staining of high FGF culture-derived d12 hEBs.....	“ 125
<b>Figure 4-30.</b> NANOG staining of high FGF culture-derived d12 hEBs.....	“ 126
<b>Figure 4-31.</b> SOX1 staining of high FGF culture-derived d12 hEBs.....	“ 127
<b>Figure 4-32.</b> SOX17 staining of high FGF culture-derived d12 hEBs.....	“ 128
<b>Figure 4-33.</b> T staining of high FGF culture-derived d12 hEBs.....	“ 129

## LIST OF TABLES

- Table 4-1.** Methyl donor composition of standard media compared to human serum levels..... pag. 75
- Table 4-2.** Densitometry analysis of *H19* expression RFLP assay.....“ 112
- Table 4-3.** Tukey-Kramer multiple comparison test on high FGF culture DNMT activity.....“ 115
- Table 4-4.** Tukey-Kramer multiple comparison test on hEBs' dimensions....“ 123

## ABBREVIATION LIST

3-DZA = 3-Deazaadenosine.  
ANOVA = Analysis Of Variance.  
ART = Assisted Reproductive Technology.  
bFGF = basic Fibroblast Growth Factor.  
BGK = BresaGen Knockout serum replacement.  
BMP = Bone Morphogenetic Protein.  
BWS = Beckwith-Wiedemann Syndrome.  
cDNA = complementary DNA.  
CM = Conditioned Medium.  
CpG = Cytosine-phospho-Guanine.  
CTCF = CCCTC binding Factor.  
CTL = Cytotoxic T Cell.  
Cys = Cysteine.  
d = day.  
D-MEM = Dulbecco's Modified Eagle Medium.  
DMR = Differentially Methylated Region.  
DMSO = DimethylSulfOxide.  
Dnmt/DNMT = DNA MethylTransferase.  
dNTP = deoxyriboNucleotide TriPhosphate.  
DOHaD = Developmental Origin of Health and Disease.  
DTT = DiThioThreitol.  
EB = Embryoid Body.  
EDTA = EtylenDiamyneTetraAcetic acid.  
EGF = Epidermal Growth Factor.  
EHS = Engelbreth-Holm-Swarm.  
ELISA = Enzyme Linked ImmunoSorbent Assay.  
ESC = Embryonic Stem Cell.  
EtOH = Ethanol.  
FA = Folic Acid.  
FBS = Foetal Bovine Serum.  
-FGF = high FGF cultures, supplemented with 100 ng/ml bFGF.  
gDNA = genomic DNA.  
GH = Growth Hormone.  
Gln = Glutamine.  
Gly = Glycine.  
GRB = Growth factor Receptor-Bound protein.  
GZMB = Granzyme B.  
GαH = Goat α-Human.  
h = human.  
HRP = HorseRadish Peroxydase.  
IAP = Intracistronic A Particle.  
ICF = Immunodeficiency-Centromeric Instability-Facial Anomalies  
ICM = Inner Cell Mass.  
ICR = Imprinting Control Region.  
IGF = Insulin-like Growth Factor.  
IGFR = Insuline-like Growth Factor Receptor.  
IsPrOH = Isopropanol.

IVF = *In Vitro* Fertilisation.  
KCNQ = Potassium Channel, voltage-gated, KQT-like subfamily.  
KO-DMEM = Knock-Out Dulbecco's Modified Eagle Medium.  
KOH = Knock-Out Dulbecco's modified eagle medium Hyclone serum.  
KSR = Knockout Serum Replacement.  
LIF = Leukaemia Inhibitory Factor.  
LOH = Loss Of Heterozygosity.  
LOI = Loss Of Imprinting.  
m = mouse.  
M6P = Mannose-6-Phosphate.  
MAT = Methionine Adenosyl Transferase.  
MEF = Mouse Embryonic Fibroblast.  
MESP = Mesodermal Posterior 1  
Met = Methionine.  
Me-THR = Methyl-TetraHydroFolate.  
MG = Methyl Group.  
MgCl<sub>2</sub> = Magnesium chloride.  
MMC = MitoMycin C.  
MMR = MisMatch Repair.  
MS = Methionine Synthase.  
MSLH = Methyl Substrate Low Hyclone serum.  
MαH = Mouse α-Human.  
NaHSO<sub>3</sub> = Sodium Bisulfite.  
NaOAc = Sodium Acetate.  
NaOH = Sodium Hydroxide.  
NDNL = Necdin-Like.  
NEAA = Non Essential Amino Acids.  
NFκB = Nuclear Factor kappa-B.  
NGF =Nerve Growth Factor.  
Oct/OCT = Octamer binding Transcription factor.  
p = passage.  
PBS = Phosphate Buffered Saline.  
PcG = Polycomb Group protein.  
PCI = Phenol Chloroform Isoamyl alcohol.  
PCR = Polymerase Chain Reaction.  
PDGF = Platelet-Derived Growth Factor.  
PEG = Paternally Expressed Gene.  
Pen/Strep = Penicillin/Streptomycin.  
PFA = para-Formaldehyde.  
PLG = Phase Lock Gel.  
PWS = Prader-Willi Syndrome.  
RαM = Rabbit α-Mouse.  
RFLP = Restriction Fragment Length Polymorphism.  
RT = Room Temperature.  
SAH = S-Adenosyl Homocysteine.  
SAHh = S-Adenosyl Homocysteine hydrolase.  
SAM = S-Adenosyl Methionine.  
Ser = Serine.  
SMAD = Sma/Mothers-Against-Decapentaplegic homologue.  
SNP = Single Nucleotide Polymorphism.  
SNRPN = Small Nuclear Ribonucleoprotein Polypeptide N.

Sox/SOX = Sex determining region of the Y chromosome-related box containing gene.  
SSEA = Stage-Specific Embryonic Antigen.  
Stat/STAT = Signal Transducer and Activator of Transcription.  
T = Brachyury.  
TBX = T Box.  
TGF = Transforming Growth Factor.  
THF = TetraHydroFolate.  
THFR = TetraHydroFolate Reductase.  
Thr = Threonine.  
TNF = Tumor Necrosis Factor.  
Usp/USP = Ubiquitin-specific processing protease.  
Vit = vitamin.  
Wsb/Nf1 = WD repeat and soxs box-containing protein /Neurofibromin.  
Zim/ZIM = Zinc finger protein imprinted gene.  
Znf/ZNF = Zinc Finger protein.  
 $\beta$ -ME =  $\beta$ -Mercapthoethanol.