Transcriptional and Epigenetic Regulation of Labour Associated Inflammatory Genes in the Amnion.

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A thesis submitted to the Faculty of Health, The University of Newcastle, New South Wales, Australia, in fulfilment of the requirements of the degree Doctor of Philosophy.

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The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968.

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Carolyn M Mitchell

April 2016

To

David

Alex, Catherine, Nicole and Dylan

In memory of

Dianne Bruce

&

Elizabeth Grace Mitchell

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List of Publications

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I attest that Research Higher Degree candidate **Carolyn Mitchell** contributed to: the experimental design and procedures; analysis and interpretation of the data; and manuscript preparation for the publication entitled:

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Additional Publications

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*indicates presenting author.

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Abbreviations

acH3	----------	Anti-acetyl-histone 3
acH4	----------	Anti-acetyl-histone 4
ACTH	----------	Adrenocorticotropic hormone
AP-1	----------	Activator Protein One
ATP	----------	Adenosine 5'- triphosphate
Aza	----------	5-aza-2-deoxycytidine
BMP2	----------	Bone Morphogenetic Protein 2
cAMP	----------	Cyclic adenosine monophosphate
CAPS	----------	Contraction Associated Proteins
CBP	----------	CREB-binding Protein
cDNA	----------	Complementary DNA
C/EBP	----------	CCAAT / Enhancer Binding Protein (NFIL-6)
ChIP	----------	Chromatin Immunoprecipitation
Cx43	----------	Connexin 43
CpG	----------	cytosine – phosphate – guanine dinucleotide
CRE	----------	cAMP Response Element
CRE/E-box	----------	cyclic AMP responsive element
CREB	----------	cAMP Response Element Binding Protein
CRH	----------	Corticotrophin Releasing Hormone
CS	----------	Caesarean Section
CTD	----------	C-terminal domain
CXCL	----------	chemokine (C-X-C motif) ligand
Dex	----------	Dexamethasone
DHEAS	----------	Dehydroepiandrosterone sulfate
DNase	----------	Deoxyribonuclease
dNK cells	----------	Decidual Natural Killer cells
DNMT	----------	DNA methyltransferase
DRB	----------	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DOHaD	----------	Developmental Origins of Health and Disease
DSIF	----------	DRB sensitivity-inducing factor
E2	----------	Estradiol
E3	----------	Estriol
EGF	----------	Epidermal Growth Factor
ENL	----------	Early Gestation Not in Labour
EMSA	----------	Electromobility Shift Assay
EP	----------	Prostaglandin E Receptor
ESR1	----------	Estrogen Receptor
ERα	----------	Estrogen Receptor alpha
ERβ	----------	Estrogen Receptor beta
FP	----------	Prostaglandin F Receptor

GA	----------	Gestational Age
GM-CSF	----------	Granulocyte-macrophage colony-stimulating factor
G-CSF	----------	Granulocyte colony-stimulating factor
GR	----------	Glucocorticoid Receptor
GRE	----------	Glucocorticoid Response Element
GROα	----------	Growth-related oncogene alpha
H3K4me3	----------	Histone-3 trimethylation at lysine 4
H3K27me3	----------	Histone-3 trimethylation at lysine 27
H5	----------	Serine-2-phosphorylated pol-II
H14	----------	Serine-5 phosphorylated pol II
HAT	----------	Histone acetyltransferase
HDAC	----------	Histone deacetylase
hnRNA	----------	Heterogenous nuclear RNA
hCG	----------	Human chorionic gonadotropin
IGF	----------	Insulin-like growth factor
IκB	----------	Inhibitor of NFκB
IKK	----------	IκB Kinase
IL	----------	Interleukin
IL-1Rα	----------	Interleukin-1 receptor alpha
INF	----------	Interferon
IP-10	----------	IFN-γ-inducible protein 10
IUGR	----------	Intra-uterine Growth Restriction
LDH	----------	Lactate dehydrogenase
LIF	----------	leucocyte inhibitory factor
LPS	----------	Lipopolysaccharide
MAPK	----------	Mitogen Activating Protein Kinase
MBD	----------	methyl-CpG binding domain
MCH	----------	Major Histocompatibility Complex
MCP-1(CCL2)	---------	monocyte chemotactic protein 1 or chemokine (C-C motif) ligand 2
miRNA	----------	Micro ribonucleic acid
MMP	----------	Matrix Metalloproteinase
NAMPT	----------	Nicotinamide phosphoribosyltransferase
NDPK	----------	Nucleotide 5'-diphosphate kinase
NELF	----------	Negative elongating factor
NFκB	----------	Nuclear Factor Kappa B
NFIL-6	----------	Nuclear Factor of Interleukin-6 (C/EBP)
NR3C1	----------	Nuclear Receptor subfamily 3, group C, member 1 or GR
NR3C3	----------	Nuclear Receptor subfamily 3, group C, member 3 or PGR
OTR	----------	Oxytocin Receptor
PBEF	----------	pre-B-cell colony-enhancing factor
PCR	----------	Polymerase Chain Reaction

