UNIVERSITA' DEGLI STUDI DI PISA
Facoltà di Scienze Matematiche, Fisiche e Naturali
Corso di Laurea Specialistica in Scienze e Tecnologie Biomolecolari
Curriculum Molecolare Cellulare

TESI DI LAUREA SPECIALISTICA

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

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ACADEMIC YEAR 2006 / 2007

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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-optimali 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 immunoistochimiche. 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.

ACKNOWLEDGEMENTS

First of all, I would like to thank Prof. Lorraine Young for having accepted me in her research group and helped me through the writing process with helpful advice. She gave me the possibility to grow both personally and professionally and I hope I have paid her a little bit back with this work. I also would also thank Prof. Renata Batistoni for being my internal supervisor and having assisted me with the presentation of the thesis. I am also very grateful to Prof. Luciana Dente and Dr. Nicolo Di Pietro for having supported me with very good references and to Prof. Paola Bagnoli for having awarded me with the Erasmus bursary. I would also like to thank Dr. Silvia Marracci and Prof. Antonella Del Corso for their co-supervision and interest about this project. I cannot avoid mentioning Mrs. Stephenie Mudd, without your constant support settling down in Nottingham would have been very hard. In regard to the research group, I would like to say thank you to all the people that made it a really nice place to stay: Dr. Alexandra Thurston for her help with bisulfite sequencing, Jayson Bispham, Emma Lucas and William Steele for their helpful advice, Sonia Sebastian for her precious help on molecular biology, Kee-Pyo Kim for his help on imprinted genes, Paul Burridge for his advice on immunohistochemistry techniques, David Anderson for his constant supply of stem cells throughout the project. I would also like to thank Noopur Dingar, Elena Matsa, Nafisa Abdul-Rhaman, Leigh Jackson and Alexander Huber for having made the office a more enjoyable place. I have to thank Dr. Virginie Sottile, Dr. Cristina Tufarelli, Dr. Hazel Cruickshanks for their support and advice through difficulties, I really appreciated your kind help. A special thanks goes to Maria Barbadillo-Munoz for always being there to help and for having taught me the basics of stem cell cultures; she has been a great friend and colleague. An

enormous thank you goes to Dr. Cinzia Allegrucci that has been a fantastic teacher and a nice friend throughout this year abroad: I will not forget all the support I received from you both at work and out of the office. Finally, I would like to spend the last words for my family: without your love and support none of this would have been possible.

I would like to dedicate this thesis to Prof. Stefania Bucci, one of the most devoted and skilful researcher I have ever met.

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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.
$MgCl_2$ = Magnesium chloride.
MMC = MitoMycin C.
MMR = MisMatch Repair.
MS = Methionine Synthase.
MSLH = Methyl Substrate Low Hyclone serum.
MαH = Mouse α-Human.
$NaHSO_3$ = Sodium Bisulfite.
NaOAc = Sodium Acetate.
NaOH = Sodium Hydroxide.
NDNL = Necdin-Like.
NEAA = Non Essential Amino Acids.
NFkB = 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 Cloroform Isoamyl alcohol.
PCR = Polymerase Chain Reaction.
PDGF = Platelet-Derived Growth Factor.
PEG = Paternally Expressed Gene.
Pen/Strep = Penicillin/Streptomicin.
PFA = para-Formaldehyde.
PLG = Phase Lock Gel.
PWS = Prader-Willi Syndrome.
RaαM = 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-Decapenthaplegic homologue.
SNP = Single Nucleotide Polymorphism.
SNRPN = Small Nuclear Ribonucleoprotein Polypetide 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 socs box-containing protein /Neurofibromin.
Zim/ZIM = Zinc finger protein imprinted gene.
Znf/ZNF = Zinc Finger protein.
β-ME = β-Mercapthoethanol.