PG	----------	Prostaglandin
PGDH	----------	15-hydroxyprostaglandin dehydrogenase
PGES	----------	Prostaglandin E synthase
PGFS	----------	Prostaglandin F synthase
PGHS2	----------	Prostaglandin H synthase-2 (now called PTGS2)
PGI_2	----------	Prostacyclin
PGR	----------	Progesterone receptor
PIC	----------	Pre-initiation complex
PKA	----------	Protein kinase A
pol II	----------	Polymerase II
PPROM	----------	Preterm premature rupture of membranes
PRA /PRB	----------	Progerterone receptor A / Progerterone receptor B
P-TEFb	----------	Positive transcription elongation factor b
PTGFR	----------	Prostaglandin F2α receptor
PTGS1	----------	Prostaglandin endoperoxide synthase-1 (PGHS1 or COX 1)
PTGS2	----------	Prostaglandin endoperoxide synthase-2
PTL	----------	Preterm labour
qPCR	----------	Qantitative polymerase chain reaction
RANTES	----------	Regulated upon activation, normal T cell expressed and secreted
RT-PCR	----------	Reverse transcription polymerase chain reaction
SAM	----------	S-Adenosylmethionine
SP1	----------	Promoter-specific transcription factor 1
SRC	----------	Steroid receptor coactivator
STAT3	----------	Signal transducer and activator of transcription 3
sTNFR	----------	Soluble tumor necrosis factor receptor
TBP	----------	TATA binding protein (TFIID)
TDG	----------	Thymine DNA glycosylase
TET	----------	Ten-eleven translocation
TGF	----------	Transforming growth factor-
TIMP-1	----------	Tissue inhibitor of metalloproteinase-1
TLR	----------	Toll-like receptor
TNF	----------	Tumour necrosis factor
TNL	----------	Term not in labour
TXA_2	----------	Thromboxane
ZEB1	----------	Zinc finger E-box-binding homeobox 1
ZEB2	----------	Zinc finger E-box-binding homeobox

ABSTRACT

Inflammatory genes are activated in the pregnant uterus at term labour producing an “acute inflammation gene expression signature” even in the absence of infection or chorio-amnionitis. The mechanisms that activate the inflammatory pathways and determine the timing of labour are unknown. The primary objective of my research presented in the thesis was to determine the molecular mechanisms that regulate the expression of labour-promoting inflammatory genes in the human fetal membranes at term parturition *in vivo*. Previous work in cell culture models suggested the involvement of the NFκB system and glucocorticoids in the upregulation of these genes including the prototypical proinflammatory gene, PTGS2, in the amnion. Epigenetic mechanisms, such as DNA methylation and histone modifications, were also hypothesised to participate in the control of proinflammatory gene activity in the fetal membranes during pregnancy.

Chromatin immunoprecipitation with freshly delivered, term amnion tissue identified TBP, NFkB p65 and p50 subunit binding at the proximal promoter of the PTGS2 gene, but binding was not associated with transcriptional stimulation. However these transcription factors stimulated IκBα gene expression in the same samples indicating the activation of NFκB signalling. Increased histone-3 and -4 acetylation was found in the proximal 1000 bp region of the PGHS-2 promoter suggesting that an open chromatin structure, permissive of gene expression, was present at term and after labour.

In short term amnion explants (≤24 h) the glucocorticoid dexamethasone decreased PTGS2 gene activity and mRNA levels. Glucocorticoid receptor-α (GRα) binding to the PTGS2 promoter decreased initiating (Ser-5) and elongating (Ser-2) pol-II phosphorylation. Accumulation of pol II in the 5’-region of the PTGS2 gene indicated post-initiation pausing. Acetylation of Histones -3 and -4 decreased, but histone-3 methylation was unchanged. The data indicated rapid PTGS2 mRNA turnover *in vivo*, which slowed quickly in the explants due to declining transcription rate and increased mRNA stability. The transcriptional mechanism thus alters profoundly *in vitro*, even in short term explant cultures.

DNA methylation of the inflammatory genes PTGS2, BMP2, NAMPT and CXCL2, and the glucocorticoid, progesterone and oestrogen receptor genes, was examined using the Methyl Profiler PCR system and bisulfite sequencing with fresh tissue samples. Variable

proportions of inflammatory and steroid receptor gene copies, to a maximum of 50.9%, were densely methylated in both amnion and decidua tissues consistent with repression. Densely methylated copy proportions were significantly different between genes showing no relationship with varying expression during pregnancy, between tissues and in individuals. Methylated copy proportions of all genes in amnion and most genes in decidua were highly correlated in individuals. DNMT1 and -3A were expressed in both tissues with significantly higher levels in the amnion at 11–17 weeks than at term.

Collectively, the data generated by my PhD research suggest that PTGS2 expression is suppressed in term amnion *in vivo* and endogenous glucocorticoid may be involved in this process. Epigenetic changes occur rapidly *in vitro* potentially influencing gene ``expression. Pol-II pausing limits PTGS2 transcriptional activity in the amnion and this regulatory mechanism is associated with the changing phosphorylation of Pol-II. The DNA methylation study found the unmethylated portion of gene copies is responsible for the regulated expression in the amnion. Dense methylation of individually variable gene copy proportions likely occurs in the first trimester amnion, influenced by DNA sequence context and affected strongly by individual circumstances.

The experiments described in the thesis provide important insights into the control of inflammatory gene activation in pregnancy, emphasising the need for techniques and approaches that detect or preserve the *in vivo* condition of the gestational tissues. Information obtained by these approaches supplements and places into context the results generated by experimental models describing molecular mechanisms potentially underpinning pregnancy maintenance and parturition.